

**CHARACTERISATION OF THE EFFECT OF STRESS ON NITROGEN METABOLISM IN THE
COMMERCIALY IMPORTANT AGAROPHYTE, *GRACILARIA GRACILIS***

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blessed to call you mum. There aren't enough superlatives to describe how amazing you are. This PhD was a dream that I shared with you Dad, and I dedicate this thesis to you.

List of abbreviations

α	alpha
β	beta
$^{\circ}\text{C}$	degrees Celsius
λ	lambda
μg	microgram(s)
μl	microlitre(s)
μm	micromole(s)
μmol	micromolar
ASW	artificial seawater
AspAT	asparatate aminotransferase
Asp	asparate
AS	asparagine synthetase
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BLAST	basic local alignment search tool
BLASTn	basic local alignment search tool nucleotide
BLASTx	basic local alignment search tool nucleotide translated
BSA	bovine serum albumin
bp	base pair(s)
cDNA	complementary DNA
EDTA	ethylenediaminetetraacetic acid (disodium salt)
Fd	ferredoxin
FAO	Food and Agriculture Organization (of the United Nations)
<i>g</i>	standard gravitational acceleration (9.81 m/s ²)
Ga	glutaraldehyde
GS	glutamine synthetase
GS ₁	cytosolic glutamine synthetase
GS ₂	chloroplastic glutamine synthetase
GOGAT	glutamate synthase
GDH	glutamate dehydrogenase
Glu	glutamate
Gln	glutamine
GOI	gene of interest
h	hour(s)
kDa	kilodalton(s)

kb	kilobase(s)
L	litre(s)
min	minute(s)
mL	millilitre(s)
mRNA	messenger RNA
MW	molecular weight
NCBI	National Centre for Biotechnology Information
NR	nitrate reductase
NiR	nitrite reductase
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NIS	non-immune serum
OAA	oxaloacetate
OG	oxoglutarate
OPP	oxidative pentose phosphate pathway
ORF	open reading frame
Ox	oxidised
PE	phycoerythrin
PET	photosynthetic electron transport
PES ASW	nitrogen containing/enriched ASW
PES-N ASW	nitrogen lacking/free ASW
PF	paraformaldehyde
PPP	protein phosphatase
PCR	polymerase chain reaction
qPCR	quantitative real-time PCR
RACE	rapid amplification of cDNA ends
Red	reduced
RG	reference gene
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
ROS	reactive oxygen species
Rubisco	1,5-bisphosphatase carboxylase/oxygenase
TCA	tricarboxylic acid
U	unit(s)

ABSTRACT

Gracilaria gracilis occurs naturally in Saldanha Bay, and was important to the South African export industry as it is a source of two important types of agar, namely food grade and sugar reactive agar. However, a number of abiotic and biotic factors, such as nutrient limitation, has virtually destroyed the *G. gracilis* resource. An understanding of the physiological response of the alga to nutrient limitation will aid in re-establishing and sustaining *G. gracilis* populations. However, modelling algal physiology and growth in response to abiotic stresses such as nutrient limitation requires an understanding of the underlying metabolic processes. The present study aimed to address this by investigating nitrogen metabolism and the mechanisms regulating nitrogen metabolism in *G. gracilis*. This was achieved by profiling changes in gene and protein expression, and activity of two major nitrogen metabolic enzymes, nitrate reductase and glutamine synthetase. Long term culture of *G. gracilis* in nitrogen replete and lacking conditions indicated that nutrient limitation causes a reduction in intracellular nitrogen and nitrogen protein stores such as phycoerythrin. When various sources were introduced to the culture medium to replenish nitrogen starved *G. gracilis*, changes in nitrate reductase and glutamine synthetase mRNA, protein and activity seemed to be dependent on the nutrient history of the cells, intracellular and extracellular nitrogen concentrations, metabolites of nitrogen assimilation and other metabolic processes such as carbon metabolism and photosynthesis. Nutrient studies suggested that multiple *G. gracilis* nitrate reductase and glutamine synthetase isoforms are present and differentially regulated via transcriptional, post-transcriptional, translational and post-translational mechanisms. Furthermore, the insensitivity of these nitrogen metabolic enzymes to ammonium inhibition and the ability to alter the GS₁:GS₂ activity ratio possibly represents adaptive strategies developed by *G. gracilis* to survive nitrogen limitation. Immunocytochemical investigations confirmed the presence of multiple nitrate reductase and glutamine synthetase isoforms. The enzymes were successfully localised to the cell wall, chloroplast and cytosol of *G. gracilis*. A novel finding was the immuno-localisation of glutamine synthetase to intracellular starch granules. Overall, findings in the current study have suggested multiple roles for these metabolic enzymes that include nitrogen assimilation/transport, cell wall biosynthesis and senescence. This study led to the development of a model of the metabolic changes that occur in nitrogen

replete and deplete *G. gracilis* and provides a firm foundation for future studies of the nitrogen stress response in *G. gracilis*. Characterisation of the *G. gracilis* nitrogen stress response may ultimately revive mariculture of this commercially important alga in South Africa.

CHAPTER 1

Literature review

1.1 INTRODUCTION

Seaweed (macro- or microalgae) are subdivided into three groups; Chlorophyta, Rhodophyta and Phaeophyta, commonly termed the green, red and brown algae, respectively (Chapman and Chapman, 1980). Macroalgae are industrially important sources of hydrocolloids (non-crystalline solids that disperse in water) (Anderson *et al.*, 1989; Glicksman, 1987). The main hydrocolloids include alginic acids in brown algae, and agars and carrageenans occurring in red algae. Some commercial uses of agar include its use as a gelling agent in foods, and in growth media for bacteriological cultures. Equally important are the carrageenans, used in confectionery items. In addition, seaweeds are used in numerous industries such as invertebrate cultivation, fertilizer production, the cosmetic industry and even as a human food source (Smith *et al.*, 1984, Oliveira and Plastino, 1994). Current research is directed toward the use of seaweed as a source of biomass in biofuel production (Ale *et al.*, 2010).

These numerous uses have made algae a focal point in aquaculture. Aquaculture based phyto-production was estimated at 15.8 million tonnes in 2008, representing US\$ 7.4 billion. This industry was estimated to have an annual growth rate of 7.7% since the 1970s. At the present time, seaweed aquaculture is dominated by East and Southeast Asian countries including China, Indonesia and the Philippines. Large scale producers outside Asia, include Chile and South Africa. Globally, Japanese kelp (*Saccharina japonica*) is the species most cultivated with *Euchema*, *Wakame*, *Gracilaria* (Rhodophyta) and *Porphyra* (commonly known as Nori) species following suit. Thus, the mariculture of seaweed can be a profitable endeavour (FAO, 2010).

1.2 GRACILARIA SPECIES. IN SOUTH AFRICA

South African *Gracilaria* species are harvested for use as a feed source for cultured abalone, but also represent a major agar source (Jaffray *et al.*, 1997). *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine *et* Farnham (class Florideophyceae, order Gracilariales,

family Gracilariaceae), an important source of food grade and sugar reactive agar, occurs naturally in Saldanha Bay, Cape Town, South Africa (Shroeder *et al.*, 2003). An active export industry dates back to 1944 (Engledov and Bolton, 1992). However, the *G. gracilis* resource was virtually destroyed after the construction of an ore jetty in 1974 (splitting the Bay into two smaller ones) and a breakwater that essentially closed the smaller bay (Figure 1) (Anderson *et al.*, 1993).

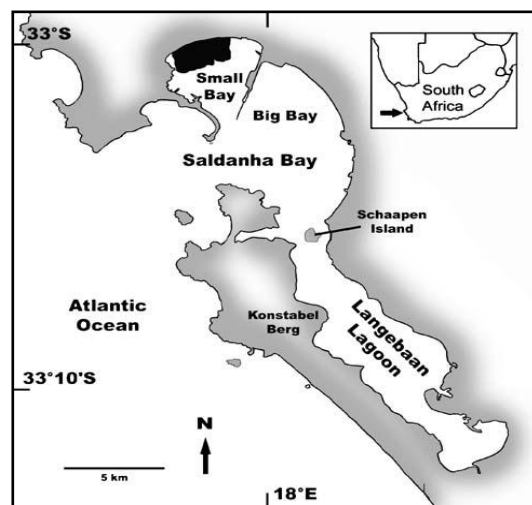


Figure 1. Map of Saldanha Bay, showing the breakwater that split the Bay into Small Bay and Big Bay (Rothman *et al.*, 2009).

Studies indicated that construction of the jetty, altered the hydrodynamics of the bay, resulting in stratification of the water column during summer (Anderson *et al.*, 1993). The result was poor nutrient exchange during the summer months that caused a 'die off' of the *G. gracilis* resource. A critical finding was the discovery that *G. gracilis* populations growing near a fish factory waste site in Saldanha Bay grew faster, and maintained growth over the summer period, while algae at a control site farther away died (Anderson *et al.*, 1999). It was determined that the input of nitrogen from the fish factory waste enabled the growth and proliferation of this group of algae. Subsequently, *G. gracilis* populations have recovered, but are continually plagued by population collapses. Although attempts at cultivating *G. gracilis* in Saldanha Bay by rope rafting (suspended culture) was found to be feasible, researchers concluded that yields could

be improved by technical research and development (Anderson *et al.*, 1996). Farming in open water subjects algae to constantly changing environmental conditions and periods of nitrogen starvation (Jaffray *et al.*, 1997). Unfortunately, exogenous inputs of nitrogen are not the answer to circumventing *G. gracilis* population collapses. Eutrophication caused by the fish waste resulted in a problem *Ulva* bloom in 1993-1994 that contaminated the *Gracilaria* and caused wash up to be discarded (Anderson *et al.*, 1996). In addition, recent studies have also defined a shift in the red macroalgal population at Saldanha Bay from *G. gracilis* to predominantly *Gracilariopsis longissima* (unpublished data). Although *G. longissima* is phenotypically indistinguishable from *G. gracilis*, the agar of the latter is of a much higher quality (Wakibia *et al.*, 2001; Rebello *et al.*, 1997).

Thus, re-establishment of the *G. gracilis* population in Saldanha Bay is of fundamental commercial importance. Studies completed on algal populations in Saldanha Bay suggest that nutrient concentration intricately controls the occurrence and survival of the alga. Therefore, an understanding of nitrogen assimilation and the regulation of nitrogen metabolism, is critical in the re-establishment of the *G. gracilis* population.

1.3 NITROGEN UPTAKE, ASSIMILATION AND INCORPORATION

Nitrogen is necessary for the synthesis of nucleotides, amino acids, phospholipids and secondary metabolites (Tschoep *et al.*, 2009). The limiting form of nitrogen in marine environments is dissolved inorganic nitrogen (DIN) and not dissolved organic nitrogen (DON). Sources of DIN are NO_3^- (most abundant), nitrite (NO_2^-) and ammonium (NH_4^+), that enter the marine environment through freshwater flow, rain and sewage release (Valiela, 1984; Chow and Oliveira, 2008). Reduced organic forms of nitrogen (amino acids and urea) as well as nitrogen released by nitrogen fixation are additional sources of nitrogen for plants and algae (Lobban and Harrison, 1994; Inokuchi and Okada, 2001). Although nitrate is more abundant in marine environments, laboratory studies established that ammonium was the preferred nitrogen source in macroalgae, as the uptake and assimilation of ammonium requires less energy (Teichberg *et al.*, 2007).

In order to utilise nitrogen, plant and algal cells need to be able to ‘sense’ and take up the required nitrogen source (Lobban and Harrison, 1994). Once nitrogen is transported into cells, it is assimilated (reduced) and incorporated into organic molecules (Figure 2). Nitrogen uptake is defined as the removal of nitrogen from the medium, while assimilation refers to the synthesis of small organic molecules, and incorporation to the synthesis of large structural macromolecules (Berges *et al.*, 1995). Factors affecting nitrogen uptake and assimilation include changes in temperature, irradiance and nitrogen concentration (Young *et al.*, 2007). Macroalgae are subjected to varying environmental changes throughout the day. For example, tidal emersions may affect nutrient availability and irradiance on time scales of hours to weeks. As environmental conditions fluctuate to varying degrees and for different periods of time, algae are required to adapt to a constantly changing environment. Therefore, in order to fully understand the regulation of nitrogen metabolism in macroalgae, one needs to investigate the responses of macroalgae to daily and seasonal fluctuations.

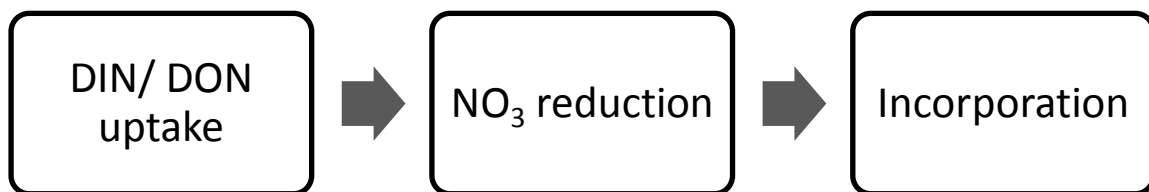


Figure 2. Simplified schematic depicting the fate of nitrogen in plant and algal cells.

Smit (2002) suggested that in order to gain an understanding of nitrogen nutrition in macroalgae, the concentration of nitrogen in the growth medium, the rate of diffusion/transport through the boundary layer (between the water column and algal thallus) and the rate at which seaweed can utilise nitrogen in metabolic processes, needed to be determined. Although the transport of nitrogen into the algal cell is not the main focus of these studies, a few important facts need to be addressed.

Regulation of nitrogen uptake is essential, as the accumulation of inorganic ions in plant cells serve as signals for nutritional and osmotic signalling as well as storage functions (Glass *et al.*, 2002). Plants and algae possess high affinity transport systems (HATS) and

low affinity transport systems (LATS) that are encoded by a number of genes. In addition, transport systems are differentially regulated, and the specificity and affinity for a particular ion differs for each transporter. For example, *G. gracilis* possesses rate-unsaturated and rate-saturated nitrogen transport systems for nitrate and ammonium (Smit, 2002). Understanding of nitrogen uptake and control is important as, in addition to initiating metabolic shifts within cells, nitrogen uptake is an indicator of the nitrogen status of the alga. For example, if nitrogen reserves are saturated and the intracellular nitrogen concentration is high, uptake and transport of nitrogen into cells may be reduced (Naldi and Wheeler, 1999). Many studies have focussed on nitrogen uptake/transport in micro- and macroalgae (Dy and Yap, 2001; Smit, 2002), but few studies have examined the assimilation of nitrogen compounds by investigating the action of assimilatory enzymes.

In plants ammonia is released and refixed numerous times by different processes that include symbiotic nitrogen fixation by nodule bacteria, the reduction of nitrate or nitrite, photorespiratory reactions and amino acid catabolism (Temple *et al.*, 1996; Oliveira *et al.*, 1997; Miflin and Habash, 2002; Barr *et al.*, 2004). Macroalgae are provided with nitrogen by these same processes with the exception of nitrogen fixation which has not been found in macroalgae (Lobban and Harrison, 1994). The reactions comprising these processes are catalysed by specific enzymes.

Following nitrate transport into the cell, nitrate is reduced to nitrite in the cytosol by nitrate reductase (NR) (Figure 3; Table 1) (Lobban and Harrison, 1994; Oliveira *et al.*, 1997). Subsequently, nitrite is transported to the chloroplast where it is reduced to ammonium by the action of the enzyme nitrite reductase (NiR). Glutamine synthetase (GS) catalyses the first step in inorganic nitrogen incorporation (Lancien *et al.*, 2000). The amination reaction catalysed by GS produces glutamine, that serves as the substrate for glutamine-oxoglutarate aminotransferase (GOGAT), also known as glutamate synthase, the next enzyme in the pathway (Figure 3) (Oliveira *et al.*, 1997). The GS-GOGAT reaction catalysed in the chloroplast, represents the first process of nitrogen assimilation, commonly termed primary nitrogen assimilation (Oliveira *et al.*, 1997). Three other enzymes that are suggested to play a role in ammonium assimilation are asparagine synthetase (AS), carbamoyl-phosphate synthase (CPSase) and glutamate

dehydrogenase (GDH) (Masclaux-Daubresse *et al.*, 2010) (Figure 3). AS uses glutamine from the GS-GOGAT cycle to produce asparagine whereas CPSase in the chloroplasts synthesises precursors for citrulline and arginine by using ATP, bicarbonate and ammonium. Mitochondrial GDH, like GS, is able to catalyse the amination of ammonium to produce glutamate (Lancien *et al.*, 2000; Masclaux-Daubresse *et al.*, 2010). Unlike GS, the reaction catalysed by GDH is reversible in that glutamate can be deaminated to produce 2-oxoglutarate and ammonium (Table 1).

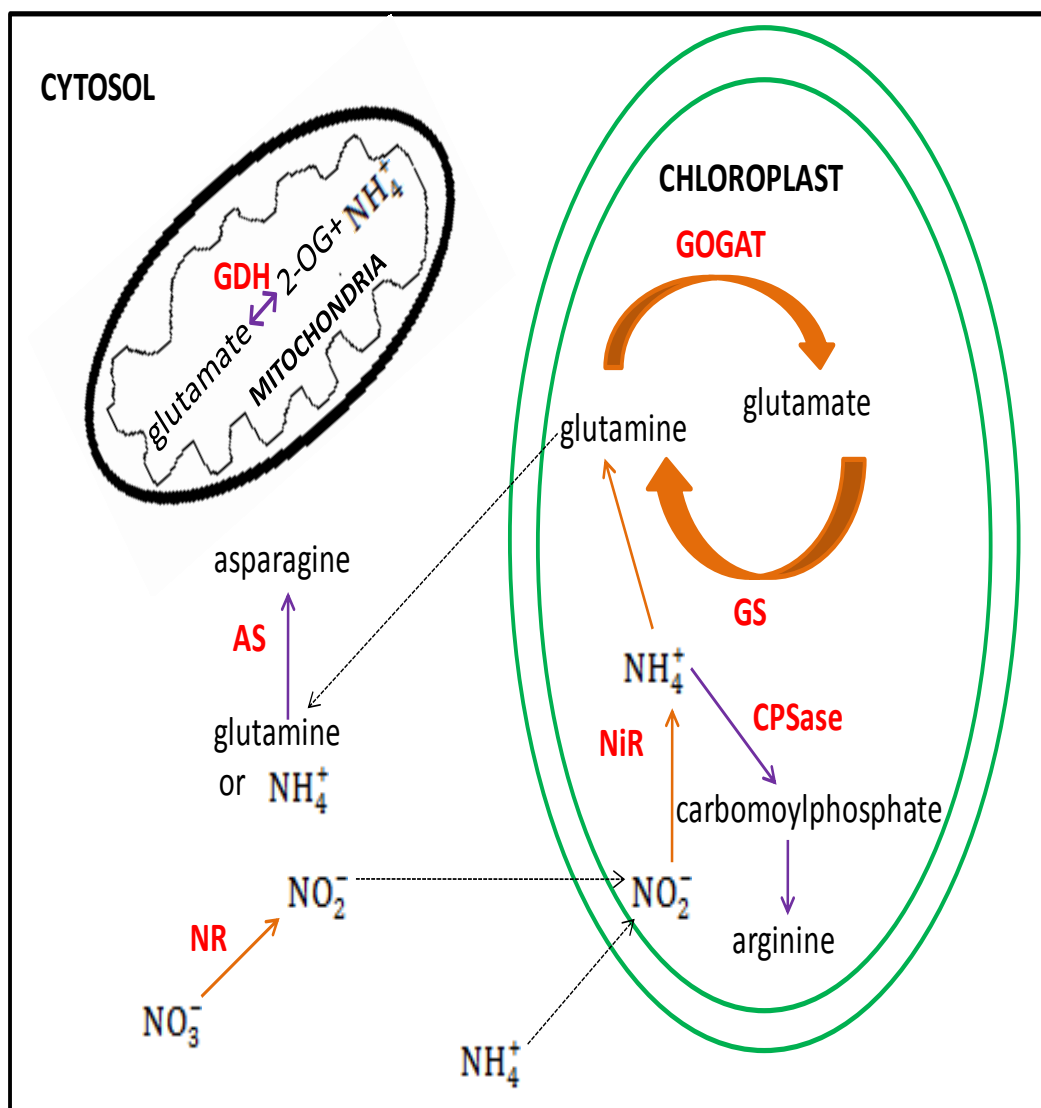


Figure 3. Simplified schematic representation of ammonium assimilation in plants and algae. The primary route of nitrogen assimilation is represented by the orange arrows. The reactions catalysed by other enzymes involved nitrogen assimilation are represented by the purple arrows. Enzyme isoforms are not depicted. (adapted from Masclaux-Daubresse *et al.*, 2010).

Table 1. Nitrogen assimilation enzymes. Common abbreviations for the assimilatory enzymes, EC numbers and the reaction each enzyme catalyses is shown.

Enzyme	Acronym	EC Number	Reaction Catalysed
Nitrate Reductase (Assimilatory)	NR	1.7.1.1/2/3	$\text{NO}_3^- + \text{NAD(P)H} + \text{H}^+ \rightarrow \text{NO}_2^- + \text{NAD(P)}^+ + \text{H}_2\text{O}$
Nitrite Reductase (Assimilatory)	NiR	1.7.1.4	$\text{NO}_2^- + 6\text{Fe}_{\text{red}} + 8\text{H}^+ \rightarrow \text{NH}_4^+ + 6\text{Fe}_{\text{ox}} + 2\text{H}_2\text{O}$
Glutamine synthetase	GS	6.3.1.2	$\text{L-Glu} + \text{ATP} + \text{NH}_4^+ \rightarrow \text{L-Gln} + \text{ADP} + \text{P}_i + \text{H}_2\text{O}$
Glutamate synthase	GOGAT	1.4.1.13	$2 \text{OG} + \text{L-Gln} + \text{NADH or Fd}_{\text{red}} \rightarrow 2 \text{L-Glu} + \text{NAD or Fd}_{\text{ox}}$
Asparagine synthetase	AS	6.3.5.4	$\text{ATP} + \text{L-Asp} + \text{L-Gln} + \text{H}_2\text{O} \leftrightarrow \text{AMP} + \text{ADP} + \text{L-Asn} + \text{L Glu}$
Carbamoyl-phosphate synthase	CPase	6.3.5.5	$2\text{MgATP} + \text{HCO}_3^- + \text{Gln} + \text{H}_2\text{O} \rightarrow 2\text{MgADP} + \text{P}_i + \text{Glu} + \text{Carbamoyl-P}_i$
Glutamate dehydrogenase	GDH	1.4.1.2/3/4	$\text{L-Glu} + \text{H}_2\text{O} + \text{NAD(P)}^+ \leftrightarrow 2 \text{OG} + \text{NH}_3 + \text{NAD(P)H}$

Glu- glutamate; Gln-glutamine; OG-oxoglutarate; OAA-oxaloacetate, Asp-aspartate; Fd-ferredoxin; Red-reduced; Ox-oxidised; HCO_3^- -bicarbonate; P_i -phosphate; Mg-magnesium. The double sided arrow (\leftrightarrow) indicates that the reaction is reversible.

GDH was originally thought to be responsible for primary nitrogen assimilation. However, this theory was dispelled after the discovery of the GS-GOGAT pathway in bacteria in the 1970s (Lobban and Harrison, 1994). It is now widely accepted that the GS-GOGAT pathway is responsible for primary nitrogen metabolism in plants, bacteria, cyanobacteria (Maurin and Gal, 1997; Gómez-Baena *et al.*, 2006), micro- and macroalgae (Taylor *et al.*, 2006). Examples include the macroalgae *Macrocystis angustifolia*, *Hinckesia mitchelliae* and *Laminaria digitata* (Lobban and Harrison, 1994). Further studies indicated that GDH only catalysed the amination

reaction under stress conditions (Lancien *et al.*, 2000). Instead, GDH primarily catalyses the deamination reaction and may act as a shunt, to divert carbon skeletons away from nitrogen metabolism toward carbon metabolism and the tricarboxylic acid (TCA) cycle (Bernard and Habash, 2009). However, the role of GDH is still widely debated. For example, in certain unicellular green algae both pathways are operational, with the GDH pathway operating when external ammonium is high, and the GS-GOGAT pathway operating when ammonium is low or cells are grown on nitrate (Lobban and Harrison, 1994).

When reviewing the different processes involved in nitrogen assimilation, it is evident that the functions and roles of nitrogen metabolic enzymes are diverse. Moreover, factors affecting their function and regulation are further complicated by the occurrence of multiple isoenzymes (for each enzyme), that are targeted to specific cellular organelles (Oliveira *et al.*, 1997). It is beyond the scope of this review to address the regulatory processes of all the nitrogen metabolic enzymes. Instead, the regulation and role of the enzymes NR and GS will be dealt with as these are two important nitrogen metabolic enzymes.

1.4 REGULATION OF NITROGEN METABOLISM WITH FOCUS ON NR AND GS

Regulatory responses require plants and algae to detect changes in the external or internal environment. The addition of nitrogen results in early/ primary responses, where changes occur as rapid or transient increases in protein synthesis that are independent of the expression of some nitrogen assimilation genes (Redinbaugh and Campbell, 1993). The response for a particular limiting nutrient is termed a 'specific response', whereas the response elicited by a number of different conditions is known as a 'general response' (Grossman, 2000). Nutrient specific responses regulate the biosynthesis of transport systems and induce changes in cellular organisation. General responses to nutrient stress, conserved among all organisms, include the cessation of cell division, the accumulation of starch or glycogen, decline in the rate of photosynthesis, modification of photosynthetic activities and modulation of metabolic processes to a reduced nutrient environment. These responses are highly regulated and when a specific deficiency is detected, modifications in metabolism are made. In

addition, although light and temperature are not specifically related to nutrient stress, their effects also integrate in acclimation processes. Mechanisms surrounding nitrate sensing and signal transduction for the expression of nitrate related genes are not well understood in plants, and even less is known in macroalgae (Vidal and Gutiérrez, 2008). However, it is known that external stimuli or stresses and nutritional status of the plant or alga, are able to modulate expression and/or activity of nitrogen transport and nitrogen metabolic enzymes by various mechanisms (Masclaux-Daubresse *et al.*, 2010). These mechanisms include transcriptional and post-translational mechanisms.

Transcriptional regulation of GS and NR is mediated by different transcription factors that can be organism specific. MYB transcription factors found in vertebrates have been identified in plants and are responsible for regulating the nitrate catabolic repression of nitrogen assimilation genes in *Arabidopsis*, as well as being a central nitrogen assimilation regulator in the unicellular red alga, *Cyanidioschyzon merolae* (Martin and Paz-Ares, 1997; Imamura *et al.*, 2010). Two more well characterised nitrogen regulation systems are the GATA family of transcription factors (include Nit), found in yeast and some fungi, and the Ntr system occurring in enteric and proteobacteria (Herrero *et al.*, 2001). Nit transcription factors responsible for nitrogen regulation in fungi have also been characterised in green algae (Marzluf, 1997; Bruhn *et al.*, 2010). In particular, NIT2 controls the expression of *NIA1* (NR) and *Nii1* (NiR) in the unicellular green alga *Chlamydomonas reinhardtii*, and NR and GS of the marine coccolithophorid *Emiliana huxleyi* (Bruhn *et al.*, 2010). In general, the model for the regulation of plant gene expression in response to nitrogen has been likened to the Ntr system in *Escherichia coli* (Lindell *et al.*, 2002). The Ntr system is responsible for the control of GS activity at the transcriptional and post-transcriptional levels. Furthermore, the signal transducing protein PII, responsible for regulation of the Ntr system, was isolated in *Arabidopsis* and the red macroalga, *Porphyra purpurea*. (Hsieh *et al.*, 1998). In cyanobacteria, the transcription of nitrogen-regulated genes is under the control of the DNA-binding protein NtcA (Capone *et al.*, 2008). NtcA belongs to the CAP (catabolite gene activator/cyclic AMP [cAMP]) family of proteins and differs from Ntr (Herrero *et al.*, 2001). In *Synechococcus* species strain PCC 7942, NtcA controls the transcription of the *nir* operon that encodes NR and NiR, the *nirBntcB* gene cluster that encodes the protein required for expression of NiR and the *glnA* gene that encodes for GS (Lee *et al.*, 1999).

Evaluation of the plastid genome of three red algae, the microalga *Cyandium caldarium* and the macroalgae *P. purpurea* and *Porphyra yezoensis*, indicated that a NtcA encoding gene was present.

Post-translational mechanisms also play an important role in regulating enzyme activity. These regulatory mechanisms are triggered by changes in light, dark, nitrate, ammonium, circadian rhythms, iron and carbon, anoxia, pH and other metabolites (Cookson *et al.*, 2005; Chow and de Oliveira, 2008). Post-translational modifications include protein degradation, redox and allosteric modulation of the NR protein (Parker and Armbrust, 2005). Post-translational control of plant NR activity has also been attributed to interaction with 14-3-3 proteins. 14-3-3 proteins belong to a family of highly conserved proteins known to play a central regulatory role in plant, fungal and mammalian cells (MacKintosh and Meek, 2001). The proposed mechanism of regulation involves phosphorylation of the NR protein by an NR kinase that allows binding of the 14-3-3 protein resulting in suppression of enzyme activity (Huber *et al.*, 1992; MacKintosh and Meek., 2001; Pozuelo *et al.*, 2001). Post-translational regulation of GS by interaction with 14-3-3 proteins has also been observed (Finnemann and Schjoerring, 2000). Similar to NR, phosphorylation of GS modifies the enzyme allowing it to interact with 14-3-3 proteins (Riedel *et al.*, 2001). However, in contrast to NR, this interaction preserved GS₂ activity of tobacco. In bacteria, post-translational regulation of GS is mediated by cumulative feedback inhibition, covalent modification, repression/derepression and metal catalysed oxidation (MCO) (Humanes *et al.*, 1995). MCO, a two step mechanism whereby the oxidation of GS causes it to become a target for enzyme degradation by proteases, has been shown to regulate GS in the unicellular green alga *Chlorobion braunii* formerly known as *Monoraphidium braunii* (Humanes *et al.*, 1995; Gómez-Baena *et al.*, 2006). In addition, the photosynthetic electron transport system has also been shown to play a role in oxidative degradation of chloroplastic GS (Palatnik *et al.*, 1999).

The mechanisms regulating nitrogen metabolic enzymes, particularly NR and GS, outlined in this review represent a brief summary of how cells modulate enzyme activity. It is beyond the scope of this review to highlight all cellular mechanisms and triggers responsible for the regulation of nitrogen metabolism. However, the numerous

transcription factors and mechanisms for post-translational modifications further illustrate the complexity of the regulation of nitrogen metabolism in all organisms. However, it is encouraging to find that similar regulatory pathways and proteins exist between plants, algae, cyanobacteria and bacteria as it gives insight into how *G. gracilis* may respond to and regulate nitrogen metabolism. In addition, these regulatory mechanisms emphasise that numerous metabolic pathways interact with one another to regulate nitrogen metabolism.

1.5 INTERACTION OF NITROGEN METABOLISM WITH OTHER METABOLIC PATHWAYS

Nitrogen metabolism is not an isolated process and nitrogen is responsible for triggering numerous metabolic responses. For example, nitrate induces the genes required for nitrate uptake and assimilation, glycolysis, oxidative pentose phosphate (OPP) pathway and organic acid synthesis, and represses phenylpropanoid metabolism (Tschoep *et al.*, 2009). Plants regulate nitrogen assimilation with regard to the availability of nitrogenous reduced compounds and metabolic carbon allocation (Llamas *et al.*, 2002). In plant and algal cells, carbon and nitrogen metabolism are intricately linked as nitrogen metabolism requires the energy, reducing power and carbon skeletons produced in carbon metabolism (Maurin and Gal, 1997; Zozaya-Hinchliffe *et al.*, 2005). The GS-GOGAT pathway represents the link between carbon and nitrogen metabolism as GS uses ATP and GOGAT requires carbon skeletons and reductant in the form of 2-oxoglutarate and reduced ferredoxin or NADH (Lancien *et al.*, 2000; Gómez-Baena *et al.*, 2006). In addition, GS isoenzymes are modulated by hexose dependant and possible hexose independent pathways (Oliveira and Coruzzi, 1999). Carbon supplied as sucrose positively affected GS gene expression which resulted in increased GS activity in *Arabidopsis*. Furthermore, when sucrose was supplied to dark adapted tobacco plants, the expression of NR and NiR was induced. Further evidence for co-regulation and interaction of carbon and nitrogen metabolism is illustrated by a study that suggests that the PII protein co-ordinates carbon and nitrogen metabolism (Lee *et al.*, 1999). A large degree of work has focussed on sugar sensing in plants and little attention has been given to amino acid sensing and signalling (Young *et al.*, 2007). Amino acids pools are important as they reflect the balance between preceding

processes that include nitrate reduction, ammonium assimilation, photosynthetic carbon fixation and subsequent processes that use amino acids, such as protein synthesis (Taylor *et al.*, 2006). Even less attention, if any, has been given to the investigation of sugar and amino acid sensing and signalling pathways in macroalgae. In addition to metabolite sensing, nitrogen assimilation may also be influenced by the TCA cycle and photosynthesis as these pathways generate ATP and reductants (Lee *et al.*, 1999; Young *et al.*, 2007). The interaction of nitrogen metabolism with other metabolic pathways is best illustrated by Figure 4.

The interactions outlined in Figure 4 represent a simplified view of nitrogen metabolism and other metabolic processes. The scheme does not account for the diversity of enzyme isoforms or their impact on nitrogen assimilation and regulation. However, it sufficiently describes the complexity of not just nitrogen metabolism, but the impact of nutrient stress or assimilation on metabolism as a whole. It illustrates how intricately connected different cellular processes are, with the purpose of maintaining homeostasis. It is not possible to review all of the enzymes or regulatory processes in adequate detail, nor is it feasible to simultaneously evaluate the expression and role of all nitrogen metabolic enzymes. Thus for the purposes of this study, it was decided that as NR and GS are two important nitrogen metabolic enzymes, their role in co-ordinating nitrogen metabolism and nitrogen stress responses in *G. gracilis* would be investigated.

1.6 RATIONALE AND AIMS OF THIS STUDY

This review has emphasised that nitrogen is an essential mineral and the critical determinant responsible for limiting plant and algal growth (Jones, 1993; Cookson *et al.*, 2005; Tschoep *et al.*, 2009). However, little is known about nitrogen metabolism in macroalgae (Lobban and Harrison, 1994). This study aims to bridge this gap by investigating the role of NR and GS in the regulation of nitrogen metabolism and nitrogen stress responses in *G. gracilis*. NR catalyses the first step in the utilisation of nitrate in the assimilation pathway (Falcão *et al.*, 2010).

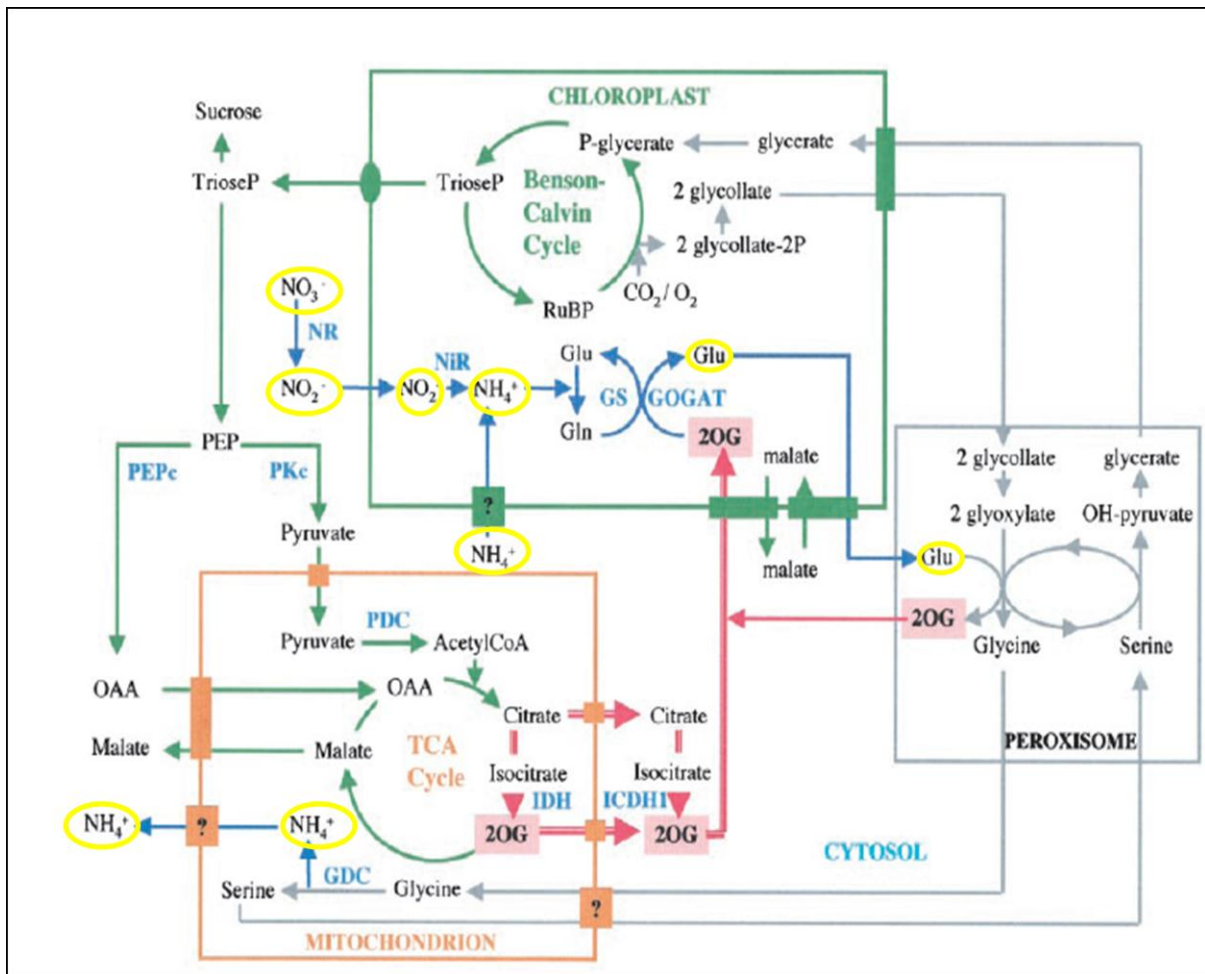


Figure 4. Simplified scheme indicating carbon and nitrogen flow between organelles. The scheme also identifies substrates that may be responsible for NR and GS regulation. Sources of nitrogen such as nitrate, nitrite, ammonium and amino acids (yellow circles) are generated by different metabolic processes within different organelles of the cell. Another important metabolite, 2-oxoglutarate (2-OG), is highlighted in pink. This metabolite indicates how the TCA cycle and reactions in the peroxisome are linked and co-ordinated with nitrogen metabolism. (adapted from Lancien *et al.*, 2000).

Furthermore, NR activity is often a reliable physiological marker that determines when environmental conditions become favourable, due to the response of the enzyme to specific nitrogen substrates or inputs (Allen *et al.*, 2006). Plants are not limited in their ability to take up nitrate, but some crop plants are limited by their ability to incorporate inorganic nitrogen into protein (Migge *et al.*, 2000; Oliveira *et al.*, 2002). Inorganic nitrogen in the form of ammonium arises from numerous metabolic pathways and processes, thereby establishing the central role of GS in nitrogen assimilation (Migge *et al.*, 2000). GS is suggested to be the rate-limiting enzyme during the incorporation of

nitrogen into protein, as it is the first major step in converting nitrogen into an organic form. Thus, NR activity is often viewed as a measurement of nitrate assimilation whereas GS activity is viewed as a measurement of total nitrogen assimilation (Thompson and Valiela, 1999).

It is suggested that macroalgae assimilate nitrogen in a similar manner to that described for phytoplankton. However, regulation of nitrogen assimilation is better understood in bacteria and plants (Maurin and Gal, 1997). Despite differences between bacteria, cyanobacteria, phytoplankton, microalgae and macroalgae, studies confirm that there are common pathways or mechanisms of regulation and therefore, models for comparison are available. Furthermore, studies in macroalgae have focussed on how different stresses affect growth or changes in intracellular nitrogen content over a growing season in the field (periods of months to years). Few studies have examined how inorganic nitrogen acquisition, storage and nitrate reduction respond to environmental conditions by monitoring gene expression (Lartigue and Sherman, 2006). At present, there is a scarcity of information regarding the molecular mechanisms of nitrogen metabolism and regulation in macroalgae, more so, the red algae. Thus, the novelty of this research is the potential information gained by using a molecular approach in a research area where molecular investigations of algal physiology and metabolism are lacking. An understanding of uptake and assimilation processes will aid in the elucidation of the impact of nitrogen stress on algal growth and proliferation (Lobban and Harrison, 1994).

Furthermore, an understanding of nitrogen metabolism and nutrient stress responses has far reaching implications. For example, *G. gracilis* is a photosynthetic organism and thus responsible for primary production in marine environments. Macroalgal primary productivity can exceed that of phytoplankton by two orders of magnitude (Naldi and Wheeler, 1999). Therefore, in addition to being a food source and shelter for marine organisms, algae are invaluable in nutrient cycling in marine environments. Algae are also useful bio-indicators (organisms that infer through changes in their biological systems the conditions in the environment) in marine environments, and can be used to determine factors such as nutrient availability in these environments (Ferrat *et al.*, 2003). Moreover, the potential commercial value of *G. gracilis* mariculture has the

ability to positively affect the South African economy. Therefore, understanding mechanisms that influence survival and orchestrate metabolic changes in this alga are important.

In conclusion, the central objective of this study was to gain an understanding of nitrogen metabolism in *G. gracilis* by focussing on expression and regulation of NR and GS. This would aid the elucidation of nitrogen stress response mechanisms in *G. gracilis*, and ultimately give insight as to how the alga can be re-established and consistently cultivated in Saldanha Bay.

The central objective was addressed by completing the following aims:

- i. Characterisation of the molecular and physiological changes that occur when *G. gracilis* is subjected to long term culture in nitrogen replete and nitrogen lacking conditions.
- ii. Determination of the effect of the re-supply of different nitrogen sources on NR and GS expression to *G. gracilis* cultured in nitrogen deplete media.
- iii. Identification of the occurrence and function of multiple NR and GS isoforms. In addition, the ecological advantage inferred to *G. gracilis* by the occurrence of multiple NR and GS isoforms, was determined.

CHAPTER 2

Identification of *Gracilaria gracilis* nitrate reductase and glutamine synthetase gene sequences.

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2.1 INTRODUCTION

GS is a ubiquitous enzyme and is possibly one of the oldest enzymes in existence (Kumada *et al.*, 1993). The importance of GS has been likened to that of Rubisco (the central enzyme responsible for regulating carbon metabolism) as the synthesis of glutamine is essential for important cellular metabolites such as nucleotides, amino acids and proteins (Unno *et al.*, 2006). Unno *et al.* (2006) stated, “the role of GS is the cornerstone of plant productivity and is therefore responsible for nitrogen nourishment of all animals on earth”. There are three types/classes of paralogous GS gene families that differ in both primary and tertiary structure (Robertson *et al.*, 2001, Bruhn *et al.*, 2010) (Figure 1). In addition, the distribution of these classes has been found to vary between the different domains of life (Ghoshroy *et al.*, 2010). Class I GS (GSI) was originally thought to occur only in prokaryotes, however, GSI forms have subsequently been identified in vascular plants (Robertson *et al.*, 2001). Class II GS (GSII) is typically found in eukaryotes, but has also been identified in soil bacteria. Finally, class III GS (GSIII) was originally only identified in prokaryotes but has since been detected in marine algae. Furthermore, GSs of photosynthetic organisms may be located to the cytosol or chloroplast (Bruhn *et al.*, 2010). For example, plant GSs are subdivided into chloroplastic (GS₂) and cytosolic (GS₁) subtypes, and GS₁ is further categorised into two groups based on the expression profile in response to the external nitrogen status, enzymatic property and physicochemical stability (Unno *et al.*, 2006). Chloroplast and cytosol GSs have also been identified in unicellular green algae (Ghoshroy and Robertson, 2011). In addition, ESTs with homology to GSII have been identified in the red macroalgae *Porphyra yezoensis*, *Porphyra haitanensis*, *Chondrus crispus* and *Gracilaria changii*.

The multiple plant class II GS isoforms are regulated in a developmental manner, have different/specific functions and their expression is tissue and organ specific (Harrison *et al.*, 2003; Bernard *et al.*, 2008). For example, there are three active GS₁ genes (gln- α , gln- β , gln- γ), in addition to a pseudogene gln- ϵ in *Phaseolus vulgaris*, three active genes in *Pisum sativum*, GS1, GS3A and GS3B, and two active (MtGSa and MtGSb) and one pseudogene MtGS_c gene in *Medicago truncatula* (Morey *et al.*, 2002). Plant GS₁ genes are encoded by a homologous multigene family whereas GS₂ is encoded by a single nuclear

gene (Morey *et al.*, 2002; Bernard and Habash, 2009). These genes encode subunits that range in size from 44-45 kDa for GS₂ and 38-40 kDa for GS₁ (Morey *et al.*, 2002; Bernard and Habash, 2009). The subunits assemble to form an oligomeric protein with a mass between 350 to 400 kDa (Miflin and Habash, 2002; Zozaya-Hinchliffe *et al.*, 2005). Early electron microscopy studies suggested that the subunits of class II GSs assembled to form an octameric protein, however, recent studies in plants, yeast and mammals has revealed a decameric arrangement of the subunits (van Rooyen *et al.*, 2011) (Figure 1). Plant GS₂ is a homo-oligomer consisting of eight identical subunits whilst GS₁ can be a homo or hetero-oligomer (Morey *et al.*, 2002; Bernard and Habash, 2009).

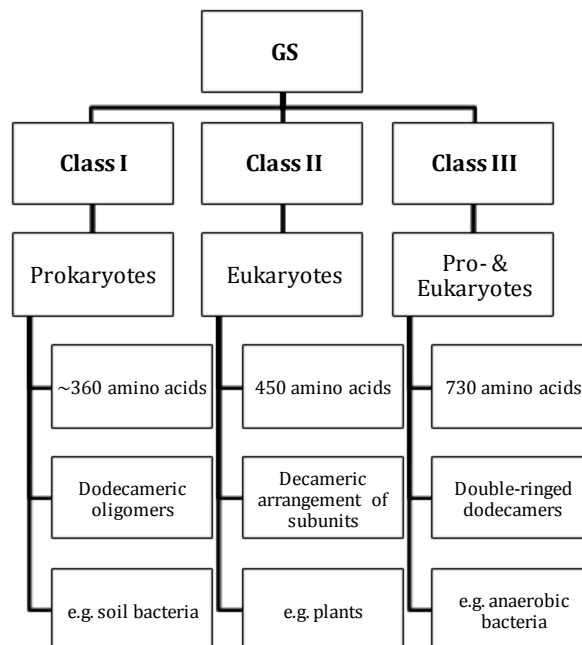


Figure 1. Simple scheme depicting the different GS classes and the organism that each class is typically found in. The three GS classes can be distinguished by the length of each subunit and differences in quaternary structure.

Unlike GS, it is widely accepted that NR is a cytosolic enzyme. However, there is evidence to suggest that the enzyme may be located in the chloroplasts or associated with the plasmalemma (Berges, 1997, Chow 2012). Subcellular localisation of NR in locations other than the cytosol need to be investigated further as less attention has been given to the role and identification of these isoforms. Two structurally different forms of NR exist in prokaryotic and eukaryotic cells; namely assimilatory and

dissimilatory NR (Berges, 1997). Dissimilatory NR is predominantly found in prokaryotes but does feature in eukaryotic metabolism. Focus will be given to assimilatory NR as it is required for nitrate reduction in plants and algae. Eukaryotic assimilatory NR is divided into three groups based on the affinity for NAD(P)H (Berges, 1997). NR isoforms specific for NADH (E.C.1.7.1.1) and NADH/NADPH (E.C.1.7.1.2.) are found in eukaryotic algae and higher plants whilst a third, NADPH (E.C.1.7.1.3.) specific form occurs only in fungi (Chow, 2012). It is commonly accepted that NR is a homodimer and each polypeptide has a molecular weight of approximately 100 kDa. However, NR from several organisms has been shown to vary in molecular weight and number of subunits (Lopes *et al.*, 2002). For example, in the green alga *C. braunii* the native protein has a molecular weight of 500 kDa that consists of eight 58 kDa subunits whereas NR of the red macroalga *Gracilaria tenuistipitata* is composed of four identical 110 kDa subunits. Furthermore, each polypeptide is associated with molybdenum, molybdopterin, iron, heme and flavin and consists of five regions known as the Mo-molybdopterin, dimer interface, NAD(P)H, cytochrome *b* and FAD domains that are able to function independently under certain conditions (Figure 2) (Berges, 1997, Campbell, 1999).

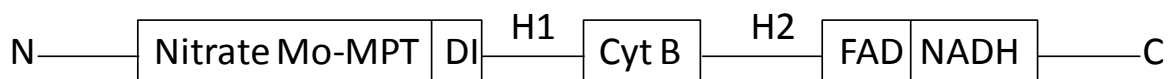


Figure 2. Model of NR enzyme. Five functional domains; Nitrate Mo-Molybdopterin (Mo-MPT) binding domain, Dimer interface (DI), Cytochrome b (Cb), FAD and NADH are outlined. Regions that share no homology to other NR forms are also indicated. These include the N-terminal (N), Hinge 1 (H1) and Hinge 2 (H2). (adapted from Campbell, 1999 and Allen and Ward, 2005)

The existence of multiple enzyme isoforms emphasises the need to identify, and characterise NR and GS gene sequences in *G. gracilis*. For example, sequence information identifies the class and subtype GS belongs to as well as the subcellular localisation of the enzyme. Furthermore, NR and GS are subject to transcriptional regulation. Thus, the identification of NR and GS genes will aid in the investigation of the regulation of these enzymes.

2.2 MATERIALS AND METHODS

2.2.1 Cloning and sequencing of NR and GS

2.2.1.1 RNA isolation

RNA isolations were completed using the peqGold Plant RNA Kit (Optima Scientific). RNA was isolated according to the manufacturer's instructions with the exception that the on column DNase treatment was omitted as tests indicated that it was inefficient at removing genomic DNA (gDNA). Following RNA isolation, RNA was quantified using a Nanodrop (ND-1000) spectrophotometer. DNase treatment followed, using DNase I (Fermentas) at a concentration of 1U DNase I/ μg RNA, according to the manufacturer's instructions. Upon the addition of DNase I, the samples were incubated at 37°C for 30 min. The DNase reaction was stopped by the addition of an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1; v/v/v) (Ausubel *et al.*, 1989). Samples were centrifuged at 4°C for 10 min at 14 000 rpm and the upper aqueous phase was transferred to a fresh microfuge tube. Thereafter, 0.1 volumes of 3 M sodium acetate (Appendix A 3.1) and 5 volumes 100% ethanol were added to the samples. RNA was precipitated by centrifugation at 4°C for 10 min at 14 000 rpm. Precipitated RNA was re-suspended in 30 μl of ultrapure RNase free water and quantified for a final time using the Nanodrop (ND-1000).

The integrity of the isolated RNA was evaluated by electrophoresis on a 1.2% (w/v) agarose formaldehyde mini gel (Sambrook *et al.*, 1989).

2.2.1.2 Conversion of total RNA to complementary DNA (cDNA synthesis)

Total RNA (2 μg) was reverse transcribed in 40 μl reactions using the Impromptu-II Reverse Transcription System (Promega) according to manufacturer's instructions with the following modifications. RNA was incubated with 1 μl Oligo(dT)₁₅ and 1 μl of random hexamers at 72°C for 5 min. A 5 min incubation on ice followed. Thereafter, a second mix containing a final concentration of 1X ImProm-II Reverse Transcriptase buffer, 3 mM MgCl₂, 0.25 mM dNTP, 2.5 U RNase inhibitor (RNasin) and 2 U reverse

transcriptase was added to RNA samples and made to a final volume of 40 µl with nuclease-free water. The reverse transcription reaction mixture was incubated at room temperature for 5 min followed by an overnight incubation at 42°C. The reverse transcriptase was inactivated by incubating the mixture at 70°C for 15 min.

2.2.1.3 Amplification of *G. gracilis* NR and GS

Numerous attempts were made to amplify the NR gene, using published degenerate primers, with limited success. However, the NR gene sequence of *G. tenuistipitata* (Falcão *et al.*, 2010) proved to be invaluable in amplifying the gene from *G. gracilis*.

Microarray analysis identified clone C234 as being upregulated when *G. gracilis* was cultured under conditions that mimic disease (Ealand, 2011). Subsequent sequencing indicated that clone C234 was likely to be GS.

2.2.1.3.1 Primer design

2.2.1.3.1.1 NR primer design

NR protein sequences were obtained from the National Centre for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov) (Table 1) and used for multiple sequence alignments using ClustalX, version 1.81 (Thompson *et al.*, 1997). Sequence alignments were required for the programme CODEHOP (Rose *et al.*, 2003) that was used to design the degenerate primers, NRF_code_1 and NR degen 2R (Table 2).

2.2.1.3.1.2 GS primer design

5' RACE was performed to obtain the full length GS gene sequence. A race primer (GS_race1) was designed according to manufacturer's specifications (Table 2). Two additional primers, GSF2 and GSR2, were also designed (Table 2). These primers were used to screen positive transformants to confirm whether RACE was successful in amplifying the target gene, before proceeding to sequencing.

Table 1. Origin and accession numbers of NR protein sequences used to design degenerate primers and for phylogenetic tree analysis.

Organism	Accession number
<i>Chlamydomonas reinhardtii</i>	AAF17595
<i>Volvox carteri</i>	CAA45497
<i>Chlorella vulgaris</i>	AAC49460
<i>Dunaliella tertiolecta</i>	AAL79356
<i>Phaeodactylum tricornutum</i>	AAV66996
<i>Cylindrotheca fusiformis</i>	AAV59538
<i>Cyanidioschyzon merolae</i>	CMG019C
<i>Gracilaria tenuistipitata</i>	ACX31
<i>Gracilaria gracilis</i>	This study
<i>Zea mays</i>	P49102
<i>Oryza sativa</i>	CAA33817.2
<i>Glycine max</i>	AAD19790
<i>Nicotiana tabacum</i>	CAA32216
<i>Arabidopsis thaliana</i>	NP177899
<i>Neurospora crassa</i>	P08619
<i>Aspergillus nidulans</i>	XP658610

Table 2. Primers designed to amplify *G. gracilis* NR and GS genes.

Primer	Primer Sequence (5'-3')	Annealing temperature (°C)	Amino acid and position in <i>G. tenuistipitata</i> NR and <i>G. gracilis</i> GS
NRF_code_1	GGTGAAGAAGACCATCGGCTTYAAYTGGGG	66	¹⁹¹ MVKQTIGFSWGC ₂₀₂
NRdegen2R	STCCGGCTTGWACCACCASSC	63	³³³ GWWYKPE ₃₄₉
GS_race1	ACGGCCGACGCTCCACGATCAGCGACG	67	³⁰³ VADRGASAV ₃₁₁
GSF2	CAATGGGAGTTCCAGATCGG	64	¹⁹¹ QWEFQIG ₁₉₇
GSR2	CTACGGCCGACGCTCCACG	62	³⁰⁶ RGASV ₃₁₁

Unless stated, the primers were designed as part of this study.

Redundancy code: Y = C/T, S = G/C and W = A/T

Numbers flanking amino acid sequences represent their position in the protein sequence.

2.2.1.3.2 PCR

2.2.1.3.2.1 PCR amplification of NR gene

cDNA was synthesised (2.2.1.2) using RNA isolated from *G. gracilis* cultured with nitrate as the sole nitrogen source. Following cDNA synthesis, the NR gene was amplified in a Bioer XP thermal cycler (Separation Scientific) according to the following parameters: denaturation at 95°C for 3 min, followed by 35 cycles at 95°C for 30 s, 58°C for 30s, 72°C for 1 min and a final extension step of 7 min at 72°C. PCR reactions contained 1 µl undiluted cDNA, 2 µl 10 X Taq polymerase buffer, 3 mM MgCl₂, 0.2 mM dNTPs, 0.2 µM of each primer and 0.5 U Supertherm Taq polymerase (Southern Cross Biotechnology) made to a final volume of 20 µl with nuclease free water.

2.2.1.3.2.2 cDNA synthesis and amplification of GS

cDNA for 5'-RACE was synthesised using the Clontech Smart™ RACE kit (Takara Bio) according to the manufacturer's instructions. Amplification of the 5'-end of the GS gene was performed according to the manufacturer's instructions. The 50 µl reaction mixture contained 2.5 µl of diluted 5'-RACE-Ready cDNA, 5 µl universal primer mix, 1 µl gene specific race primer (GS_race_1), 5 µl 10X Advantage 2 PCR Buffer, 1 µl dNTP mix (10 mM) and 1 µl 50X Advantage 2 Polymerase Mix. Reactions were performed using the XP thermocycler (Bioer) with the following cycling conditions: 5 cycles at 94°C for 30 s, 72°C for 3 min, then 5 cycles at 94°C for 30 s, 70°C for 30 s, 72°C for 3 min followed by 25 cycles at 94°C for 30 s, 68°C for 30 s and 72°C for 3 min.

2.2.1.4 Cloning and transformation

NR and GS PCR products were electrophoresed on a 1.2% (w/v) agarose gel in 1X Tris Acetate EDTA (TAE) buffer (Sambrook *et al.*, 1989). Amplified products were visualised using a long wavelength UV light box (Biorad GelDoc EQ-system™, Biorad). The correct band size was excised and DNA extracted using the Biospin Gel Extraction kit (Bioer Technology Co., LTD) according to the manufacturer's instructions. The purified products were used in cloning reactions.

The Fermentas InsTAclone™ PCR cloning Kit (Fermentas) was used to clone NR and GS PCR amplicons according to the manufacturer's instructions. After the PCR products were ligated into the pTZ57R/T vector, 10 µl of the ligation was used to transform 50 µl *Escherichia coli* DH5α competent cells using the heat shock method (Sambrook *et al.*, 1989). Thereafter, transformed *E. coli* cells were inoculated onto Luria agar (LA) (Appendix A 1.1) medium supplemented with IPTG (0.5 mM), 0.2 % X-gal (w/v) and 100 µg/ml ampicillin (Amp). Positive transformants (white colonies) were selected and inoculated onto LA–Amp medium. Plasmid DNA was isolated from a 5 mL culture using the Qiagen Plasmid Mini Kit (Whitehead Scientific) according to the manufacturer's instructions. M13 forward and reverse primers were used to confirm the presence of the insert. In the case of GS, GS specific primers GSF2 and GSR2 were used to confirm that the cloned product was indeed the GS gene. PCR was completed according to the described (2.2.1.3.2.1) cycling conditions with the exception that 1 µl of plasmid DNA was used as the template and annealing temperatures were 55 and 62°C for M13 (F/R) and GSF2/R2 primer sets, respectively.

Plasmids containing the correct size insert, 443 and 1000 bp for NR and GS, respectively, were sequenced. Sequencing was performed by Macrogen (Korea) using M13 forward and reverse primers. Sequences were edited and analysed using the software Chromas version 2.1 (Technelysium PTY, Ltd).

2.2.2 Bioinformatic analysis

Sequence identities were determined using the BLASTx algorithm available on the NCBI website (www.ncbi.nlm.nih.gov/blast). Conserved domains and open reading frames were determined using domain finder and ORF-finder features on the NCBI website (www.ncbi.nlm.gov), respectively. Sequences were aligned using the DNAMAN (version 4.1.21; Lynnon Biosoft) multiple sequence alignment algorithm. Protein sequences were downloaded from the global NCBI database and were used to construct neighbour joining evolutionary trees for the NR (Table 1), GSI and GSII genes (Table 3), and GS using the MEGA software (MEGA version 5) (Tamura *et al.*, 2011).

Table 3. Nomenclature and accession numbers of GS protein sequences used for phylogenetic analysis of the GSI and GSII genes.

Protein	Organism	Accession number
MEDTR-Ia	<i>Medicago truncatula</i>	AJ238212
RHIME-3	<i>Sinorhizobium meliloti</i>	P38094
RHILP-3	<i>Sinorhizobium leguminosarum</i> <i>by phaseoli</i>	AAC62223.1
PYRFU	<i>Pyrococcus furiosus</i>	Q05907
METVO	<i>Methanococcus voltae</i>	P21154.1
HALVO	<i>Haloferax volcanii</i>	P43386.2
BACSU	<i>Bacillus subtilis</i>	P12425.3
STRVR-I	<i>Streptomyces</i> <i>viridochromogenes</i>	Q05542.1
ANASP	<i>Anabaena PCC7120</i>	P00964.1
RHIME-I	<i>Sinorhizobium meliloti</i>	Q59747
AZOVI	<i>Azobacter vinelandii</i>	P22248.1
SALTY	<i>Salmonella typhimurium</i>	POA1P6.2
RHIME-II	<i>Sinorhizobium meliloti</i>	P45626.1
BRAJA-II	<i>Bradyrhizobium japonicum</i>	P04772.2
RHILP-II	<i>Rhizobium leguminosarum</i> <i>by</i> <i>phaseloi</i>	Q02154.3
FRAAL-II	<i>Frankia alni</i>	P20478.2
STRVR-II	<i>Streptomyces</i> <i>viridochromogenes</i>	P19432.1
DROME-2	<i>Drosophila melanogaster</i>	P20478.3
XENLA	<i>Xenopus laevis</i>	P51121
HUMAN	<i>Homo sapiens</i>	P15104.4
YEAST	<i>Saccharomyces cerevisiae</i>	P32288
CHLRE-1	<i>Chlamydomonas reinhardtii</i>	Q42688.1
MEDTR-IIa	<i>Medicago truncatula</i>	Y10267.1
ARAB	<i>Arabidopsis thaliana</i>	AED93955.1
MAIZE	<i>Zea Mays</i>	BAA03433.1
PEA	<i>Pisum sativum</i>	AAB03493.1

2.3 RESULTS AND DISCUSSION

2.3.1 PCR amplification and sequence analysis of *G. gracilis* NR

Despite several attempts to amplify the NR gene, only a partial fragment was obtained. Degenerate primers were designed by aligning full length NR protein sequences of plant, green algal and the unicellular red alga, *C. merolae*. However, inclusion of the *G. tenuistipitata* NR to the alignment indicated that a region spanning the dimer interface region, which is conserved in these other organisms, is not conserved in the red macroalga. Primers were originally designed to a conserved methionine-rich region located in the dimer interface, indicating why PCR amplification was unsuccessful. Another difficulty was finding a region of conservation that favoured primers with low degeneracy. To overcome this problem, numerous primers were designed and their “suitability” was determined using the BLASTn tool to search the NCBI GenBank database. Primers that were most similar to NR and possessed low E-values were selected. PCR amplification using the degenerate primers NRF_code_1 and NR degen2R yielded a 443-bp fragment from *G. gracilis* cDNA (Figure 3). The amplicon was sequenced and identified as NR based on its similarity to other NR sequences in the NCBI GenBank (Table 4). The *G.gracilis* NR sequence was most similar (homology 90%) to NR of the red macroalga, *G.tenuistipitata*. Hereafter, the *G. gracilis* NR gene sequence will be referred to as *GgNR*.

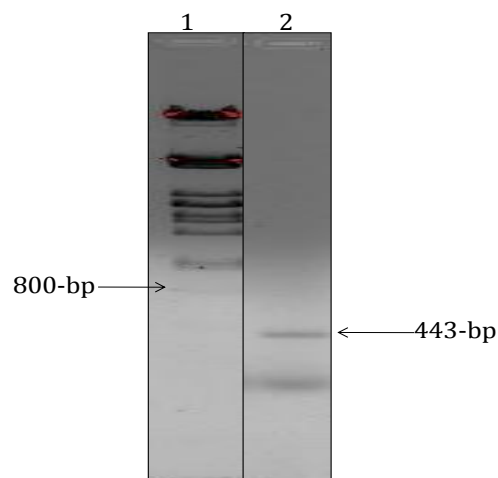


Figure 3. PCR amplification of the partial *GgNR* gene in *G. gracilis*. Lane 1: Lamda *Pst*I molecular weight marker, Lane 2: 443-bp NR gene fragment amplified from cDNA using NRF_code_1 and NR degen2R primers.

Table 4. Sequences identified by BLASTx that displayed significant homology to *GgNR*.

Species	Accession number	% Sequence similarity to <i>G. gracilis</i> NR	% Overlap	E value
<i>Gracilaria tenuistipitata</i>	ACX31652.1	90	99	4e-95
<i>Chlamydomonas reinhardtii</i>	XP 001696697.1	58	99	1e-52
<i>Volvox carteri</i>	XP 002955156.1	57	99	2e-51
<i>Chlorella vulgaris</i>	ABJ91208.4	54		3e-51
<i>Brassica rapa</i>	AAY86186.1	55	99	2e-50
<i>Nicotiana attenuata</i>	AAS19200.1	55		2e-49
<i>Heterosigma akashiwo</i>	ACS44801.1	52	99	2e-49
<i>Solanum tuberosum</i>	BAB93534.1	57	99	3e-49
<i>Beta vulgaris</i>	ABW05098.1	55	99	2e-48
<i>Cucumis sativus</i>	ADK77877.1	55	99	3e-48

% Overlap refers to the percentage overlap between *G. gracilis* NR and NR of species listed in the table.

An alignment of the partial *G. gracilis* NR protein sequence with full length NR protein sequences of plants and algae, indicated that the molybdenum domain and the start of the dimer interface had been amplified (Figure 4). This observation was further validated using the conserved domains function in NCBI, which indicated that both the partial *GgNR* DNA and *in silico* determined protein sequence shared homology to the sulfite oxidase (SO) superfamily molybdopterin (Moco) binding domain (Figure 5). The Moco binding domain is a characteristic of a variety of oxireductases that include NR and SO (Campbell, 1999). The molybdopterin cofactor (Moco) binding domain of *G. gracilis* NR possessed 7 of the 12 conserved amino acid residues found in the Mo-MPT domain. The amino acids His294 and Lys312 are responsible for MPT binding while Gly308 functions to bind the molybdenum (Mo) metal ion in *Arabidopsis* NR.

Finally, alignment of *GgNR* with the full length *G. tenuistipitata* DNA sequence indicated that approximately 530-bp and 1724-bp of sequence must be amplified at the 5' and 3' ends, respectively, in order to obtain the full length *GgNR* coding sequence. The full

length sequence would allow the identification of possible regulatory sites as well as other important amino acids. However, despite the limitation of the truncated sequence, there is sufficient sequence information to allow basic transcriptional studies to be conducted using real time polymerase chain reaction (qPCR).

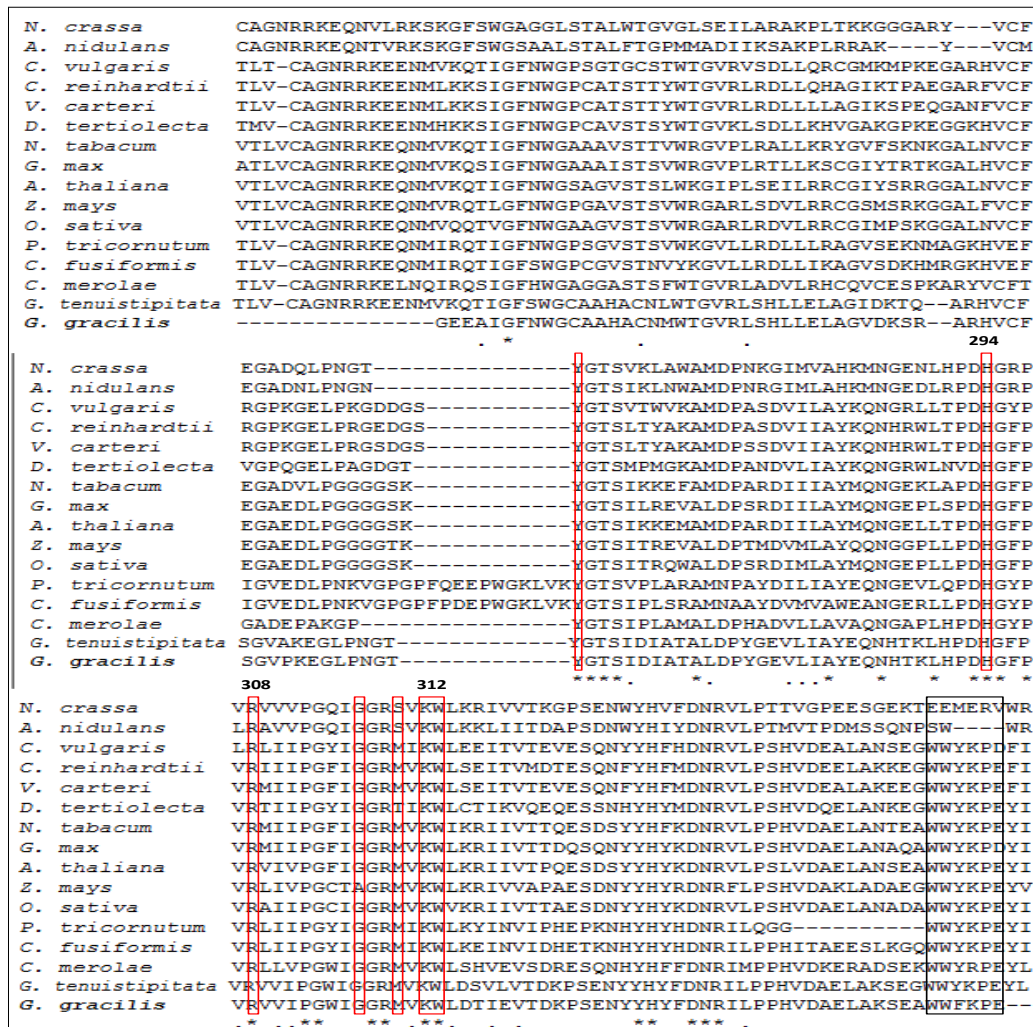


Figure 4. Protein sequence alignment of *G. gracilis* NR with other NR protein sequences from vascular plants (*Glycine max*, *Arabidopsis thaliana*, *Nicotiana tabacum*, *Zea mays* and *Oryza sativa*), green algae (*Chlorella vulgaris*, *Chlamydomonas reinhardtii*, *Volvox carteri* and *Dunaliella tertiolecta*), red algae (*Gracilaria tenuistipitata* and *C. merolae*), fungi (*Aspergillus niger* and *Neurospora crassa*) and diatoms (*Phaeodactylum tricornutum* and *Cylindrotheca fusiformis*). Alignments were completed in DNAMAN. Amino acid residues conserved in molybdopterin binding domain (Mo-MPT) are highlighted by red box, while the first six amino acids that are homologous to the Dimer Interface Region (DIR) are highlighted by the black box. (*) Indicates positions which have a fully conserved residue and (.) indicates greater than 75% homology. Numbers indicate amino acid positions of *NIA2* (GenBank Accession No. J03240) in *A. thaliana*.

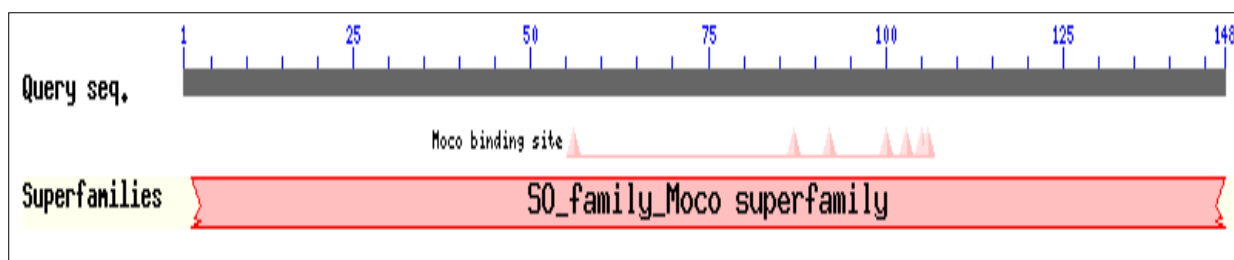


Figure 5. The conserved domain search tool in NCBI indicated that the deduced *G. gracilis* NR protein sequence (query) shared homology with the SO superfamily. Triangles represent 7 of the 12 residues conserved in the Mo-MPT domain.

2.3.2 PCR amplification and sequence analysis of *G. gracilis* GS

An alignment of the partial GS C234 sequence, to full length GS₁ and GS₂ gene sequences, indicated that the 5' region of the sequence remained to be determined. A review of GS sequences indicated that the coding sequence of GS₁ and GS₂ isoforms differ at the 5' and 3' end, however, most variation occurs at the 5' end (Parry *et al.*, 2000). In addition, different GS subtypes can be distinguished by examining the 5'-end as it contains the chloroplast signalling peptide.

Total RNA was isolated from *G. gracilis* and subsequently converted to cDNA. This cDNA served as the template for 5'RACE which yielded a 1000-bp fragment (Figure 6) that was identified as GS via PCR screens of positive transformants with the primers GSF2 and GSR2 (data not shown). Following sequencing, the 1000-bp fragment was assembled with the partial C234 sequence using DNAMAN (Lynnsoft) and yielded an approximately 1300-bp fragment. This fragment was confirmed to be GS based on its similarity with other GS sequences in the NCBI Genbank database (Table 5). *G. gracilis* GS was most similar (homology 76%) to the GS sequence of the red macroalgae *Gelidium crinale*. A blast search, using the conserved domain tool, indicated that the 1300-bp fragment represented a full length GS gene that showed homology to the glutamine synthetase beta-grasp domain (Gln-synt_N superfamily) and the glutamine synthetase catalytic domain (Gln-synt_C superfamily) (Figure 7).

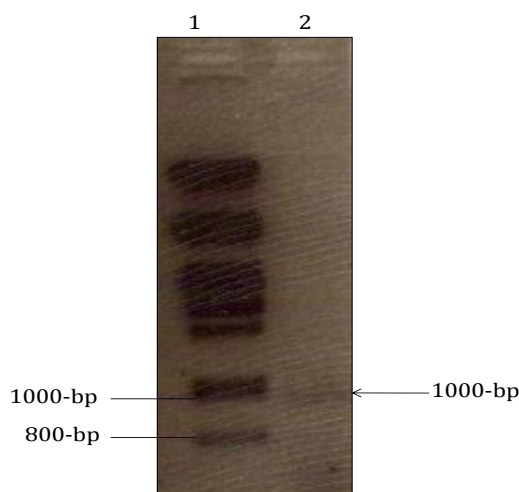


Figure 6. PCR amplification of the partial *G. gracilis* GS gene. Lane 1: Lamda *Pst*I molecular weight marker, Lane 2: 1000-bp GS gene fragment amplified from 5'-RACE ready cDNA.

Table 5. Sequences with significant homology to *G. gracilis* GS following BLASTx analysis

Species	Accession number	% Sequence similarity to <i>G.</i> <i>gracilis</i> GS	% Overlap	E value
<i>Gelidium crinale</i>	AAK60408.1	76	75	9e-123
<i>Dixoniella grisea</i>	ADX97445.1	65	72	3e-101
<i>Selaginella moellendorffii</i>	XP 002977477.1	57	76	2e-88
<i>Stevia rebaudiana</i>	ABF06665.1	60	73	4e-88
<i>Avicennia marina</i>	AAK08103.1	58	78	4e-88
<i>Populus trichocarpa</i>	XP 002312733.1	56	83	7e-88
<i>Raphanus sativus</i>	BAA04996.1	59	76	2e-87
<i>Arabidopsis thaliana</i>	NP 198576.1	57	76	2e-87
<i>Glycine Max</i>	AAG24873.1	60	76	3e-87
<i>Hevea brasiliensis</i>	AAB61597.1	57	76	4e-87

% Overlap refers to the percentage overlap between *G. gracilis* GS and the GS of species listed in the table.

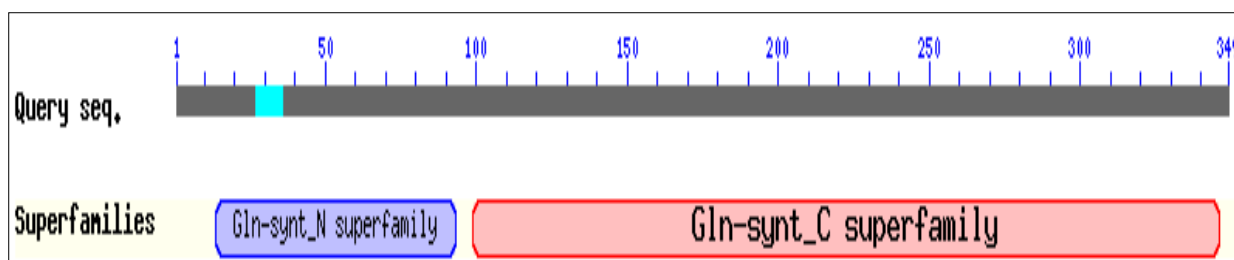


Figure 7. Conserved domain search tool in NCBI detected the beta-grasp (Gln-synt_N) and catalytic (Gln-synt_C) domains in the deduced *G. gracilis* GS protein sequence (query).

Analysis of the 1300-bp GS DNA sequence indicated that it contained an open reading frame (ORF) of 1050-bp that was terminated with an in-frame TGA stop codon (Figure 8). The ORF encodes a polypeptide with a predicted molecular weight of approximately 38 kDa which is in agreement with the molecular weights of higher plant cytosolic GS isoforms (Mathis *et al.*, 2000). Analysis of the complete *G. gracilis* GS protein sequence using the Signal P 4.0 Server (Petersen *et al.*, 2011) indicated that it lacked a chloroplastic transit peptide sequence. Instead, a GS₁ signature sequence was detected (Figure 8) (Zhang *et al.*, 2006). These findings indicate that the amplified fragment is likely to be a cytosolic GS and will be referred to as *GgGS₁*.

An alignment of *GgGS₁* with different plant GS isoforms was completed to identify amino acids that may be involved in substrate affinity and stability of the enzyme (Figure 9). The residues Asp 52, Cys 87 and His 242, are thought to play a role in transferase activity, thermal stability and glutamate binding at the active site of plant GS, respectively (Bernard *et al.*, 2008). Since these residues were conserved in *G. gracilis*, it is likely that they may have similar functions in the alga. The *Arabidopsis* GS gene has a Ser residue at position 171 that was shown to be a feature of high substrate affinity GS₁ isoforms. Furthermore, an Ile at position 154 in the maize GS gene was shown to confer heat stability to the GS protein (Bernard *et al.*, 2008). As *GgGS₁* does not possess serine or isoleucine residues at these amino acid positions, it may be unlikely that this isoform has high substrate affinity or heat stability. However, a more detailed investigation of other algal GS sequences is required in order to confirm this.

AGATTCTAACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGTACGCGGGGCCCC	1
TCACCACCACCTCCACCTCTCGCGCCTCTTCCACACTTTCTTGTCTACCCCGTGCACC	61
CACC ATG TCTGCAATCGACGCCCTTCTCAACCTGCAAGTCTCGGACGGCTGCATGGCCGA	121
M S A I D A L L N L Q V S D G C M A E	
GTACG TCTGGATCGACGGCTTCGGCAAGCTCCGCGAAAGGGCCGTAAGCTCTCCTTTGT	181
Y V W I D G F G K L R G K G R K L S F V	
GCCCT CATCGCCGTCCGATCTGCCGGACTGGA ACTACGACGGCAGCTCAACTGGCCAGGC	241
P S S P S D L P D W N <u>Y D G S S T G Q A</u>	
GCCAGGCGT GATTCCGAGGTCATCATCAAGCCG CAGGCCATCTTCCCTGATCCGTTCCG	301
<u>P G A D S E V I I</u> K P Q A I F P D P F R	
CGGCGCTCCAACGTGCTCGTCATGTGCGACACCTACACCCGGAAGGCGCCCGCTGCC	361
G A P N V L V M C D T Y T P E G A P L P	
CACCAATACTCGCCACGCCTGCGCCCTCGTCATGGAGGAGGCCAAGGAGCTCGTGCCGTG	421
T N T R H A C A L V M E E A K E L V P W	
GTTCCGGCTGGAGCAGGAGTACTTCTCATCAACCCTGCCACTGAGAAGCCTGTGGGCTT	481
F G L E Q E Y F L I N P A T E K P V G F	
CCCCAAGGAAGGGGAACCGCGCCGAGGGGCCGTA CTACTGTGGTGTGTCTGGCTCTAA	541
P K E G E P A P Q G P Y Y C G V S G S K	
GGTGT TTGGTTCGCGATGTGGTTGACGCC ATTTCAAGGCGTGTGTATGCTGGGCTCAA	601
V F G R D V V D A H F K A C V Y A G L K	
GGTATCGGGAATTAACGCCGAGGTGGCGCCGGGCAATGGGAGTTCAGATCGGACCGGC	661
V S G I N A E V A P G Q W E F Q I G P A	
AGTGGGCATCGAGGAAGCGATCACATGTGGATGGCTCGCTACCTGCTGGAGCGAGTGGC	721
V G I E E G D H M W M A R Y L L E R V A	
GGAATTGAAGGGTTGGGACATCAACTACGACCCGAAACCTATCGAGGGTGACTGGAACGG	781
E L K G W D I N Y D P K P I E G D W N G	
CTCTGGCTGCCACGCCA ACTTCTCCACCAAGCCGATGAGAGAGCAGGGGGATATGAGAA	841
S G C H A N F S T K P M R E Q G G Y E K	
ACACATCGTGCCGGCGCTGGAGAAGCTGGCGAAGAAGCACAGGAGCATATTGCCCGTA	901
H I V P A L E K L A K K H K E H I A A Y	
TGGCTCCGGAACGAGAAGAGGTTGACTGGAAAACGACGAGACAGCTTCTATCGAACAGTT	961
G S G N E K R L T G K H E T A S I E Q F	
CAAGTGGGGCGTTCGCTGATCGTGGAGCGTCGGCCGTAGTGGGCAGCGACACTAAAGAGAA	1021
K W G V A D R G A S A V V G S D T K E K	
GGGCTACGGCTACTTTGAGGATCGCCGACCGCCGAAACTGCGACCCGTACGTGGTGCAC	1081
G Y G Y F E D R R P A G N C D P Y V V T	
CAGAATGCTGGTGCAGACGTGCTGTCTGGAGTGA AAGAGCGCGTGGAATGCGAACAA	1141
R M L V Q T C C L E *	
ACCAGTCAAGTTCATAGAGCACAAGTGCAGGAGAAACGGTTGATCGTCCGCACATCATTTT	1201
CGTCGCTTCGTTTTACTAGTACAAAAACAACAGTAAACATGCGCGCCACGCTGCGCGCG	1261
CTGTATCTACAAA	1321

Figure 8. Nucleotide and deduced amino acid sequence of GS from *G. gracilis*. The *GgGS₁* sequence was obtained by aligning the partial fragment obtained from 5' RACE and the GS sequence of clone C234. Nucleotides are numbered positively in the 5' to 3' orientation starting with the first nucleotide in the 5' untranslated region and ending with the 3' polyadenylated tail. The predicted open reading frame is indicated by black uppercase letters. The deduced amino acid sequence is shown below the nucleotide sequence beginning with the first in-frame methionine (M) and ending with the TGA termination codon (*). The GS₁ signature is underlined and the GS ATP binding signature is highlighted in red.

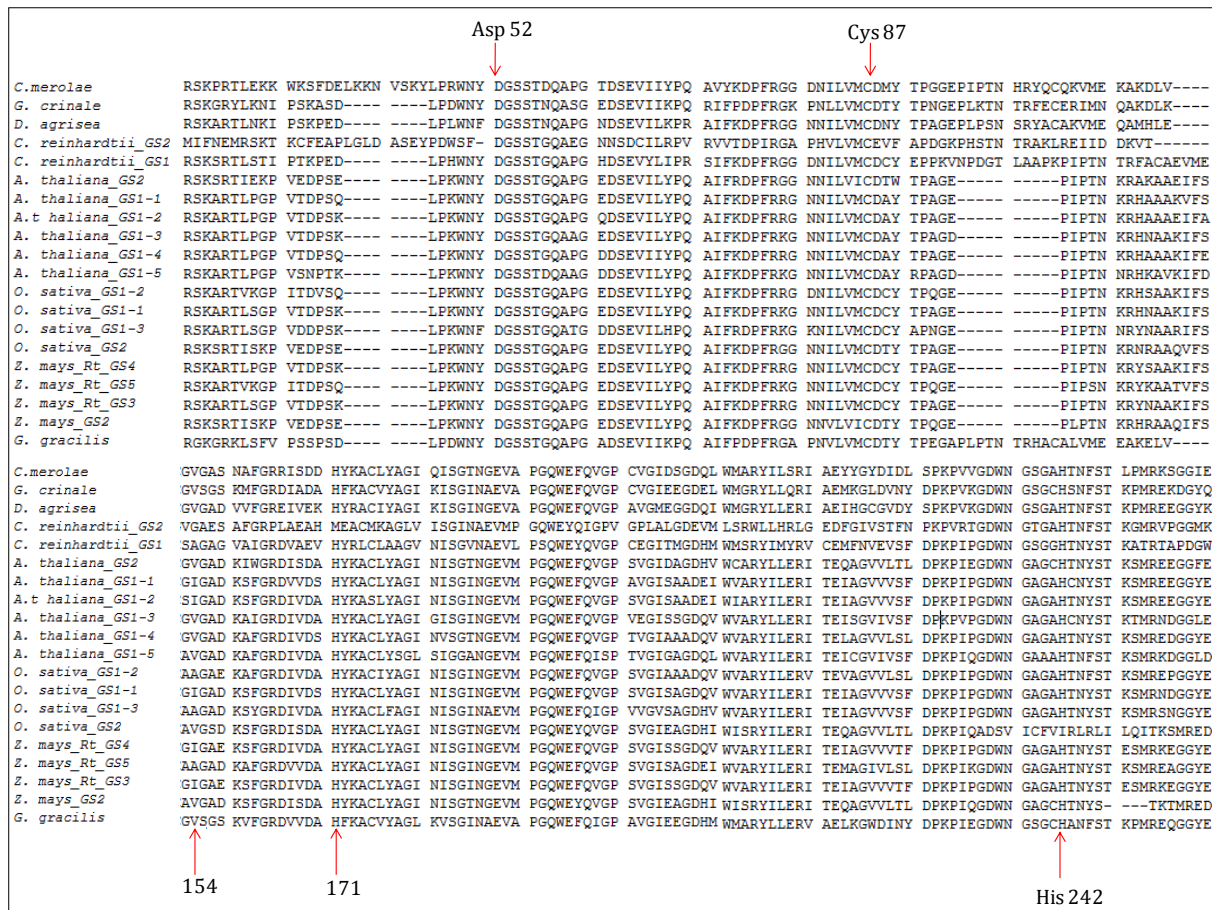


Figure 9. Multiple sequence alignment of *C. merolae* (GS₁), *G. crinale* (GS₁), *D. grisea*, *C. reinhardtii* (GS₂), *C. reinhardtii* (GS₁), *A. thaliana* (GS1-1), *A. thaliana* (GS1-2), *A. thaliana* (GS1-3), *A. thaliana* (GS1-4), *A. thaliana* (GS1-5), *O. sativa* (GS1-2), *O. sativa* (GS1-1), *O. sativa* (GS1-3), *O. sativa* (GS₂), *Z. mays* (Root GS4), *Z. mays* (Root GS5), *Z. mays* (Root GS3), *Z. mays* (GS₂) and *G. gracilis* GS. Important residues are indicated by the red arrows. Residues 154 and 171, are important in determining the substrate affinity of each isoform (Bernard *et al.*, 2008). Asp 52, Cys 87 and His 242 are other important amino acids.

2.3.3 Phylogenetic analysis of GgNR and GgGS₁

2.3.3.1 Phylogenetic analysis of GgNR

The partial amino acid sequence obtained from *in silico* translation of the *GgNR* gene was aligned with full length protein sequences of NR genes from various organisms in the GenBank database, including the red unicellular microalgae *C. merolae* and the red macroalga *G. tenuistipitata*. The alignment was performed using the ClustalW program in MEGA 5 (Tamura *et al.*, 2011). A matrix with 16 sequences and 179 positions was generated from an initial alignment with the full length protein sequences and distance

based neighbour-joining trees were constructed (Figure 10). Bootstrap analysis was carried out based on 1000 re-sampled datasets. Phylogenetic analysis indicated that GgNR was more closely related to other rhodophyte sequences, in particular the red macroalga *G. tenuistipitata*, with strong bootstrap support (100). GgNR was not closely related to green algal, diatom, vascular plant and ascomycete NR sequences. Each of these groups formed distinct monophyletic clades.

These findings are in agreement with other studies that observed that rhodophyte NR did not share close homology with diatoms, green algae and vascular plants (Allen *et al.* 2005). While a short partial protein sequence was used to construct the phylogenetic tree, the results observed in this study confirm those of Falcão *et al.* (2010), who analysed a larger portion (approximately 686 amino acids) of the same set of protein sequences. The authors showed that rhodophyte NR formed a distinct clade that was separate from other taxonomic groups. Although NR is not generally used as a molecular marker for phylogenetic analyses, findings by Falcão *et al.* (2010) and Allen *et al.* (2005) are coherent with the relationship inferred by other valid markers such as rRNA genes. The current study strongly supported these results, as a short sequence of 179 amino acids produced a robust tree, emphasising that although NR is not generally used as a marker for phylogenetic analysis, it could potentially be useful.

2.3.3.1 Phylogenetic analysis of *GgGS₁*

Initially, to confirm that the amplified *GgGS₁* sequence belonged to the Class II group of GS genes, an alignment of the *in silico* determined *GgGS₁*, GSI and GSII full length protein sequences was performed using the ClustalW program in MEGA 5. Neighbour-joining trees indicated that the deduced *GgGS₁* sequence grouped with other known GSII sequences, with strong bootstrap support (100) (Figure 11). The grouping of *GgGS₁* with GSII class proteins further extends the observation that the majority of eukaryotic GSs belong to this class (Robertson *et al.*, 2001).

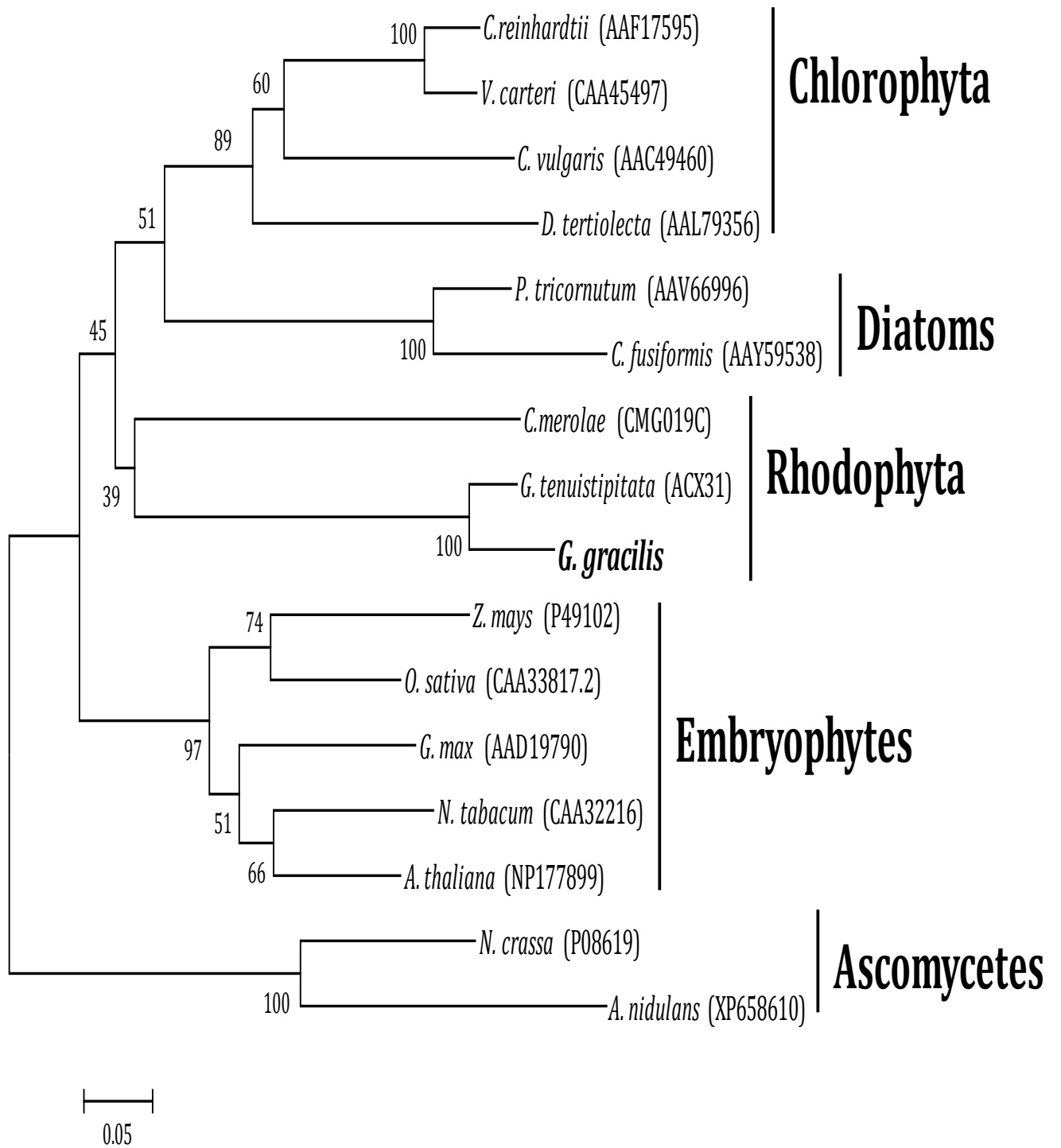


Figure 10. Unrooted phylogenetic tree using NR protein sequences. The tree was based on 179 conserved amino acids and was obtained using the neighbour-joining method. The bootstrap values refer to 1000 re-sampled datasets and values are shown on branches. The bar represents 0.05 amino acid substitutions per amino acid position. All sequences were retrieved from GenBank with the exception of *C. merolae* which was retrieved from <http://www.ddbj.nig.ac.jp/>. Accession numbers are indicated in brackets.

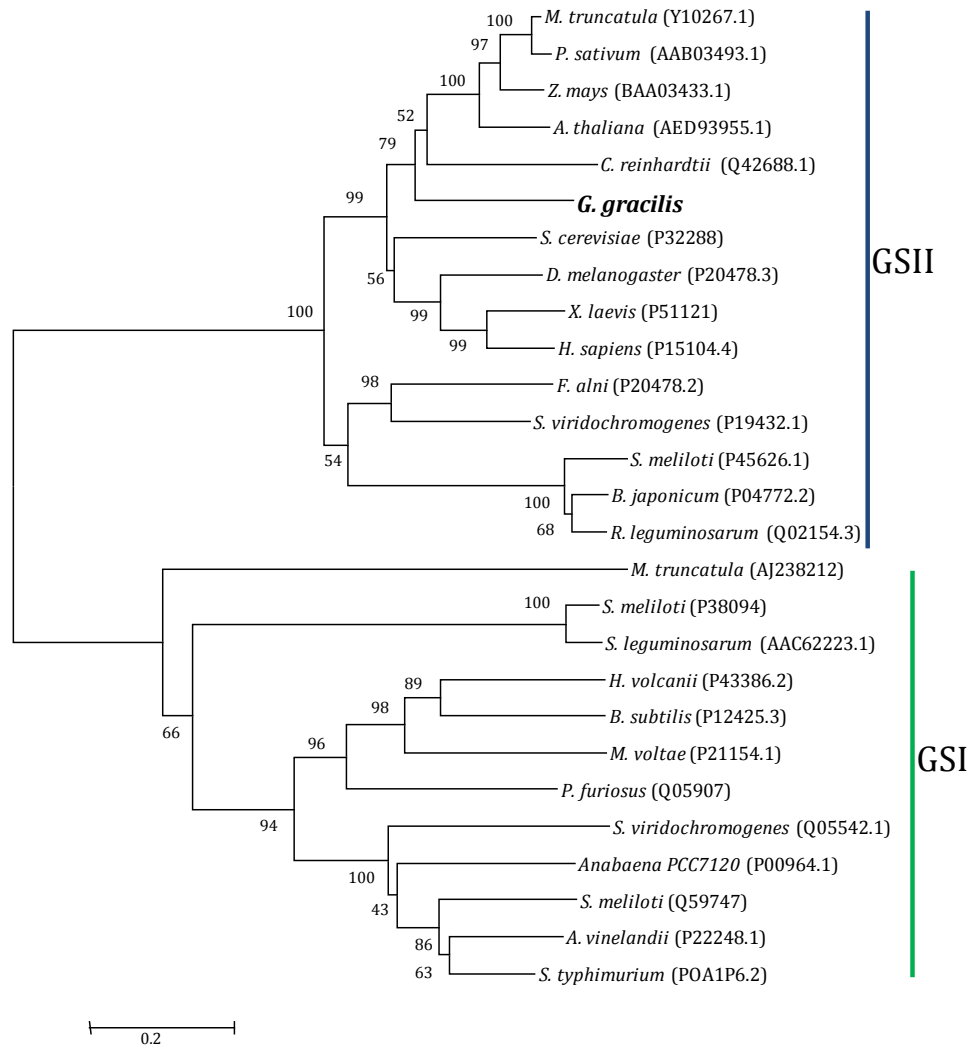


Figure 11. Unrooted phylogenetic tree using full length GSI and GSII protein sequences. The tree was based on 759 conserved amino acids and was obtained using the neighbour-joining method. The bootstrap values refer to 1000 re-sampled datasets and values are shown on branches. Two distinct groups can be seen in the phylogenetic tree, the vertical blue and green lines indicate the class II and class I GS genes, respectively. The bar represents 0.2 amino acid substitutions per amino acid position. All sequences were retrieved from GenBank. Accession numbers are indicated in brackets.

A neighbour-joining tree of GgGS₁ and other GS II protein sequences indicated that plant and red algal sequences formed two separate clades (Figure 12). Within the plant GS clade, chloroplast, cytosol and root isoforms generally tended to group together. A distinct grouping of chloroplast and cytosol GS was not observed for the algal GS isoforms. GgGS₁ was more closely related to other rhodophyte GS sequences that included the unicellular red algae *Dixoniella grisea* and *C. merolae*, with greatest homology to the cytosolic GS sequence of the red macroalga *G. crinale*.

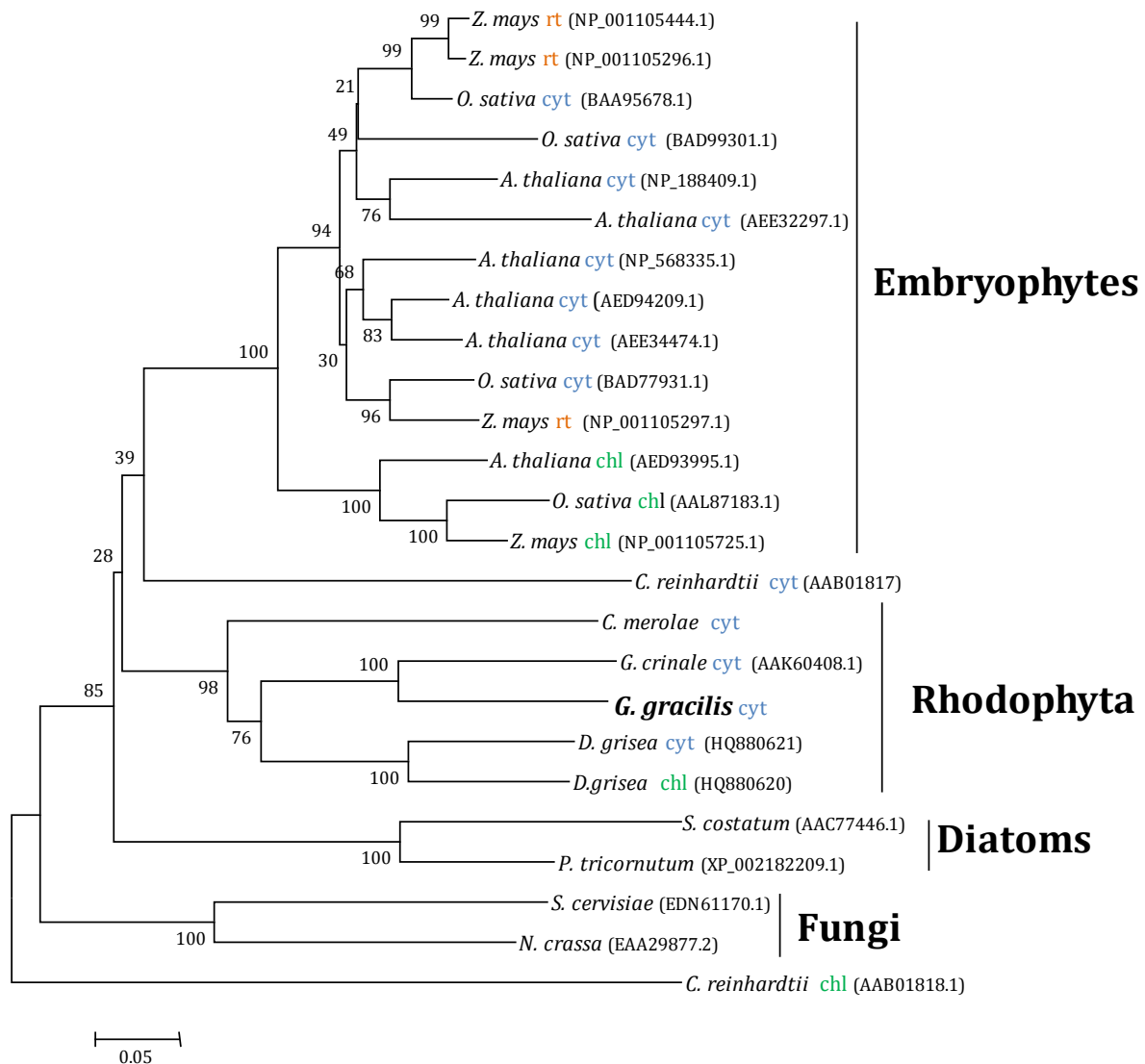


Figure 12. Unrooted phylogenetic tree using full length GSII protein sequences. The tree was based on 398 conserved amino acids and was obtained using the neighbour-joining method. The bootstrap values refer to 1000 re-sampled datasets and values are shown on branches. Chloroplastic (cp), cytosolic (cyt) and root (rt) GS genes are indicated in green, blue and orange, respectively. The bar represents 0.05 amino acid substitutions per amino acid position. All sequences were retrieved from GenBank with the exception of *C. merolae* that was retrieved from <http://www.ddbj.nig.ac.jp/>. Accession numbers are indicated in brackets.

In summary, the NR and GS genes were amplified from *G. gracilis*. Identification of these genes is invaluable to the broader objectives of this study, and sets a firm foundation for the identification of other *G. gracilis* NR and GS isoforms in future studies.

CHAPTER 3

In vitro* activity and daily oscillations of nitrate reductase and glutamine synthetase in *Gracilaria gracilis

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3.1 INTRODUCTION

Enzymes are useful for characterising the metabolic responses of different organisms to various environmental stimuli. Two important enzymes responsible for coordinating nitrogen metabolism are NR and GS (Yuan *et al.*, 2001; Granbom *et al.*, 2004). In the past, studies used non-metabolisable substrates such as radiolabelled chlorate to measure nutrient transport/assimilation. However, uptake kinetics for radiolabelled substrates differed when compared to the metabolically active substrate, and represented nutrient transport and incorporation rates only when the assay conditions resembled those of the natural environment (Lomas, 2004). Measuring the activity of nitrogen metabolic enzymes such as NR and GS overcomes the problems associated with traditional methods that use non-metabolisable substrates (Lomas, 2004). As changes in irradiance, periodic exhaustion of nitrate and the presence of alternative nitrogen sources modulate NR and GS activity, it is likely that changes in enzyme activity may serve as indicators of changing environmental conditions (Berges *et al.*, 1995). Thus, when investigating algal physiological and metabolic changes in response to nutrients, measurement of enzyme activities is invaluable (Berges *et al.*, 1995; Young *et al.* 2007).

Both *in vivo* and *in vitro* assays have been used to measure NR and GS activity (Corza and Neill, 1991; Rees *et al.*, 1995). *In vivo* or *in situ* assays as they are known, measure enzyme activity without extracting protein (Berges, 1997). Instead, algal cells are permeabilised to allow the release of reaction products. Disadvantages of this method are: the thallus of macroalgae such as *G gracilis* may be thick and resistant to permeabilisation; assay conditions are not controlled; and results may be difficult to reproduce as substrates are not present in saturating amounts. In addition, enzyme activities may be affected by diffusion rates of substrates into and out of the cells (Lartigue and Sherman, 2002). The majority of *in situ* NR assays employ *n*-propanol (a chemical responsible for the disruption of membranes and lipids) that may cause bleaching of the tissue, thereby allowing nitrite released into the external environment to be taken back up by the thallus (Lobban and Harrison, 1994). Consequently, *in situ* assays do not always represent a true reflection of *in vivo* conditions. Conversely, *in vitro* assays offer more controlled conditions in that temperature, pH and substrate

concentrations are fixed. *In vitro* assays measure enzyme activities from total protein extracts, sometimes referred to as crude enzyme extracts. Disadvantages associated with this choice of assay method are: post-translational modifications such as phosphorylation state may or may not be preserved upon protein extraction and non-optimised assay conditions can cause instability of the enzyme following extraction (Lomas, 2004).

It was decided that *in vitro* assays would be performed to measure NR and GS activity in this study. NR catalyses the reduction of nitrate to nitrite, using NAD(P)H as the electron donor (Campbell, 1999). The commonly employed colorimetric *in vitro* NR assay method is an indirect measure of NR activity that measures nitrite concentrations spectrophotometrically (Chow *et al.*, 2007; Chow, 2012). GS is able to catalyse several reactions (equations 1-3) but the formation of γ -glutamyl hydroxamate is commonly used for determining enzyme activity (Vorhaben *et al.*, 1973). Equations 1-3 represent the biosynthetic, transferase and forward reaction assays, respectively (Slawyk and Rodier, 1988).

1. L-glutamate + ATP + NH₃ → L-glutamine + ADP + Pi
2. L-glutamate + ATP + NH₂OH → γ -glutamyl hydroxamate + ADP + Pi
3. L-glutamine + NH₂OH → γ -glutamyl hydroxamate + NH₃

A few factors must be considered when using crude extracts to assay GS biosynthetic, transferase and forward activity. Equation 1 represents the “true” GS reaction and the biosynthetic assay colorimetrically measures the concentration of phosphate released by the hydrolysis of ATP. However, adenosine triphosphatase (ATPase) activity in crude enzyme extracts may lead to non-specific ATP hydrolysis. Non-specific ATP hydrolysis can in turn interfere with the biosynthetic assay and cause an overestimation of GS activity. A disadvantage of the transferase reaction is that several glutaminases and amidases are able to catalyse the same reaction, once again introducing the possibility of overestimating GS activity. Therefore, the forward reaction assay was chosen to measure *G. gracilis* GS activity. The forward reaction assay is an alternative colorimetric method that is based on the biosynthetic reaction assay but produces a more stable final product than the biosynthetic reaction assay. The problem of non-specific ATP

hydrolysis is overcome by measuring the formation of γ -glutamyl hydroxamate rather than inorganic phosphate (Slawyk and Rodier, 1988).

Standard extraction and assay procedures are not always applicable when measuring NR and GS activities in different algal species (Chow *et al.*, 2004). For example, the extraction and assay protocol for measuring NR activity in *Porphyra* species was not suitable for measuring maximal NR activity of *Enteromorpha* species (Lobban and Harrison, 1994). When completing *in vitro* assays, the activity or effectiveness of an enzyme in catalysing a specific reaction is determined by numerous factors that include physiological buffers, pH and temperature. These parameters can be tested, allowing researchers to establish the ideal conditions required for measuring maximal enzyme activity. In addition, circadian regulation of plant and algal NR and GS has been observed (Chow *et al.*, 2004; Wise and Hooper, 2006). Thus, the examination of diel changes in enzyme activity allows a researcher to determine at which point during the photoperiod maximal enzyme activity is detected. This is useful when completing growth experiments as it allows a suitable sampling time to be established.

Therefore, the aim of this study was to first optimise the *in vitro* NR and forward GS assays to ensure that maximal activity of these enzymes can be measured in crude protein extracts. Second, the daily oscillation of NR and GS activity was evaluated to establish: i) the ideal sampling time and ii) to test whether *G. gracilis* NR and GS enzymes are subject to circadian regulation.

3.2 MATERIALS AND METHODS

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

3.2.1 Source and pre-culture of the seaweed

G. gracilis was obtained from the Irvine and Johnson Abalone Culture Division, Danger Point, Gaansbaai, South Africa. *G. gracilis* was washed in filtered seawater to remove sediment and visible epiphytes, including small isopods and crustaceans. After washing, seaweed was maintained in 45 L tanks containing filtered seawater in a 15°C constant temperature room with a 16 h photoperiod, until required.

3.2.2 Experimental design

3.2.2.1 Enzyme optimisation experiments

G. gracilis was cultured in 1 L Erlenmeyer flasks (Figure 1) containing artificial sea water (ASW) (Appendix A2.1) supplemented with PES-N (Appendix A2.2) medium. Nitrate in the form NaNO_3 (Appendix A2.7) was added at a concentration of $500 \mu\text{M NO}_3^-$ and served as the sole nitrogen source in the NR assay optimisation experiments. The culture medium for the GS assay optimisation experiments contained ASW supplemented with PES-N media containing $200 \mu\text{M NH}_4^+$ in the form NH_4Cl (Appendix A2.8) as the sole nitrogen source. The cultures were initiated with a 5 g fresh weight (FW) inoculum of alga per 0.8 L culture medium. Cultures were continuously aerated and illuminated with white fluorescent light (LUMILUX cool white 840; $45 \text{ photons m}^{-2}\text{s}^{-1}$) at a 16:8 h light-dark (LD) cycle. The temperature was maintained at $18 \pm 2^\circ\text{C}$.

Algae were cultured in ASW for three days before experiments were performed to allow acclimation to the photoperiod. Thereafter, the culture medium was changed and PES-N supplemented ASW containing the appropriate nitrogen source was added. Thalli were sampled 24 h after the addition of nitrogen (3 h after the start of the light period).

3.2.2.2 Oscillation of NR and GS activity over a 24 h period

Algae were cultured as described (3.2.2.1) with the exception that cultures were initiated with a 2 g (FW) inoculum of alga per 0.8 L culture medium. Algae were acclimatised for one week in PES (Appendix A 2.3) supplemented ASW before the NR and GS activity was evaluated.

The time 0 sample was taken at 21h00 and the time 24 h sample at 21h00, 24 h later. The lights illuminating the flasks automatically switch on at 05h00 and this corresponds to time 8 h in the experiments. Algae were flash frozen in liquid nitrogen, and NR and GS activity was assayed immediately utilising optimised protein extraction and assay conditions (3.2.5).



Figure 1. Experimental setup for culturing *G. gracilis*. Flasks containing algae were arranged horizontally in front of fluorescent lights (a). Thalli were aerated through plastic tubes attached to a central air pipe (b). Aeration caused bubbling and allowed the thalli to tumble, ensuring that the entire thallus received equal illumination.

3.2.3 Preparation of total protein/crude enzyme extract

Following sampling, NR and GS activity was assayed immediately. *G. gracilis* thalli were blotted dry and total protein extracts for NR and GS assays were obtained by homogenising thalli in a mortar and pestle using liquid nitrogen. Thereafter, powdered algal material was suspended in the appropriate extraction buffer: NR_{opt} Buffer (3.2.5.1) and GS_{opt} Buffer (3.2.5.2) for NR and GS, respectively.

Cell debris was removed by centrifugation at 12 000 $\times g$ for 15 min at 4°C. Total protein extracts were kept on ice until assayed. Total soluble protein content was determined using Bradford reagent (Bio-Rad) according to the manufactures instructions. Bovine serum albumin (BSA) (Roche) served as the standard protein.

3.2.4 *In vitro* enzyme assays

All enzyme assays were performed in triplicate. Enzyme assays were conducted in assay mixes that contained the same physiological buffer and pH used to extract the protein.

3.2.4.1 Nitrate reductase

NR activity was determined as described by Chow *et al.* (2007) with modifications. The assay reaction mixture consisted of 100 μg total protein, 0.25 mL NR assay mix (20 mM KNO₃, 0.1 mM EDTA, 0.1 mM NADH and 50 mM Tris, pH 8) made to a final volume of 0.5 mL with 0.1 M Tris pH 8. The enzymatic reaction proceeded at 37°C for 30 min. The reducing donor, NADH, was added at time zero to initiate the reaction. Controls that lacked NADH were prepared. The reactions were stopped by the sequential addition of 0.25 mL of 1% (w/v) sulphanilamide (Appendix A 4.5) followed by 0.25 mL of 0.02% (w/v) *n*-(1-naphthyl) ethylenediamine dihydrochloride (Appendix A 4.6). Precipitates were removed by centrifugation at 12 000 $\times g$ for 5 min at 20°C. Nitrite concentrations were determined by measuring absorption at 540 nm in a spectrophotometer (Beckman DU530). Nitrite (Sigma) was used to construct a standard curve. One unit of NR was defined as the amount of enzyme required to produce 1 μmol of nitrite per min at 37°C.

3.2.4.2 Glutamine synthetase

GS activity was determined on the basis of Shapiro and Stadtman (1970b) with modifications. The assay reaction mixture consisted of 100 µg total protein and 0.2 mL GS forward assay mix (94 mM hydroxylamine, 112 mM MgCl₂, 340 mM monosodium glutamate, 24 mM ATP and 50 mM Tris, pH 7.5) made to a final volume of 0.5 mL with 0.1mM Tris pH 7.5. The reaction was initiated by the addition of ATP (Sigma) and the enzymatic reaction was allowed to proceed for 30 min at 25°C. Controls that lacked glutamate were prepared. The reaction was stopped by the addition of 1 mL of GS assay stop mix (Appendix A 4.7). The assay mixture was centrifuged at 12 000 *x g* for 5 min at 20°C to remove precipitates. The appearance of γ-glutamyl hydroxymate (GGH) was determined by measuring the absorbance at 540 nm. GGH (Sigma) was used to generate a standard curve. One unit of GS was defined as the amount of enzyme required to produce 1 µmol of GGH per min at 25°C.

3.2.5 Enzyme optimisation experiments

Extraction buffer composition was initially based on those described by Chow *et al.* (2007) (Buffer A: 200 mM Phosphate buffer, 5 mM EDTA, 1 mM DTT; pH8) and Shapiro and Stadtman (1970b) (Buffer B: 50 mM Imidazole buffer, 1 mM EDTA, 1 mM MgCl₂, 10 mM β-mercaptoethanol; pH7.7) for NR and GS, respectively.

3.2.5.1 Optimisation of NR extraction and assay conditions

The effect of different physiological buffers on NR activity was initially determined. NR was extracted and assayed in 0.2 M phosphate buffer, 50 mM hydroxymethyl aminomethane (Tris) and 50 mM imidazole buffer (pH 8). In these tests, several 1 g pieces of thallus from a single culture were weighed, blotted dry and ground with liquid nitrogen separately. The powdered algal material was re-suspended in 2 mL of the appropriate physiological buffer and enzyme activity was measured in triplicate. Thereafter, the extraction buffer, Buffer A, was amended and contained 50 mM Tris instead of 200 mM phosphate.

The effect of pH and temperature on NR activity was then determined. A pH range between 6.5-9 pH units and four temperatures, 15, 25, 30 and 37°C were tested. Five grams (FW) of thallus was ground in a pestle and mortar with liquid nitrogen and the powdered algal material subsequently re-suspended in 10 mL of amended Buffer A.

Finally, the effect of the additives EDTA (Merck) and DTT (Roche) was tested. Enzyme activity was assayed in amended Buffer A (50 mM Tris, pH 8) containing either 5 mM EDTA, 1 mM DTT or 5 mM EDTA and 1 mM DTT at 37°C for 30 min. Once again, 1 g of thallus from a single culture was weighed, blotted dry and processed as described above.

Hereafter, NR protein was extracted in the optimised extraction buffer, NR_{opt} Buffer (50 mM Tris, 5 mM EDTA, pH8) and enzyme activity assayed at 37°C for 30 min.

Finally, the effect of storage of thallus samples after freezing with liquid nitrogen was determined. After culturing, thalli were weighed, blotted dry and frozen in liquid nitrogen. Samples were assayed immediately, and 24 h and 48 h after storage at -80°C. One gram of thallus was ground in liquid nitrogen and re-suspended in 2 mL of NR_{opt} Buffer.

3.2.5.2 Optimisation of GS extraction and assay conditions

Enzyme optimisation experiments for GS were performed in the same manner as described for NR assay optimisations (3.2.5.1). The only exceptions were the types of physiological buffers and additives tested.

The addition of imidazole, Tris or HEPES to the extraction buffer was examined. Subsequently GS extractions were performed with amended Buffer B that contained 50 mM Tris instead of 50 mM imidazole.

Optimal pH and temperature were tested as described (3.2.5.1) with the exception that total protein was extracted using the amended Buffer B.

The effect of amending Buffer B with EDTA (1 mM), MgCl₂ (1 mM) or β-mercaptoethanol (10 mM) on GS activity was also examined. Subsequent GS protein extractions were completed using the optimised GS extraction buffer, GS_{opt} Buffer (0.1 M Tris, 10 mM β-mercaptoethanol, pH 7.5), and GS was assayed at 25°C for 30 min.

Similar to NR, the effect of storage of *G. gracilis* thalli at -80°C was determined on GS activity. Algal material was re-suspended in GS_{opt} buffer and assays were performed in triplicate for 30 min at 25°C.

3.3 RESULTS

Initially, *G. gracilis* protein extraction and enzyme assays were performed according to the conditions outlined by Chow *et al* (2007) and Shapiro and Stadtman (1970b) for NR and GS, respectively. After each optimisation experiment was completed, extraction buffers and assay conditions were amended accordingly.

3.3.1 Optimal physiological buffer

The three most common physiological buffers used to extract and assay NR and GS were tested.

Maximal NR and GS activity was recorded in Tris buffer (Table 1). In comparison to Tris buffer, NR activity was reduced by approximately 50% and 11% in phosphate and imidazole buffer, respectively. Similarly, extraction in Hepes and imidazole buffer resulted in a 40% and 50% decrease in GS activity.

Table 1. NR and GS activity in total protein extracts of *G. gracilis* prepared in various buffers.

Physiological Buffer	Relative Enzyme Activity (%)	
	NR	GS
Phosphate	47.2	-
Imidazole	89.9	-
Tris	100	-
Imidazole	-	48.8
Tris	-	100
Hepes	-	59.3

The NR and GS activities in Hepes, imidazole and phosphate buffer were expressed as a fraction of the activity in Tris buffer since the latter was maximal and thus set at 100%. Means (three measurements from the same crude extract).

3.3.2 Optimal pH

The effect of pH on NR and GS activity was determined. NR and GS proteins were extracted in Tris-based extraction Buffer A and Buffer B, respectively. Assay mixes were calibrated to the pH being tested and enzyme activity was measured.

Maximal NR activity was observed at pH 8 (Figure 2), whilst maximum GS activity occurred at pH 7.5 (Figure 3). NR activity was reduced by 10% at pH 7.5 and 8.5 and 20% at pH 9. Compared to the maximum GS activity detected at pH 7.5, a reduction in activity of 35% occurred at pH 7. When pH values exceeded 7.5 units, a 50% reduction in GS enzyme activity was observed.

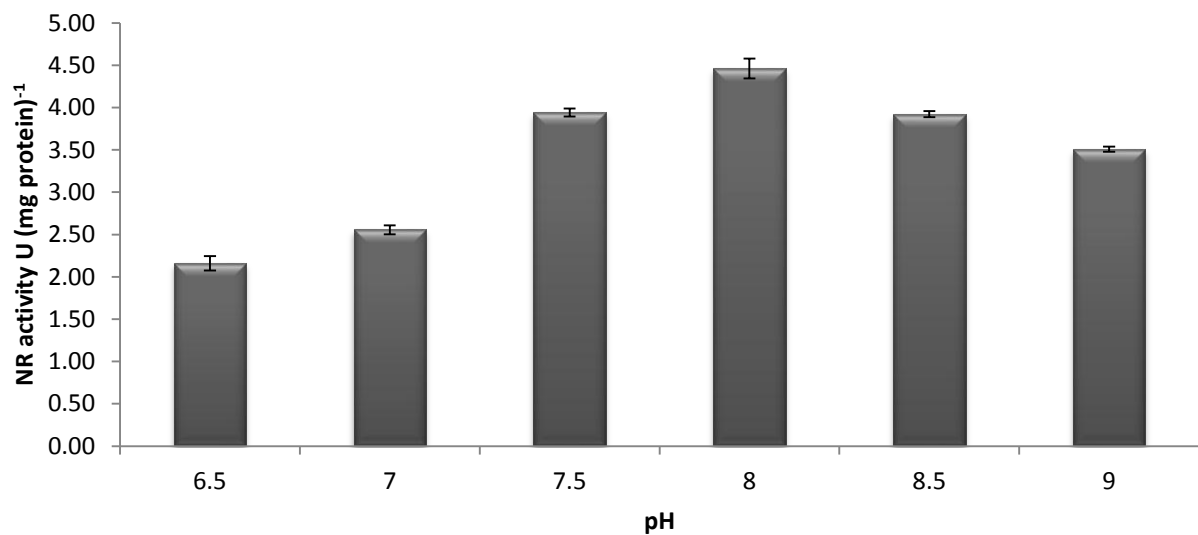


Figure 2. Effect of pH on *G. gracilis* NR. Bars represent mean activity \pm SD (three measurements from the same crude extract).

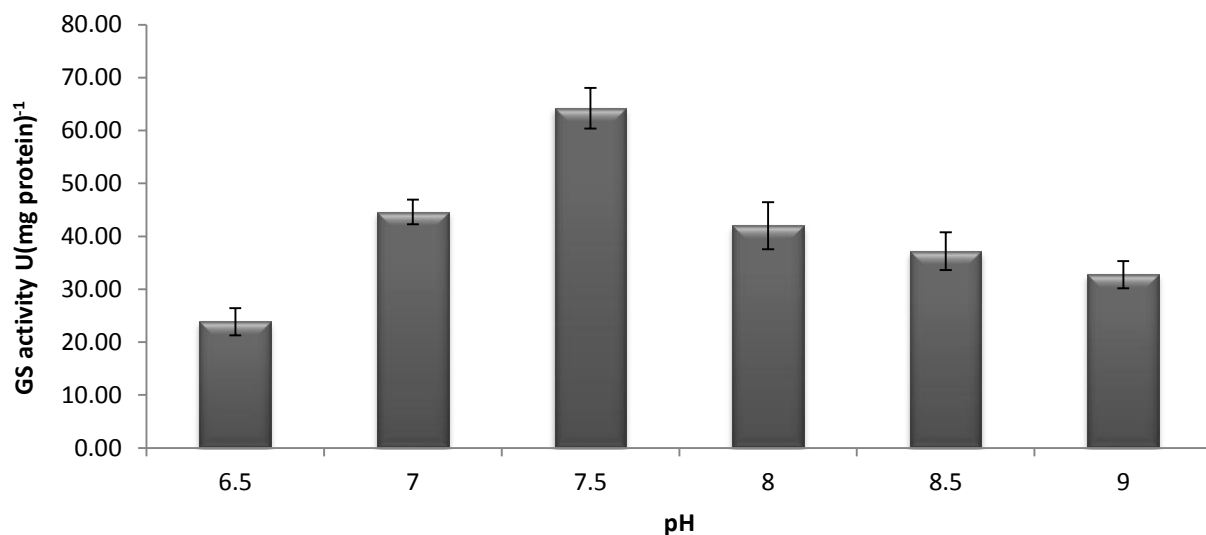


Figure 3. Effect of pH on *G. gracilis* GS. Bars represent mean activity \pm SD (three measurements from the same crude extract).

3.3.3 Optimal temperature

The effect of temperature on NR and GS activity was evaluated. *G. gracilis* NR and GS temperature optima were determined by extracting total protein extracts in amended Buffer A (50 mM Tris, pH 8) and Buffer B (0.1 M Tris, pH 7.5), respectively. Assay mixes were adjusted to the determined optimal pH and enzymes were assayed at differing temperatures.

Maximal NR and GS activity occurred at 37°C (Figure 4A) and 15°C (Figure 4B), respectively. In addition, both enzymes were thermotolerant. However, NR was more tolerant to high temperatures as a reduction in GS activity of 45% occurred at 37°C.

3.3.4 The effect of additives on enzyme activity

Additives are generally employed to stabilise enzymes upon extraction. The effectiveness of commonly used additives in preserving enzyme activity was determined. In these experiments, NR was extracted in amended Buffer A (50 mM Tris, pH 8) and assayed for 30 min at 37°C. GS was extracted in amended Buffer B (0.1 M Tris, pH 7.5) and assayed for 30 min at 25°C.

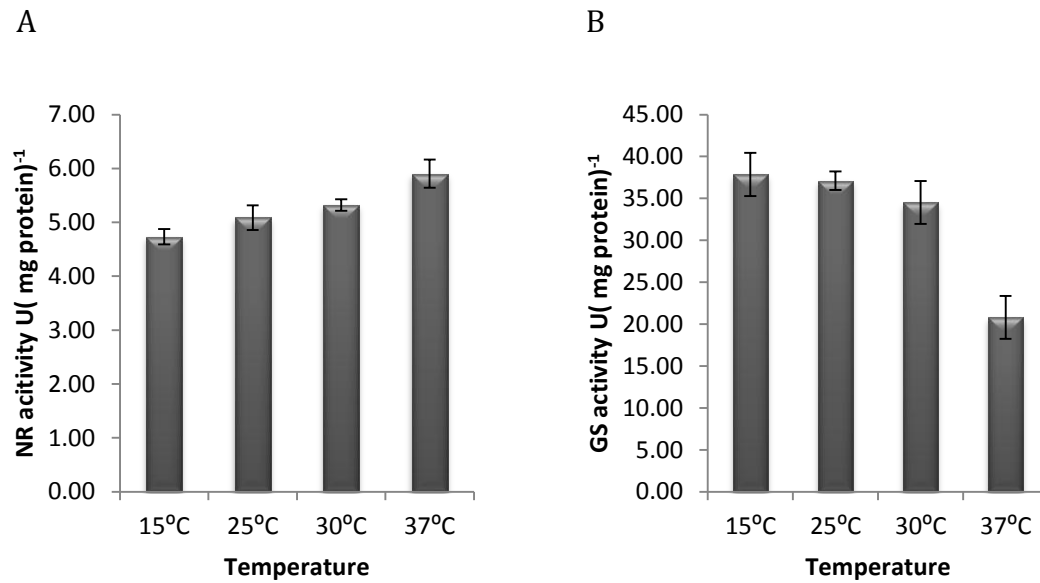


Figure 4. Effect of temperature on *G. gracilis* (A) NR and (B) GS activity. Bars represent mean activity \pm SD (three measurements from the same crude extract).

In comparison to the Tris control, NR activity increased by approximately 60% when the extraction buffer included 5 mM EDTA (Table 2). The addition of DTT had no effect on NR activity, whilst a combination of DTT and EDTA resulted in an 8% increase in NR activity compared to the Tris control.

Compared to the Tris control, none of the additives enhanced GS activity (Table 2). The inclusion of β -mercaptoethanol to the extraction buffer resulted in an approximately 10% increase in GS activity. The addition of 1 mM EDTA had no effect on GS, while the addition of 1 mM $MgCl_2$ resulted in a 7% decrease in GS activity.

Table 2. Effect of various additives on *G. gracilis* NR and GS activity.

Additive	Relative Enzyme activity (%)	
	NR	GS
Tris control	100	100
EDTA & DTT	108.3	
DTT	101.5	
EDTA	159.3	100.8
MgCl ₂		93.2
β-mercaptoethanol		109.6

NR and GS activities are expressed as a percentage of the NR and GS activity in the Tris control which lacks additives. Means (three measurements from the same crude extract).

Having determined the optimal extraction and assay conditions for NR and GS, NR and GS proteins were extracted in NR_{opt} Buffer (3.2.5.1) and GS_{opt} Buffer (3.2.5.2), respectively. In addition, NR and GS assays were performed at the optimal pH and temperature.

3.3.5 Effect of storage on enzyme activity

Prolonged storage of algal samples may cause a reduction in enzyme activity. Therefore, the stability of *G. gracilis* NR and GS enzymes following storage at -80°C was tested.

Freezing and storage of *G. gracilis* thalli has an effect on NR and GS activity. Both enzymes maintained high activities after 24 h (Table 3). In comparison to time 0, NR and GS activity was reduced by 15% and 7%, respectively, after storage at -80°C for 24 h. After 48 h, NR and GS activity was reduced by approximately 24% in comparison to time 0.

Table 3. The effect of prolonged storage at -80°C on *G. gracilis* NR and GS activity.

Storage at -80°C (h)	Relative enzyme activity (%)	
	NR	GS
0	100	100
24	86.2	93.1
48	78.6	75.8

NR and GS activities at 24 and 48 h are expressed as a percentage of the NR and GS enzyme activity at time 0. Means (three measurements from the same crude extract).

3.3.6 Oscillation of NR and GS activity over a 24 h period

The oscillation of *G. gracilis* NR and GS activity during a 24 h photoperiod was evaluated.

No distinct oscillation pattern was observed in NR (Figure 5A) and GS (Figure 5B) activity over a 24 h photoperiod. The activity of both enzymes seemed to exhibit diurnal variation and fluctuated throughout the 24 h period. The addition of light seemed to stimulate NR activity as an approximately 45% increase in NR activity was observed 6 h (T14) after the onset of light (Figure 5A). However, with the exception of T14, no appreciable difference in NR or GS activity was detected between thalli sampled in the light phase and those sampled in the dark phase. This indicated that it was likely that NR and GS activity may not be subject to circadian regulation

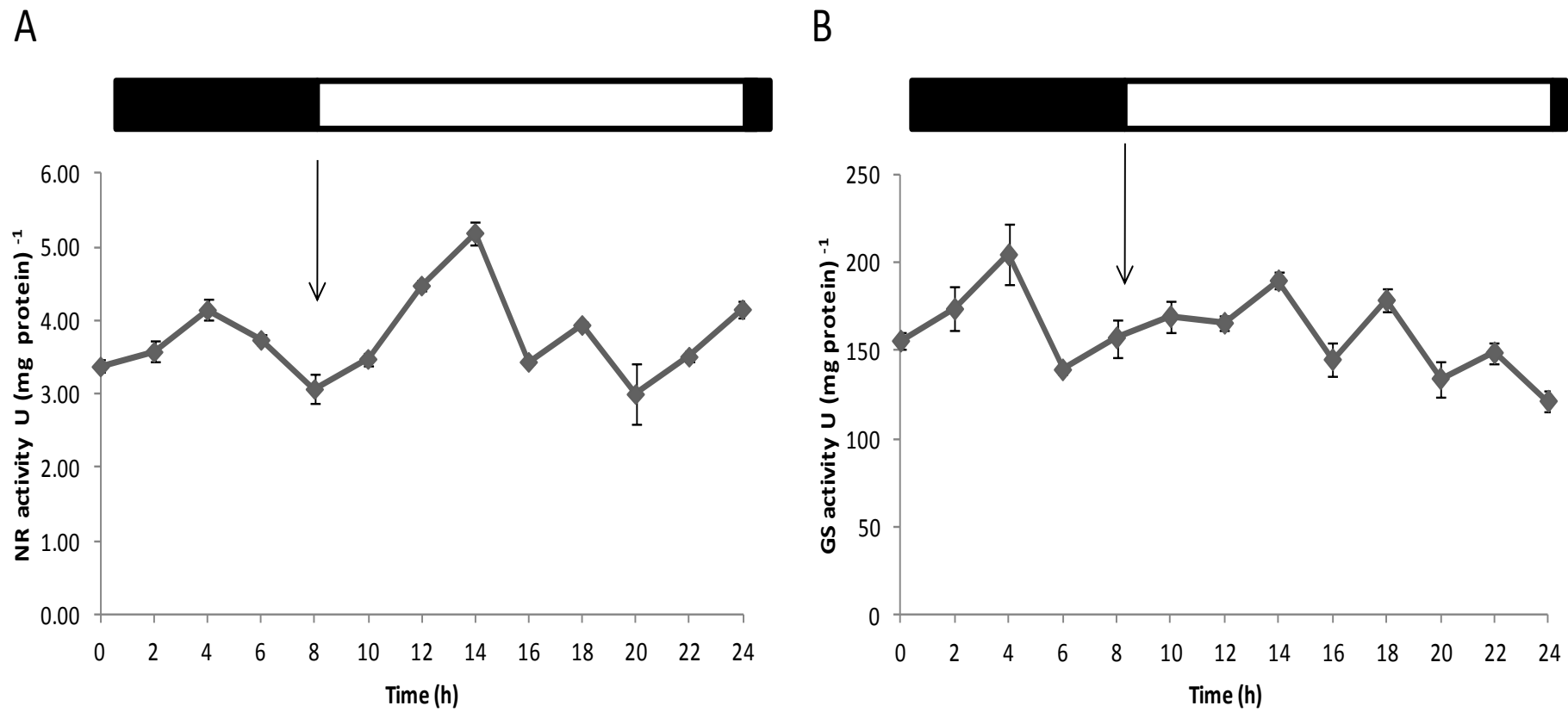


Figure 5. Oscillation of *G. gracilis in vitro* (A) NR and (B) GS activity when cultured under a LD(16:8) photoperiod. Black filled bars represent the dark phase and white filled bars represent the light phase. Points are mean activities \pm SD (three measurements from the same crude extract). Arrow indicates start of the light phase.

3.4 DISCUSSION

Enzymes act as biological catalysts within cells and are a diverse group of proteins responsible for the catalysis of numerous metabolic reactions (Mathews and van Holde, 1990). *In vitro* enzyme assays are often employed to gain an understanding of how enzymes are regulated *in vivo* and establish metabolic responses occurring within cells. Successful *in vitro* assays require determination of the optimal extraction and assay conditions that ensure maximal enzyme activity.

Maximal NR and GS activities were detected when total protein was extracted and assayed in Tris buffer. The pKa of Tris is 8.3 whilst those of phosphate, imidazole and HEPES buffer are 6.86, 7 and 7.6, respectively. The optimal pH for NR and GS is close to the pKa of Tris buffer. When the optimal pH of an enzyme is similar to the pKa of the physiological buffer, drifts in pH away from the pH optimum do not occur as the enzymatic reaction proceeds. This is important for enzyme assays as enzymatic substrates or products can cause pH shifts (Mathews and van Holde, 1990).

The optimal pH, required for maximal *G. gracilis* NR activity is in agreement with previous findings for the red macroalgae *Kappaphycus alvarezii* (Granbom *et al.*, 2004), *Gracilaria chilensis* (Chow *et al.*, 2004) and *Gracilaria caudata* (Chow *et al.*, 2007). Maximal *G. gracilis* GS activity was detected at pH 7.5 and is in agreement with pH optima determined for GS of higher plants (Cabello *et al.*, 1994). This pH value is the midpoint value between the pH of the cell cytoplasm (pH 7) and chloroplast stroma (pH8) (Ahmad and Hellebust, 1993). Two isoforms of GS have been detected in higher plants and green algae (Ahmad and Hellebust, 1987). The work of Ahmed and Hellebust (1987) demonstrated that different GS isoforms had different pH optima. GS₁ had a pH optimum closer to the pH of the cell cytoplasm and GS₂ had a pH optimum closer to the pH of the chloroplast stroma. In the current study, enzyme activity was measured using a total protein extract that contained both isoforms. The use of total protein extracts may skew enzyme activity data as increased pH could favour the activity of one isoform over the other. This hypothesis can be confirmed by purifying both isoforms and completing biochemical analysis on each.

The response of *G. gracilis* NR and GS to different assay temperatures was comparable to other red macroalgae. Tolerance of *G. gracilis* NR activity to a wide range of temperatures is congruent with findings in *K. alvarezii* (Granbom *et al.*, 2004), *G. chilensis* (Chow *et al.*, 2004) and *G. caudata* (Chow *et al.*, 2007). However, unlike its red macroalgal counterparts, *G. gracilis* NR maintained high activities at 37°C, whereas NR activity of these algae steadily declined from 25°C onwards. *G. gracilis* NR seems to be thermotolerant, much like the NR found in chlorophytes and higher plants (Granbom *et al.*, 2004). GS activity was also detected over a wide range of temperatures. However, unlike NR, GS activity decreased when the temperature was greater than 30°C.

A study by Granbom *et al.* (2004) suggested that pH and thermotolerance of NR in *K. alvarezii* was an adaptation to growth in the lower intertidal zone, allowing the alga to survive daily changes in the natural environment. Similar evidence suggests that pH and thermotolerance of *G. gracilis* NR and GS is an adaptation to the external environment. *Gracilaria* species are known to grow and survive in a wide range of temperatures (Santelices and Doty 1989). In addition, heat stress experiments performed by McLachlan (unpublished data) revealed that *G. gracilis* cultured for 3 weeks at 30°C maintained viability and growth, but eventually died when cultured at 35°C. As *G. gracilis* is located in the intertidal zone, thermo- and pH- tolerance of these enzymes may also be an adaptation to constant changes in the external environment.

Additives are generally included in extraction buffers with the intent to stabilise the enzymes that are going to be assayed. This preserves the native structure of the target enzyme, allowing easy assay of the enzyme. Furthermore, the effects of additives are species specific (Berges and Harrison, 1995). The inclusion of EDTA and DTT to extraction buffers minimises protein degradation (Chow *et al.*, 2007). Studies indicate that the presence of Mg²⁺, even when present in small amounts, may inhibit NR activity. As EDTA is a known metal chelator, it is likely that the increased *G. gracilis* NR activity occurred as a consequence of Mg²⁺ chelation. DTT had no real effect on *G. gracilis* NR activity. The effectiveness of DTT in stabilising NR proteins seems to be dependent on the organism that the protein is isolated from (Berges and Harrison, 1995). For example, flagellate NR required only cysteine whereas diatom species required DTT to stabilise NR and maintain activity. Therefore, DTT may not be required to stabilise

G. gracilis NR. The slight increase in GS activity in the presence of the reductant β -mercaptoethanol is in agreement with studies conducted on the unicellular marine alga *Phaeodactylum tricornutum* Bohlin (Rees *et al.*, 1995) and *C. reinhardtii* (Florencio and Vega, 1983). Thiol compounds such as β -mercaptoethanol are known to act as inhibitors of phenolic reactions (Cabello *et al.*, 1994). Therefore, increased GS activity could be attributed to stabilisation of GS following extraction by the protective action of thiols against phenolics. The addition of Mg^{2+} reduced *G. gracilis* GS activity whilst EDTA had no effect on enzyme activity. Since EDTA is a metal chelator, an improvement of GS activity is expected. However, the presence of multiple GS isoforms may explain why EDTA did not affect GS activity and further investigation is required. In addition, studies have shown that different metal ions and metal ion concentrations have varying effects on GS activity, depending on the type of GS assay employed (MacParland *et al.*, 1976, Yuan *et al.*, 2001). For example, biochemical analysis of purified soya-bean root nodule GS₁ (MacParland *et al.*, 1976) and the unicellular cyanobacterium *Synechococcus* RF-1 (Yuan *et al.*, 2001), indicated that Mg^{2+} increased GS biosynthetic activity. Conversely Mn^{2+} was the most effective metal ion for GS transferase activity, but strongly inhibited GS biosynthetic activity of *Synechococcus* RF-1. Thus, testing a range of Mg^{2+} concentrations or different metal ions may reveal the type of metal ion and concentration required to enhance *G. gracilis* GS activity when performing the forward reaction assay.

Optimisation experiments established the optimal extraction and assay conditions required for the isolation and assay of stable NR and GS proteins. In addition to stability upon extraction, both NR and GS showed minimal loss of enzyme activity after storage at $-80^{\circ}C$.

Once optimal extraction and assay conditions were established, the oscillation of *G. gracilis* NR and GS activity over a 24 h period was examined. NR is regulated by light in addition to other factors that include carbon skeletons and amino acids (Granbom *et al.*, 2004). Unlike *G. tenuistipitata* (Lopes *et al.*, 2002), *K. alvarezii* (Granbom *et al.*, 2004) and *G. chilensis* (Chow *et al.*, 2004), *G. gracilis* NR activity was not distinctly reduced in the dark. The pattern of *G. gracilis* NR activity is in agreement with studies performed in the dinoflagellate *Gonyaulax polyedra* (Harrison, 1976) and the unicellular green alga

Chlorella species (Berges, 1997). These studies demonstrated that light was not essential for NR gene expression and nitrate assimilation as long as sufficient carbohydrates were available. Furthermore, evidence of dark induction of NR in other marine chlorophytes is established (Berges, 1997). Therefore, it is likely that high *G. gracilis* NR activity measured in the dark phase may be attributed to dark assimilation of nitrate. In addition, high *G. gracilis* GS activity during the dark phase is congruent with findings in the cyanobacterium *Prochlorococcus* species strain PCC 9511 (El Alaoui *et al.*, 2001). Enzyme activity of GS isoforms are differentially regulated by light (Florencio and Vega, 1983; Maurin-Defossez and Gal, 1998). GS₂ exhibits highest activity in light, whilst GS₁ has been reported to increase in the dark. Therefore, it is possible that the observed increase of *G. gracilis* GS activity may be attributed to the GS₁ isoform. However as a crude extract was used for the enzyme assays, biochemical separation of the different isoforms would need to be completed before any conclusions can be made.

Apart from light, circadian regulation of NR (Chow *et al.*, 2004) and GS activity (García-Fernández *et al.*, 1995; Wise and Hooper, 2006) in plants and some algae has been established. Three diel patterns of NR activity have been documented (Berges *et al.*, 1995). The first pattern describes increased NR activity that peaks once or twice in the light but is low in the dark. Many macroalgae, microalgae, marine phytoplankton and higher plants exhibit pattern one. The second pattern is the monotonic increase of NR activity in the light and decrease in the dark. This pattern has been documented in barley leaves. Pattern three is characterised by an increase in NR activity just before dawn, followed by a decline in NR activity during the day. Pattern three was observed in *E. huxleyi* and some marine phytoplankton species. Different patterns of expression suggest that no universal diel pattern for NR activity exists. Researchers have suggested that a second midday peak could correspond to a peak in photosynthesis. Conversely, some have argued that differences in NR patterns of expression may be a consequence of the types of assays used (*in vitro* vs. *in vivo*) and the sampling frequency. For example, sampling may be insufficient to allow detection of both peaks in some studies. *G. gracilis* NR seems to exhibit a variation of pattern one and three, with high NR activities in the dark and a possible double peak in the middle of the light period. However, sampling

intervals in different studies differ, making comparison between studies difficult (Berges *et al.*, 1995).

Although *G. gracilis* NR diel pattern has similarities to those documented for other plants and algae, it must be stressed that this study has not fully tested whether *G. gracilis* NR or GS activity is under the control of a biological clock. Circadian regulation of *K. alvarezii* NR activity was only observed when the alga was cultured at high light intensities of 100 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$. Low light, 25-55 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$, comparable to the light intensities used in the present study, caused a fade out or dampening of the circadian rhythm of NR activity in this red macroalga. Therefore, it is possible that a similar dampening of the circadian rhythm in NR activity occurred in *G. gracilis*. In contrast, NR activity of *G. chilensis* irradiated at 77 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ was not regulated by a biological clock, but was stimulated by light (Chow *et al.*, 2004). Lack of circadian regulation at high light intensities is also supported by studies completed on the eelgrass *Zostera marina* (Touchette and Burkholder, 2007) and the brown macroalgae *L. digitata* and *Fucus* species (Young *et al.*, 2007). Furthermore, in the unicellular green alga *C. braunii*, circadian regulation of GS activity was dependent on the length of the light:dark cycles and growth or age of the culture (García-Fernández *et al.*, 1995). Researchers proposed that alleviation of circadian control is advantageous in a nitrogen limited environment as it allows the alga to assimilate nitrogen whenever it becomes available (Young *et al.*, 2007). Instead, NR activity may be dependent on either light or light regulated metabolites. *Fucus* species inhabit the higher intertidal zone and experience prolonged exposure to air, so there is no advantage to using light-dark cues to synchronise nitrogen metabolism. These macroalgae are more responsive to tidal changes and are able to maintain high NR activity at night to compensate for light availability and periods of emersion. In view of these findings, there is also evidence to suggest that the lack of circadian control of NR and GS activity in response to light and dark cues may be an adaptive advantage to *G. gracilis*. *G. gracilis* is found at depths of 2-20 meters along the north coast of Saldanha Bay and is subject to periods of emersion and differences in irradiance (Anderson *et al.*, 1996). Similar to *Fucus* species and *Z. marina*, lack of circadian control of *G. gracilis* NR and GS may be an adaptation to growth in a constantly changing environment where nitrogen is temporally available. In addition, GS activity in sunflower roots was dependent on photoperiodically induced

processes (Knight and Weissman, 1982). GS activity of sunflower roots and *C. braunii* was modulated by rhythmic changes in the ratio of ATP/ADP and AMP (Knight and Weissman, 1982; García-Fernández *et al.*, 1995). Thus, many factors are responsible for modulating NR and GS activity. On the whole, further investigation is required before it can be concluded with certainty whether or not *G. gracilis* NR and GS are regulated by an endogenous biological clock.

In summary, *G. gracilis* NR and GS seem to be quite robust proteins that tolerate a wide range of pH and temperature shifts and exhibit high activity throughout the photoperiod. In addition, extraction buffers included minimal additives but resulted in the extraction of stable enzymes that could be easily assayed. The ability to successfully extract and assay these enzymes will allow NR and GS regulation to be investigated under different nitrogen conditions, and consequently, elucidate their role in nitrogen assimilation in *G. gracilis*.

Chapter 4

Investigation of the effect of long term culture of *Gracilaria gracilis* under nitrogen replete and deplete conditions

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4.1 INTRODUCTION

The majority of growth studies performed on plants, microalgae and macroalgae have focussed on the response of these organisms to an input of nitrogen, specifically nitrates. In marine estuaries, eutrophication is responsible for increased algal blooms that reduce the growth and persistence of other marine organisms (Anderson *et al.*, 1996). In addition, macroalgae are limited by a lack of nitrogen at some point of the year as a result of seasonal changes (Chow and de Oliveira, 2008). In comparison to microalgae, macroalgal nitrogen starvation kinetics and its implications on photosynthesis and metabolism are not well understood (Young *et al.*, 2009). Although our understanding of the regulatory mechanisms controlling nitrogen metabolism in plants has advanced, little is known about nitrogen metabolism and its regulation in macroalgae. Thus, it is important to assess the effect of nitrogen input and nitrogen starvation on nitrogen cycling in macroalgae.

Previous studies indicated that *G. gracilis* exhibited distinct physiological responses when cultured in nitrogen starved conditions (Gebrekiros, 2003). The effect of nitrogen starvation stress on *G. gracilis* growth was evaluated by measuring the concentration of nitrogen in the growth medium and thallus. In addition, differential screening analysis of a full length cDNA library (generated from RNA extracted from *G. gracilis* cultured under nitrogen deplete conditions) identified several genes that were differentially expressed in response to nitrogen starvation. Lebi (2006) continued this work, but employed microarray analysis to identify genes up-regulated in response to nitrogen depletion.

Gebrekiros (2003) and Lebi (2006) evaluated overall gene expression and were successful in establishing the physiological responses of *G. gracilis* cultured under nitrogen deplete conditions. Their investigations revealed the many metabolic processes required for *G. gracilis* acclimation and survival in a nitrogen deplete environment. However, neither study characterised the response of specific genes to nitrogen stress in *G. gracilis*. The current study is a continuation of the work started by Gebrekiros (2003) and Lebi (2006). In the present study nitrogen metabolism and its regulation in *G. gracilis* was assessed by evaluating the role of NR (activity is an index of

nitrate incorporation) and GS (a central enzyme for nitrogen incorporation) (Migge *et al.*, 2000). Before addressing nitrogen assimilation, incorporation and regulation in macroalgae, a few considerations must be kept in mind.

Unlike unicellular microalgae, multicellular macroalgae have large vacuoles that enable the alga to take up nitrogen even when it is not needed for metabolism (Inokuchi and Okada, 2001). This adaptation complicates the study of nitrogen assimilation and incorporation, and the function of enzymes involved in these processes. The 'luxury consumption' and storage of nitrogen as proteins, pigments or amino acids is an adaptation that allows macroalgal survival when nutrients are limiting (Lignell and Pedersén, 1987). Thus, measurement of storage metabolites that include thallus nitrogen and carbon, tissue nitrate and ammonium, and carbohydrates is useful when determining the physiological state of cells. In addition, measuring metabolite pools allows the overall health status of the alga to be assessed, as changes in metabolite pools is an indication of how the alga is responding to the surrounding environment or environmental stress. Moreover, changes in chemical or biochemical constituents may cause changes in the regulation of NR and GS (Redinbaugh and Campbell, 1993; Oliveira and Coruzzi, 1999). For example, nitrate induces NR synthesis in plants whereas the absence of nitrate makes NR susceptible to degradation (Berges, 1997), sucrose and 2-oxoglutarate stimulate GS₁ and GS₂ (Mifflin and Habash, 2002) and the ratio of cellular Gln:Glu modulates GS activity (Tschoep *et al.*, 2009). These factors ultimately determine how the alga may respond and adapt to changes in the environment.

Thus, if we are to expand our knowledge of nitrogen utilisation in *G. gracilis* one needs to consider metabolite changes, gene expression and regulation, as well as the relationship, if any, between these variables. This type of approach would allow a global understanding of nitrogen assimilation and utilisation within *G. gracilis* to be achieved. Similar approaches were used in studies of the macroalgae *Enteromorpha lingulata* and *Gracilaria pisillum* (Lartigue and Sherman, 2006), *L. digitata* and *Fucus* species (Young *et al.*, 2007), *Ulva lactuca* (Teichberg *et al.*, 2007) and *G. chilensis* (Chow and de Oliveira, 2008). However, these studies examined either NR or GS activity in comparison to metabolite changes and nitrogen sources. Few studies have evaluated the expression of NR and GS by monitoring mRNA and protein abundance, in addition to the

measurement of enzyme activities. Evaluation of gene expression at the mRNA, protein and activity levels is important. Several factors control translation levels, an increase in the number of mRNA transcripts does not always correlate with an increase in protein, and increases in protein does not necessarily result in increased enzymatic activity (Peat and Tobin, 1996). In addition, plant nitrogen status regulates NR via transcription, post-transcription and post-translational modifications (Klein *et al.*, 2000), whilst GS regulation is mediated at transcription, post-transcriptional processing and stabilisation of transcripts, translational, subcellular localisation, processing/modification of the GS polypeptide, assembly of the active enzyme and post-translationally through enzyme degradation (Cren and Hirel, 1999). Thus, evaluation of NR and GS transcript and protein abundance, in addition to enzymatic activity, is invaluable in understanding nitrogen metabolism and regulation in *G. gracilis*.

The aims of the present study were to investigate the response of *G. gracilis*, cultured for an extended period of time in nitrogen replete (enriched, containing nitrogen) and nitrogen deplete (lacking, nitrogen starved) conditions, to changes in the external environment and internal metabolites by evaluating the expression of NR and GS. Identification and amplification of *GgNR* and *GgGS₁* genes (Chapter 2), optimisation of NR and GS *in vitro* assays (Chapter 3) and commercially available antibodies to both NR and GS enabled all of these parameters to be tested. The current study is one of the first, if not the first, study to examine macroalgal nitrogen metabolism and the alga's response to nitrogen stress by monitoring the expression of NR and GS at all molecular levels, as well as determining whether changes in metabolite pools impact gene expression. Ultimately, an understanding of the effect of differing nutritional conditions on nitrogen metabolism will aid in elucidating the nitrogen stress response mechanisms of *G. gracilis*.

4.2 MATERIALS AND METHODS

Media and solutions, and supplementary information relating to experiments completed in Chapter 4 can be found in Appendix A, B and C.

4.2.1 Nitrogen enrichment and starvation experiments

4.2.1.1 Source and pre-culture of the seaweed

G. gracilis was sourced and pre-cultured as described (3.2.1).

4.2.1.2 Experimental design

The experimental design was similar to that described in section 3.2.2.1. The cultures were initiated with a 3.9-4.2 g (FW) inoculum of alga per 0.8 L culture medium. The culture medium for nitrogen replete and nitrogen starvation experiments contained ASW supplemented with PES (nitrogen replete) (Appendix A 2.3) and PES-N (nitrogen lacking/free) media, respectively. PES supplemented ASW contained nitrate supplied as NaNO_3 and ammonium supplied as $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$. Antibiotic treatment of thalli was omitted as previous findings indicated that axenic cultures exhibited abnormal growth when thalli were cultured in nitrogen lacking media (Gebrekiros, 2003). However, germanium oxide (Appendix A 2.9) was added to culture media to limit the growth of diatoms. The addition of germanium oxide has no adverse effect on algal growth.

Prior to the start of the nitrogen experiments, algae were acclimated to the photoperiod of 16:8 (L:D) by pre-culturing in PES supplemented ASW for 7 days. Throughout the experimental period (including 7 day acclimation period), media was changed every two days. In addition, flasks were rinsed with water once a week to remove debris and other agents such as diatoms that caused fouling of the flasks.

Following the 7 day acclimation, thalli for nitrogen starvation experiments were transferred to nitrogen free culture media and sampled after 2, 6, 10, 14 and 18 days of

culture. Day 7 cultures growing in nitrogen replete media represented the control (designated Time 0). The response of *G. gracilis* to continued culture in nitrogen replete conditions was also evaluated. Thus, following 7 days of acclimation, thalli were maintained in PES media and sampled after 2, 6, 10, 14 and 18 days of culture.

After sampling (5 h after start of the light period), thalli were blotted dry, weighed, flash frozen in liquid nitrogen and stored at -80°C until required. Three biological repeats were performed for each nitrogen experiment.

4.2.2 Physiological tests

4.2.2.1 Tissue analysis

A CHNS analyser was used to measure the amount of nitrogen and carbon in each thallus sample. Pieces of thalli, approximately 1 mg fresh weight, were removed from each flask and prepared for analysis by drying at 60°C overnight (O/N). Operation of the elemental analyser (CHNS-O Thermo) was performed by the Microanalysis Centre (Department of Chemistry, University of Cape Town).

4.2.2.2 Pigment analysis

Pigment was isolated according to the method described by Beer and Eshel (1985) with modifications. Approximately 0.2 g of seaweed was ground in liquid nitrogen, re-suspended in 1 mL phosphate buffer and centrifuged for 5 min at 14 000 rpm. Thereafter, the supernatant was transferred to a fresh 1.5 mL microcentrifuge tube and the absorbance was measured using a spectrophotometer (Beckman DU530). Phycoerythrin (PE) concentrations were determined according to the following equation:

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

4.2.2.3 Biomass accumulation

Biomass was determined by weighing thalli after blotting dry on paper towel. The final FW was compared to the initial FW and the difference in weight represented the increase in biomass.

4.2.2.4 Determination of growth rate

Specific growth rate (SGR) of *G. gracilis* was calculated and expressed as the percent FW mass increase per day according to the following formula:

$$SGR = \frac{\ln(W_2/W_1)}{n} \times 100$$

Where W_1 represents the initial weight (g), W_2 the final weight (g) and n the total number of days since the start of the experiment (Smit and Bolton, 1999).

4.2.3 Total RNA isolation and conversion to complementary DNA (cDNA synthesis)

Total RNA, from nitrogen replete and nitrogen starved thalli, was extracted and DNase treated (2.2.1.1). The efficiency of the DNase treatment was evaluated by testing for the presence of genomic DNA (gDNA) in DNase treated RNA samples by performing quantitative real time PCR (qPCR) using the Oligo dT Sybr Green Sensi mix kit (Quantace) (Appendix C 1.2). Total RNA (200 ng, DNase treated) was added to a qPCR reaction mix containing 0.25 μ l 50 x SYBR Green solution, 6.25 μ l of 2 x SensiMix and 0.25 μ l 10 μ M GS_1 gene specific primers (Table 1) made to a final volume of 12.5 μ l with nuclease-free H_2O . *GgGS₁* gene specific primer sets are able to amplify the *GgGS₁* gene product from a gDNA template. Thus, if the DNase treatment was effective, no amplification of *GgGS₁* should occur, as DNase treated RNA samples should be free of gDNA. A positive control reaction was performed using gDNA as the template and nuclease free H_2O was added to the no template control. Cycling parameters consisted of an initial denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 10 s, 62°C

for 15 s and 72°C for 20 s, with a final extension period of 5 min at 72°C. Amplification plots were automatically generated by the Rotor-Gene™ Series Software (version 1.7).

Total RNA, free of gDNA, was reverse transcribed to cDNA as described (section 2.2.1.2) and used immediately. In addition, a reaction excluding reverse transcriptase (No RT) was performed for each biological repeat.

4.2.4 Quantitative Real Time PCR (qPCR)

Before changes in *GgNR* and *GgGS₁* transcriptional expression could be determined using qPCR, a suitable reference gene (RG) for normalisation needed to be established (Appendix B 1). A putative serine protease (*PSP*) proved to be suitable for use as a RG.

Primers (Table 1) specific to *GgNR* and *GgGS₁* genes were designed from the *GgNR* and *GgGS₁* gene sequences (Chapter 2.). The abundance of the *GgNR* and *GgGS₁* gene transcripts was determined in separate reactions as described (4.2.3). Triplicate qPCR reactions containing 1 µl of undiluted cDNA as template were performed. Control qPCR reactions for each gene, that included a no template (NTC) as well as the no RT control (both in duplicate), were run concurrently.

Table 1. Gene specific qPCR primers and sequence information.

Name	Primer Sequence (5'-3')	T _m (°C)	Reference
NR_qPCR_F1	GTGTGCTTCTCGGGCGTG	53.9	This study
NR_qPCR_R1	CGGGAAGCCATGGTCCGG	56.2	This study
GS ₁ _qPCR_F2	GTGCCGGCGCTGGAGAAG	63.6	This study
GS ₁ _qPCR_R2	ACGGCACGACGCTCCACG	61.6	This study
PSP_qPCR_F	GGCATTTCCTGCTTGAAAGG	52.8	Christopher Ealand (PhD thesis, 2011)
PSP_qPCR_R	AACTGCGGCGTCAATCTG	50	Christopher Ealand (PhD thesis, 2011)

Cycling parameters remained the same as those described (4.2.3) with the exception that annealing temperatures were 59, 58 and 62°C for *PSP*, *GgNR* and *GgGS₁*, respectively. A standard curve was constructed by using a ten-fold serial dilution series (1 to 0.000001 ng) generated with plasmid DNA harbouring a cDNA insert of each gene. Dissociation (melt) peaks, reaction efficiencies (E) correlation efficiencies (R²-value) and Ct values were determined using the Rotor-Gene™ Series Software (version 1.7, Corbett Research) (Appendix C 1.3 & 1.4).

GgNR and *GgGS₁* expression was normalised to *PSP* expression. Time 0 thalli served as the calibration point. Expression was calculated according to the Pfaffl method (Pfaffl, 2001) using the following equation:

Fold Change expression

$$= \text{Efficiency}_{GOI}^{(\text{Calibrator-sample})} / \text{Efficiency}_{RG}^{(\text{calibrator-sample})}$$

where *GOI* and *RG* refer to gene of interest and reference gene, respectively.

Gene expression at each sample point was represented as the *n*-fold change relative to the calibration/control sample.

4.2.5 Isolation of *G. gracilis* protein

Proteins were extracted according to the method described by Ingle *et al.* (2005) with slight modifications. Approximately 0.5 g of algal tissue was ground in liquid nitrogen in a sterile pestle and mortar. The powdered algal tissue was transferred to a microfuge tube containing 1 mL of extraction buffer (Appendix A 5.1) and vortexed. Samples were then centrifuged for 5 min at 10 000 x *g* and the supernatant transferred to a fresh microfuge tube. An equal volume of phenol (pH 8) was added. Samples were vortexed once again and then centrifuged for 1 min at 10 000 x *g*. Approximately 80% of the upper aqueous phase was removed and discarded. The remaining bottom layer was re-extracted with an equivalent volume of extraction buffer. Samples were once again vortexed and centrifuged for 1 min at 10 000 x *g*. The aqueous phase (top layer) was removed and discarded once again. The remaining organic bottom layer was split into 500 µl aliquots and the protein was precipitated by the addition of 5 volumes 0.1 M

ammonium acetate (Appendix A 5.2) to each aliquot. Protein was allowed to precipitate overnight at -20°C. Following precipitation, each aliquot was centrifuged at 10 000 x *g* for 5 min and the supernatant discarded. Samples were concentrated in a single microfuge tube. For example, if sample one had three aliquots, after centrifuging aliquot 1, aliquot 2 was added to the remaining protein pellet from aliquot 1 and centrifuged once again. This process was repeated until all aliquots were combined resulting in a single protein pellet for each sample. Pellets were then subjected to a series of washes that included a 0.1 M ammonium acetate wash followed by an 80% (v/v) ice-cold acetone wash. Pellets were allowed to dry for 10 min and then solubilised in urea lysis buffer (ULB) (Appendix A 5.3).

Protein concentrations were determined using Bradford reagent (Bio-Rad) according to the manufacturer's instructions with modifications. Protein samples were diluted in ULB (final volume 20 µl). A volume of 80 µl 0.1 M HCl (Appendix A 5.4) was added to the samples followed by 900 µl of Bradford reagent. Samples were vortexed and incubated at room temperature for 5 min before the absorbance at 595 nm was measured using a spectrophotometer (Beckman DU530). A standard curve was constructed using a BSA (Roche) dilution series.

Thereafter, protein quality and integrity was evaluated by performing SDS-PAGE according to Laemmli (1970). Protein samples were stored at -80°C until western hybridisation analysis could be performed.

4.2.6 SDS-PAGE and western hybridisation analysis

Before completing western hybridisation analysis on *G. gracilis* samples, the cross reactivity of commercial antibodies (Appendix B2.1) and the optimal amount of protein (Appendix B 2.2) required to visualise *G. gracilis* NR and GS proteins was tested. Total protein extracted from *Arabidopsis* leaves served as a positive control. Findings indicated that the commercial AS08 210 NR (Agrisera) and GLN1-2 (Agrisera) antibodies cross reacted with *G. gracilis* NR and GS polypeptides, respectively.

4.2.6.1 SDS-PAGE

Total protein extracted from *G. gracilis* at each sampling point for each treatment was resolved by performing SDS-PAGE. Resolving gels containing 10 and 12% (v/v) acrylamide solution (40% (w/v), Sigma) were used to separate proteins for NR and GS evaluation by western hybridisation analysis, respectively. A third gel, containing 10% acrylamide solution was also prepared. Protein samples were diluted in 5X SDS sample application buffer (SAB) (Appendix 5.5) to a final concentration of 5 µg/µl. Therefore, a single protein-SAB master mix was prepared for each sample point. Proteins were equally loaded and electrophoresed at 100 V until the desired degree of separation was reached. Forty (equivalent to 8 µl) and thirty micrograms (5 µl) of total protein was loaded for NR and GS analysis, respectively. Thirty micrograms of protein was also loaded in each well of the third gel that was stained with coomassie solution (0.05 % (w/v) Coomassie Brilliant Blue R-250; 50 % methanol; 10 % glacial acetic acid; 40 % water) according to Laemmli (1970b). The coomassie stained gel served as an indication of equal loading and tested the accuracy of the dilutions (Appendix C 2.1).

4.2.6.2 Western hybridisation analysis

Following SDS-PAGE, protein gels required for western hybridisation analysis of NR and GS were rinsed in sterile distilled water. Thereafter, protein gels were equilibrated in Towbin buffer (Appendix A 5.10). Proteins were then transferred onto nitrocellulose membranes (Protran, Separation Scientific) using a Mini Trans-Blot cell (Bio-Rad) for 1 h at 100 V at 4°C. After transfer, nitrocellulose membranes were stained with Ponceau S solution (Appendix A 5.6), to assess whether proteins were completely and equally transferred (Appendix C 2.1). Membranes were then washed in 1X TBS to remove the Ponceau S stain. Western hybridisation was performed by immersing the membranes in blocking buffer (Appendix 5.9) for 1 h at room temperature. NR and GS antibodies were diluted 1:1000 and 1:10 000 in blocking buffer, respectively. NR and GS membranes were incubated separately O/N in the appropriate antibody dilution at 4°C with shaking. Following incubation in primary antibody, the membranes underwent four 15 min washes in blocking buffer. The secondary antibody, peroxidase labelled goat anti-rabbit IgG (H+L) liquid conjugate (KPL), was diluted 1:20 000 in blocking buffer and

added to the membranes. The membranes were incubated with the secondary antibody for 2 h at 4°C and subsequently washed four times for 15 min each time in 1X TBST (Appendix 5.8). A final 5 min wash in 1X TBS was performed. NR and GS proteins were detected using the Immun-Star™ WesternC™ Chemiluminescent Kit (Bio-Rad) according to the manufacturer's instructions. Chemiluminescent signals were visualised using the Molecular Imager ChemiDoc XRS+ system (Bio-Rad). The immunoblots were exposed for 1800 s with one image captured every 30 s to detect NR, whereas GS detection required the immunoblots to be exposed for 300 s with one image captured every 5 s. The density (intensity.mm⁻²) of each positive signal was calculated using the Image Lab Software (version 2.0.1, BIORAD). Densities of the positive NR and GS signals at day 2, 6, 10 and 14 were calibrated to Time 0. Expression was expressed as the *n*-fold change increase/decrease of NR or GS protein at each time point relative to Time 0 .

4.2.7 NR and GS enzyme assays

NR and GS were extracted and assayed immediately after sampling as described (3.2.3 and 3.2.4).

4.2.8 Statistical analysis

Statistical analysis was performed using SigmaStat version 3.1 (Systat Software™ Inc. GmbH, 2004). One way Anova was used to determine statistical differences in data from the physiological assays and gene expression studies. Log10 transformations were conducted on data that was not normally distributed. Nitrogen percentage data that was not normally distributed were transformed by square rooting. P<0.05 was used to define statistical significance. Descriptive statistics (mean, standard deviation and standard error) were completed using Microsoft Excel (Microsoft Office 2007, Microsoft Corporation).

4.3 RESULTS

4.3.1. Physiological response of *G. gracilis* to growth in nitrogen deplete and nitrogen replete conditions

The physiological response of *G. gracilis* to culture in a nitrogen replete and nitrogen deplete environment was established by measuring tissue nitrogen, C:N ratio, biomass, specific growth rate and visual examination of the physical appearance of the thalli. Nitrogen starved thalli started to fragment after fourteen days of culture in nitrogen free conditions, resulting in insufficient sample at day 18 to complete enzyme assays and other tests. Thus, data obtained from two weeks of culture in nitrogen replete and nitrogen free media was evaluated.

4.3.1.1 Physical appearance of *G. gracilis*

Thalli starved of nitrogen turned green and bleaching at the tips was observed after two weeks of nitrogen limitation (Figure 1A), with some samples eventually fragmenting. Conversely, thalli supplied with nitrogen maintained the brown to red colour for the duration of the experiment (Figure 1B).

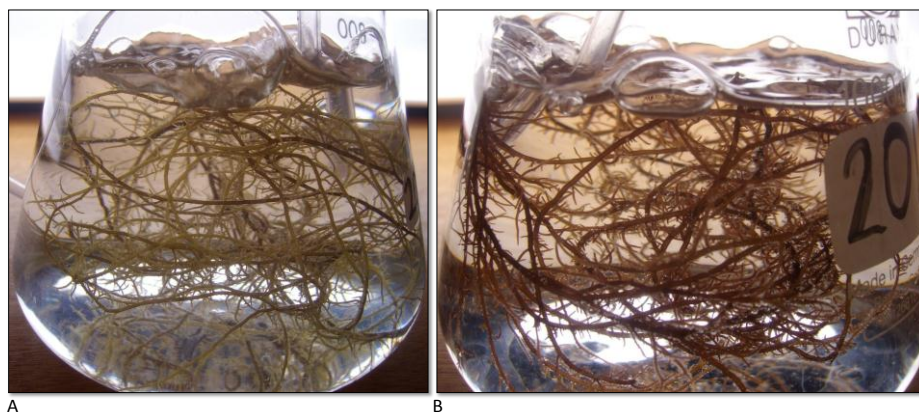


Figure 1. Illustration of the difference in the overall appearance of *G. gracilis* thalli after fourteen days of cultivation in (A) nitrogen deplete (PES-N) and (B) nitrogen replete (PES) culture medium.

4.3.1.2 Tissue nitrogen content

The intracellular nitrogen content of *G. gracilis* decreased when the alga was cultured in nitrogen deplete conditions for fourteen days (Table 2). In comparison to the control, the nitrogen content of thalli was statistically lower after two days of culture in nitrogen deplete media (Tukey, $P < 0.007$). Following fourteen days of culture in nitrogen deplete medium, a 70% reduction in intracellular nitrogen was observed in thalli. According to Smit *et al.* (1997), a thallus nitrogen percentage of 1.7% is an indication that *G. gracilis* is nitrogen limited, whereas 2.3% represents nitrogen replete thalli. Therefore, *G. gracilis* cultured in nitrogen deplete medium was nitrogen limited from day 6 onwards (Table 2). Indeed, the extremely low total nitrogen content of the thalli suggests that *G. gracilis* could be termed severely nitrogen limited. Although a reduction in thallus nitrogen also occurred in algae supplied with nitrogen, tissue nitrogen percentages of 2% or more were maintained for the majority of the experimental period (Table 2).

4.3.1.3 C:N ratio

The C:N ratio was determined to evaluate the nitrogen status of the alga as a ratio greater than ten indicates that the alga is nitrogen limited, whereas a ratio less than ten indicates nitrogen storage (Jones, 1994). Accordingly, the C:N ratio of thalli cultured in nitrogen deplete media indicated that the alga was nitrogen limited at the start of the experiment (Table 2). *G. gracilis* cultured in nitrogen replete conditions also exhibited increased C:N values, with values indicating nitrogen limitation after 10 and 14 days of cultivation. These observations were contrary to the tissue nitrogen content of the alga.

4.3.1.4 Phycoerythrin content

Phycoerythrin (PE) concentrations were determined as PE represents the major nitrogen store in most macroalgae (Lee, 1999). The greatest reduction in PE concentration was detected in thalli that were starved of nitrogen (Table 2). In comparison to the control, PE was reduced by 70% (Tukey, $P = 0.05$) at day 14. No

statistically significant reduction in PE was observed in alga cultured in nitrogen replete conditions.

Table 2: Changes in thallus nitrogen, C:N ratio, pigment and protein pools of *G. gracilis* cultured in either nitrogen lacking (PES-N) or nitrogen enriched (PES) ASW. Data represent the mean nitrogen, C:N ratio, pigment and protein ($n = 3$).

		Monitoring Index		
		Tissue N (%)	C:N Ratio	Phycocerythrin (PE) (mg/ml)
Treatment	Culture Time (days)			
PES-N cultured thalli	0	2.85	10.98	0.41
	2	2.11*	14.13	0.31
	6	1.49*	20.69	0.31
	10	1.05*	30.42	0.28
	14	0.89*	35.53	0.13*
PES cultured thalli	0	3.31	8.52	0.55
	2	3.08	9.36	0.58
	6	2.69	10.60	0.56
	10	1.99	13.35	0.44
	14	2.33	12.27	0.47

Asterisk (*) represents statistically significant differences (One-Way ANOVA, $P < 0.05$) compared to the control (Time 0).

4.3.1.5 Effect of nitrogen starvation and enrichment on *G. gracilis* biomass and growth rate.

4.3.1.5.1 Biomass

G. gracilis grew and accumulated biomass throughout the experimental period when cultivated in nitrogen deplete (Figure 2A) and nitrogen replete media (Figure 2B). However, biomass accumulation of thalli cultured in nitrogen deplete media reached a plateau towards the end of the experimental period, whilst biomass of thalli supplied with nitrogen continued to increase. In comparison to the control, thalli starved of nitrogen increased in biomass at day 10 (Tukey; $P < 0.05$). Thalli supplied with nitrogen

followed the same trend as algae starved of nitrogen, with a statistically significant increase (Tukey; $P < 0.05$) in biomass occurring at day 14 compared to the start of the experiment, day 0.

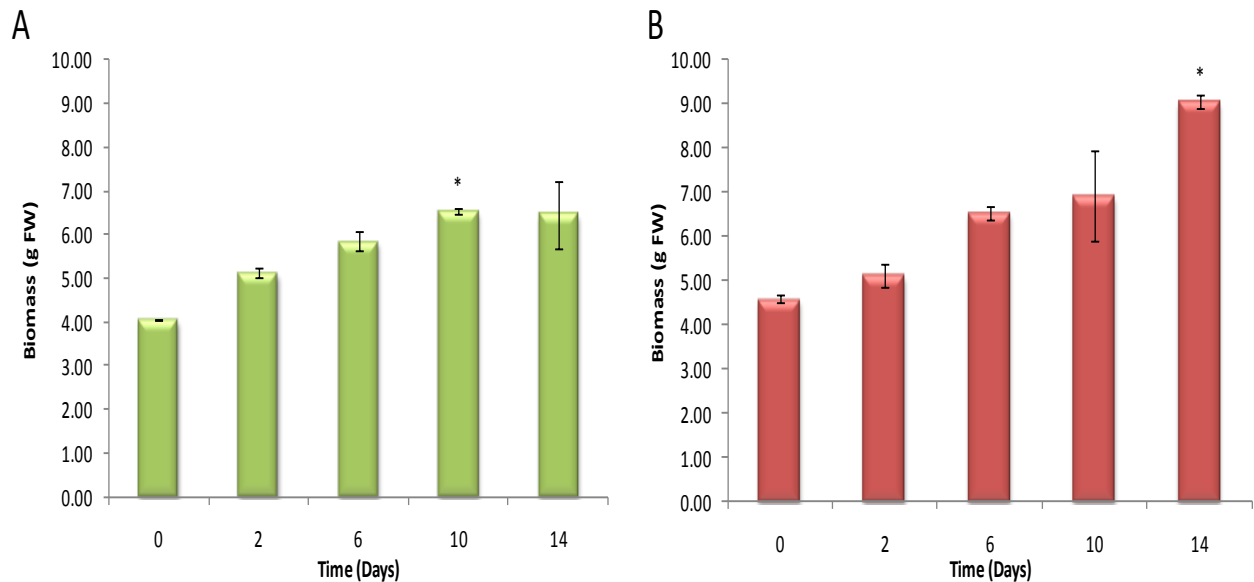


Figure 2. Biomass accumulation of *G. gracilis* over fourteen days of culture in (A) nitrogen deplete (PES-N) and (B) nitrogen replete (PES) media. Bars represent mean biomass (g FW) \pm SE ($n = 3$). The asterisk (*) indicates significant differences at each time point compared to the control (Time 0) (One-Way ANOVA, $P < 0.05$).

4.3.1.6.2 Specific Growth Rate

Contrary to results obtained for biomass analysis, the specific growth rate (SGR) of both algae starved of (Figure 3A) and supplied with (Figure 3B) nitrogen decreased over the experimental period. A greater than 50% reduction in SGR of thalli cultured in nitrogen deplete and replete media occurred at day 14 and day 10, respectively.

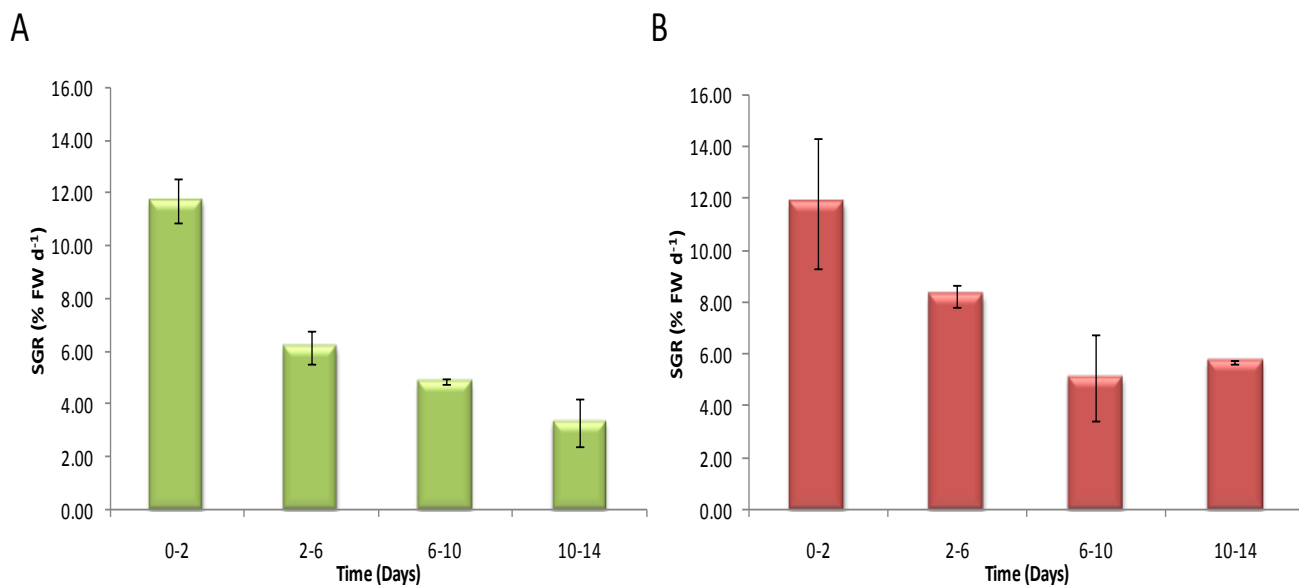


Figure 3. Specific growth rates (SGR) of *G. gracilis* over fourteen days of culture in (A) nitrogen deplete (PES-N) and (B) nitrogen replete (PES) conditions. Values represent the mean percent decrease per day \pm SE ($n = 3$).

4.3.2. *G. gracilis* NR and GS expression during culture in nitrogen deplete and replete conditions

The effect of nitrogen absence and input on NR and GS activity, transcript and protein abundance was evaluated by enzyme assays, qPCR and western hybridisation analysis, respectively.

4.3.2.1 Total NR and GS enzyme activity

NR activity of algae starved of nitrogen was statistically lower (Holm–Sidak, $P < 0.01$) than the control from day 6 (Figure 4A). As expected, NR activity of thalli supplied with nitrogen was high throughout the experimental period (Figure 4B). A striking difference between thalli cultured in nitrogen deplete and replete media was the difference in NR activity at day 0. This difference is likely due to the fact that different ‘batches’ of *G. gracilis* were used in nitrogen starvation and nitrogen enrichment studies

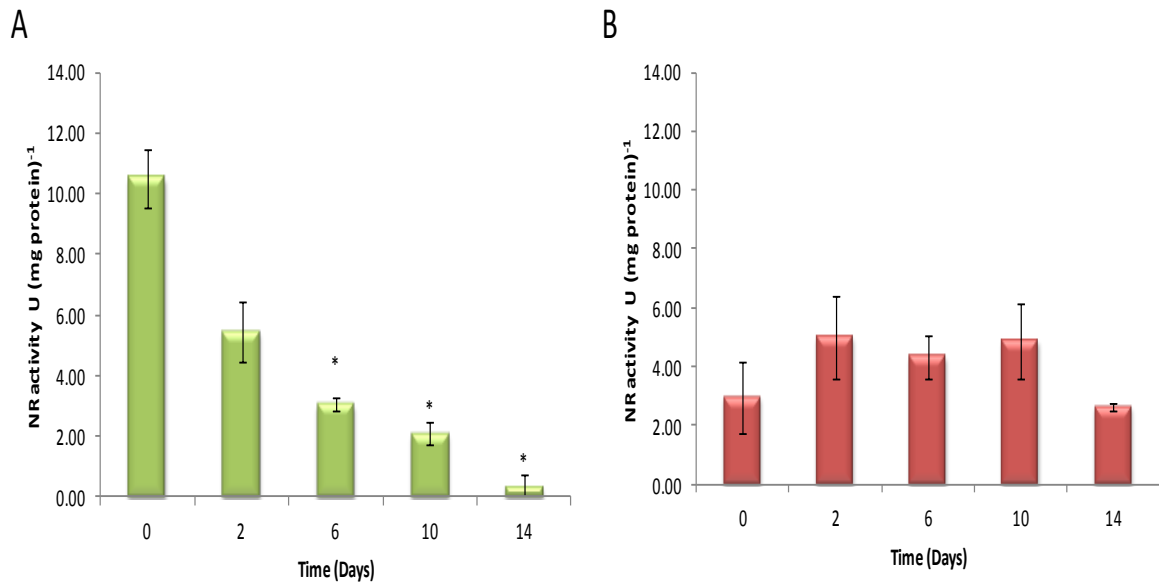


Figure 4. *G. gracilis* NR activity over fourteen days of culture in (A) nitrogen deplete (PES-N) and (B) nitrogen replete (PES) media. The mean activity is represented \pm SE ($n = 3$). Asterisk (*) represents statistically significant differences (One-Way ANOVA, $P < 0.05$) from the control (Time 0).

Total GS activity in thalli starved of nitrogen did not differ to the control (Figure 5A). However, the total GS activity increased by approximately 73% at day 14. Conversely, prolonged culture in nitrogen replete media resulted in a decrease in the total GS activity (Figure 5B). Compared to the control, GS activity of algae supplied with nitrogen was statistically lower (Holm-Sidak, $P < 0.05$) at days 6, 10 and 14.

4.3.2.2. *GgNR* and *GgGS₁* transcript abundance

GgNR transcript levels of thalli starved of nitrogen were lower than the control (Figure 6A). In general, transfer to nitrogen deplete medium resulted in a greater than 70% reduction in *GgNR* transcript levels. Prolonged culture in nitrogen replete conditions had no effect on *GgNR* gene expression (Figure 6B).

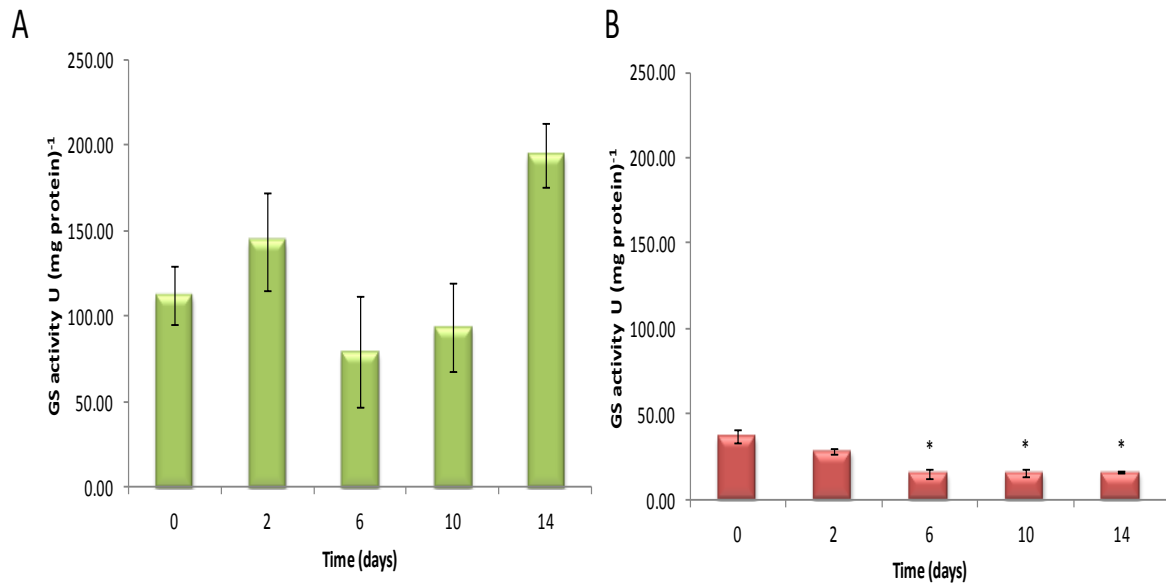


Figure 5. *G. gracilis* total GS activity over fourteen days of culture in (A) nitrogen deplete (PES-N) and (B) nitrogen replete (PES) media. The mean activity is represented \pm SE ($n = 3$). Asterisk (*) represents statistically significant differences (One-Way ANOVA, $P < 0.05$) from the control (Time 0).

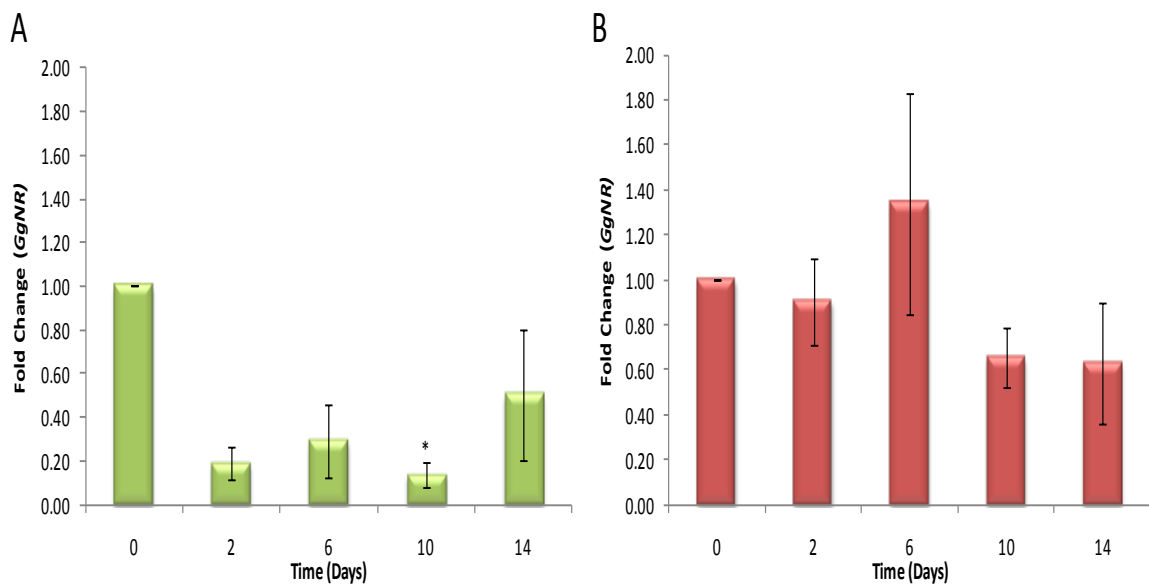


Figure 6. *G. gracilis* *GgNR* gene expression over fourteen days of culture in (A) nitrogen deplete (PES-N) and (B) nitrogen replete (PES) media. Expression data is the mean fold change in expression relative to the control (Time 0) \pm SE ($n = 3$). An asterisk (*) represents statistically significant (One-Way ANOVA, $P < 0.05$) differences at each time point compared to the control (Time 0).

GgGS₁ expression remained unchanged upon transfer to nitrogen deplete medium (Figure 7A). Similarly, *GgGS₁* transcript levels were constant in *G. gracilis* cultured in nitrogen replete media for ten days (Figure 7B). However, *GgGS₁* gene expression increased 2-fold and 3-fold in thalli starved of and supplied with nitrogen, respectively, after fourteen days.

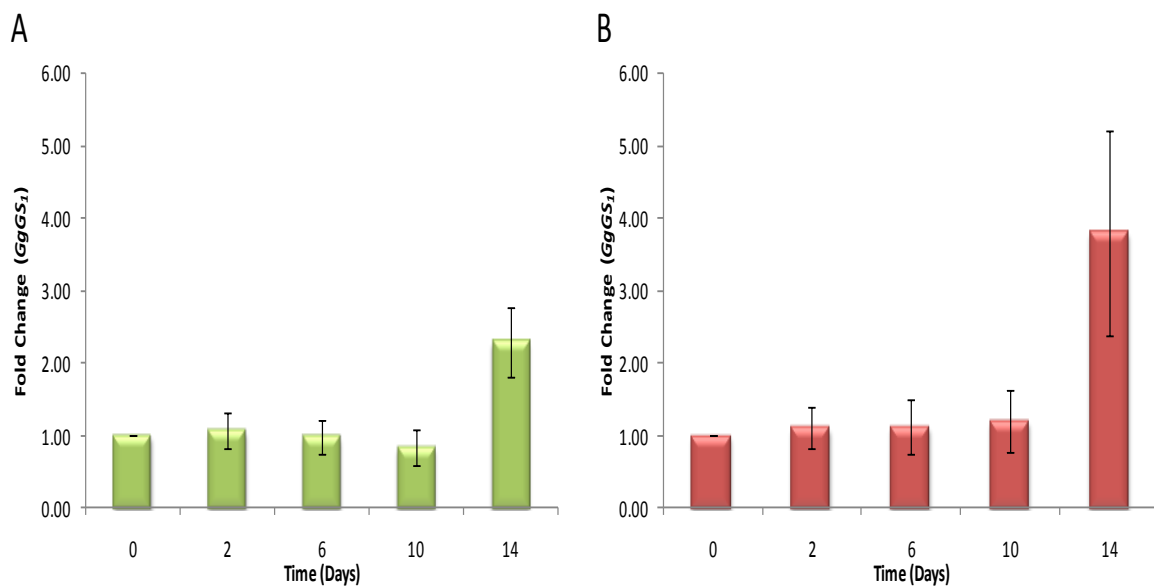


Figure 7. *G. gracilis* *GgGS₁* gene expression over fourteen of culture in (A) nitrogen deplete (PES-N) and (B) nitrogen replete (PES) media. Expression data is the mean fold change in expression relative to the control (Time 0) \pm SE ($n = 3$).

4.3.2.3. NR and GS protein expression

NR protein dropped below the detection level after two days of cultivation in nitrogen deplete media (Figure 8A). NR was detected throughout the fourteen day period when *G. gracilis* was cultured in nitrogen replete conditions (Figure 8B). A 2-fold increase in NR was observed at day 2 and increased further at day 6 and day 10 before a decrease was observed. Quantitative analysis confirmed that NR protein levels of algae cultured in nitrogen replete media increased at days 2, 6 and 10 compared to day 0 (Figure 8C).

The polyclonal commercial GS antibody, GLN1-2, employed for evaluating GS protein expression, cross reacted with *G. gracilis* GS₁ and GS₂ proteins. GS₂ was the predominant

isoform detected in when *G. gracilis* was starved of (Figure 9A) and supplied with (Figure 9B) nitrogen. GS₁ was detected after fourteen days of nitrogen starvation (Figure 9A). In comparison to the control, a 2.5-fold (Tukey, P<0.05) increase in GS₂ expression occurred after six days of culture in nitrogen deplete media (Figure 9C). After six days of culture in nitrogen deplete media, GS₂ decreased with the lowest amount of protein detected at day 14. GS₂ protein levels remained unchanged in *G.gracilis* cultured in nitrogen replete media (Figure 9D).

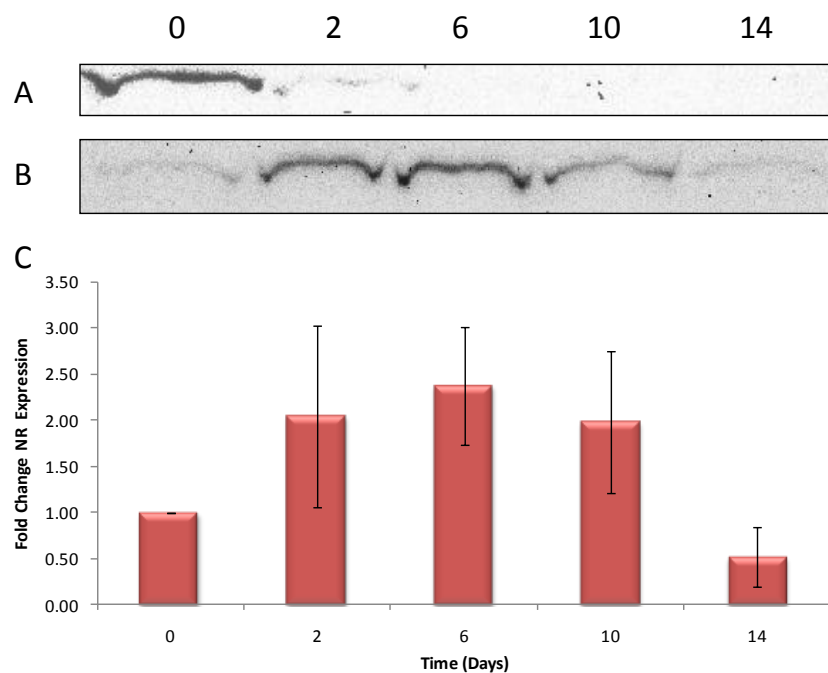


Figure 8. Western hybridisation analysis of NR protein expression in *G. gracilis* cultured in (A) nitrogen deplete (PES-N) and (B) nitrogen replete (PES) ASW over fourteen days. Forty micrograms of protein was separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with a 1:1000 dilution of AS08 210 NR primary antibody and a 1:20 000 dilution of the secondary antibody (alkaline phosphatase goat anti-rabbit IgG conjugate). A density (intensity.mm⁻²) for each positive signal was calculated using the Image Lab Software (version 2.0.1, BIORAD). Densities of the positive NR signals at day 2, 6, 10 and 14 were calibrated to the density of NR at Time 0. The graph (C) represents the fold change increase/decrease of NR at each time point relative to Time 0 ± SE (*n* = 3) of *G. gracilis* cultured in PES ASW.

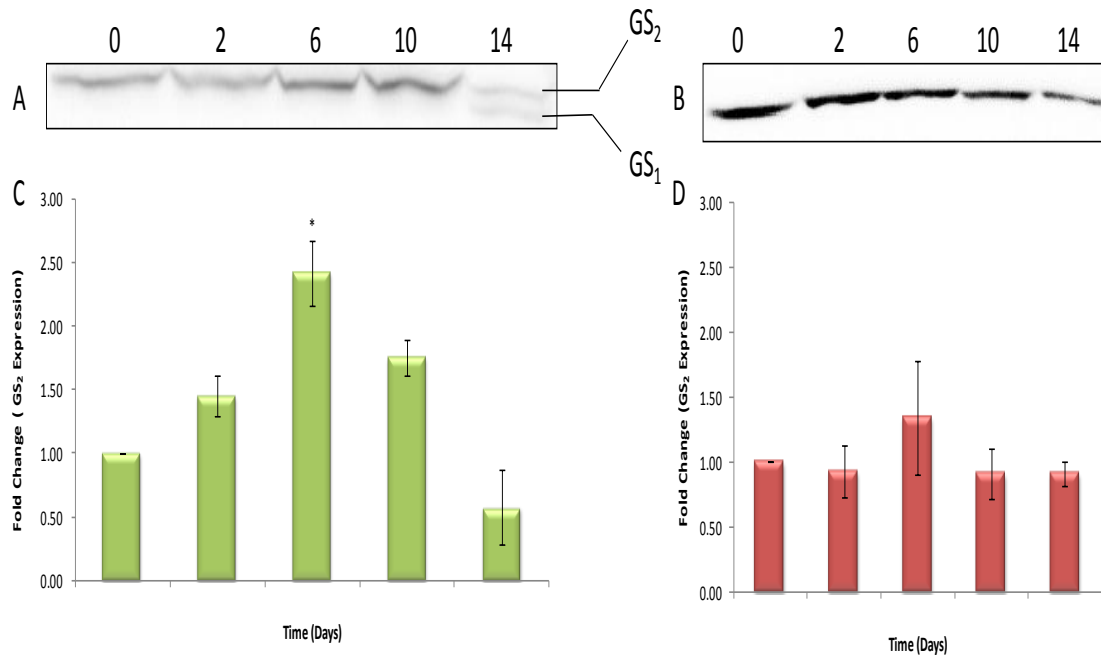


Figure 9. Western hybridisation analysis of GS₂ protein expression in *G. gracilis* cultured in (A) nitrogen deplete (PES-N) and (B) nitrogen replete (PES) ASW over fourteen days. Thirty micrograms of protein was separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with 1:10 000 dilution of GLN1-2 GS primary antibody and a 1:20 000 dilution of the secondary antibody (alkaline phosphatase goat anti-rabbit IgG conjugate). A density (intensity.mm⁻²) for each positive GS₂ signal was calculated using the Image Lab Software (version 2.0.1, BIORAD). Densities of the positive GS₂ signals at day 2, 6, 10 and 14 were calibrated to the density of GS₂ Time 0. The graphs represents the fold change increase/decrease of GS₂ at each time point relative to Time 0 ± SE (*n* = 3) of *G. gracilis* cultured in (C) PES-N and (D) PES ASW.

4.4 DISCUSSION

4.4.1 Analysis of physiological changes that occur when *G. gracilis* is cultured in nitrogen deplete and replete conditions

Physiological changes are indicative of metabolic changes within cells and are useful when determining the nitrogen status of macroalgae (Naldi and Wheeler, 1999). Observed decreases in thallus nitrogen, increased C:N ratio and discolouration of thalli in nitrogen starved growth conditions in the present study, are in agreement with findings by Gebrekiros (2003) and Lebi (2006). Measurement of intracellular tissue nitrogen content is a useful indicator of algal health status and growth (Hanisak, 1979). A reduction or increase in intracellular nitrogen content indicates whether intracellular nitrogen stores are being depleted or replenished in response to changes in the external environment. Furthermore, the measurement of intracellular nitrogen concentration is an established method for the determination of 'critical nutrient' concentrations for terrestrial plants, aquatic vascular plants and macroalgae (Hanisak, 1979). When intracellular nitrogen concentration falls below the 'critical nutrient' concentration, the alga is termed nitrogen limited (Smit *et al.*, 1997). Prolonged culture of *G. gracilis* in nitrogen deplete and replete conditions reduced intracellular nitrogen but algae starved of nitrogen became nitrogen limited whereas algae supplied with nitrogen did not. However, according to C:N ratios, both thalli starved of nitrogen and thalli supplied with nitrogen were nitrogen limited even though the thallus nitrogen concentration was greater than 1.7%. According to Hanisak (1979), C:N ratios should be interpreted with caution as this ratio, although important for indicating physiological changes in algae, is not an indication of the critical nitrogen concentration as shifts in carbohydrate metabolism impact the ratio even when nitrogen is not limiting. Thus, an increased C:N ratio in thalli supplied with nitrogen is likely to be a consequence of a shift in carbohydrate metabolism and not nitrogen limitation. Since biomass:volume was not kept constant for the duration of the experiment, reduced thallus nitrogen of *G. gracilis* supplied with nitrogen may be attributed to either restricted water movement, dilution of tissue nitrogen as biomass increased or the excretion of dissolved organic nitrogen during active growth as observed in *U. lactuca* (Teichberg *et al.*, 2007).

Pigments and proteins are also important nitrogen stores (Naldi and Wheeler, 1999). Phycobiliproteins that form part of the light harvesting complex are composed of phycocyanin and phycoerythrin, and represent the pigment storage forms in red algae (Lee, 1999). More specifically, phycoerythrin is the major nitrogen store in most macroalgae. These pigments are stored when nitrogen is available and used when nitrogen is depleted (Rico and Fernández, 1996). In some red macroalgae phycoerythrin can account for almost 60% of the total soluble protein and is responsible for the red colour (Gebrekiros, 2003). During starvation, PE is broken down and used as a nitrogen source. The reduction in phycoerythrin is responsible for the discolouration of *G. gracilis* thalli from the red-brown to green colour. A reduction in photosynthetic pigment content has also been documented when *Gracilaria gaditana* was cultured in low nitrogen growth conditions (Andria *et al.*, 1999). Phycoerythrin is also an important source of nitrogen during the early phase of nitrogen limitation in *Gracilaria tikvahiae* (Rico and Fernández, 1996). Thus, the considerable reduction in phycoerythrin in addition to the low intracellular nitrogen concentration after fourteen days of nitrogen starvation, may suggest that *G. gracilis* entered the late phase of nitrogen limitation.

In addition to metabolite changes, culture in nitrogen replete and starved conditions impacted *G. gracilis* biomass accumulation and growth rates. The accumulation of biomass in nitrogen deplete conditions is in agreement with findings of *Arabidopsis* plants grown under low nitrogen conditions (Tschoep *et al.*, 2009). The continued increase in biomass while specific growth rates decrease is a characteristic feature of how organisms respond to nutrient stress (Grossman, 2002). A shift in metabolism and metabolic machinery occurs where algal cells are able to grow and accumulate biomass, but cell division ceases and organisms enter into an almost dormant or semi-dormant phase that allows survival in unfavourable conditions. In addition, decreased growth rates in nitrogen replete *G. gracilis* thalli is not uncommon. A study by Touchette and Burkholder (2007) indicated that when the eelgrass *Z. marina*, adapted to living in nutrient limited environments, was exposed to a nitrate enriched environment, growth rates decreased as a consequence of unsaturated uptake and assimilation of nitrogen that depleted the available carbon reserves. As ammonium uptake in *G. gracilis* is not rate saturated (Smit, 2002), it is possible that reduced *G. gracilis* growth rates in

nitrogen replete culture conditions may be a consequence of reduced carbon concentrations due to the unsaturated uptake of nitrogen. However, unsaturated uptake of nitrogen is unlikely as reduced carbon stores would have resulted in lower C:N ratios. Furthermore, maximal *G. gracilis* growth rates were observed when the alga was cultivated in 1200 μM ammonium (Smit *et al.*, 1997), illustrating that *G. gracilis* has a larger nitrogen storage capacity than the eelgrass. Therefore, it is more likely that the growth rate of nitrogen replete *G. gracilis* increased up to a point and then declined in a similar manner to what has been observed in other algae growing in nitrogen rich environments (Lignell and Pedersén, 1987). As the SGR will only continue to increase until a saturation point of nitrogen is reached (Gebrekiros, 2003), once the saturation nitrogen concentration is reached, nitrogen assimilation is reduced and the result is a reduction in growth rate. Ammonium transported into the cells is likely to be stored as protein or other metabolites and thus contribute to the increased biomass, further illustrating the different shifts in metabolic machinery that allows the survival of *G. gracilis* in differing nutrient conditions.

4.4.2 Analysis of the effect of cultivation of G. gracilis in nitrogen replete and deplete conditions on NR and GS gene expression and regulation

An understanding of the regulation of gene expression is essential to understanding how metabolic shifts are mediated in response to changes in the external environment and internal changes within the cell. For example, nitrate assimilation genes can be induced in order to allow acclimation to nitrogen limited conditions (Imamura *et al.*, 2010). Genes strongly induced during nitrogen deficiency in cyanobacteria, included GS, PII and a small polypeptide protein required for phycobiliprotein degradation (Muro-Pastor *et al.*, 2001). Thus, gene expression analysis compliments the information gained from physiological responses.

G. gracilis NR transcript levels, protein abundance and activity was similar to findings in nitrate deficient and high nitrate replete plants (Klein *et al.*, 2000). The persistence of NR transcripts, protein and activity in *G. gracilis* cultured under nitrogen replete conditions was not unexpected, as the alga was continuously supplied with nitrogen. Reduction of NR protein and activity after fourteen days suggested that *G. gracilis* had

sufficient nitrogen reserves, thereby circumventing the need for continued nitrogen assimilation. These findings are in agreement with high nitrate grown cultures of the red macroalga *Gelidium pusillum* (Lartigue and Sherman, 2006) and *C. reinhardtii* (Llamas *et al.*, 2002). In comparison to *G. gracilis* cultured in nitrogen replete conditions, the detection of NR activity throughout the starvation period was unexpected as no external nitrogen (nitrate) source was available. Different responses of NR activity to nitrogen starvation have been reported (Berges *et al.*, 1995). NR activity either increased after nutrient exhaustion as seen in *Chlorella* species, *C.reinhardtii* and several species of marine phytoplankton, or a constant decline in NR activity was observed after nitrogen depletion. Increased NR activity may occur if algae are initially cultured in ammonium and then transferred to medium lacking nitrogen, resulting in the release of ammonium inhibition of NR and a subsequent increase in NR activity. However, the general trend of *G. gracilis* NR activity resembled the constant decline pattern. These findings are comparable with findings in nitrogen starved *G.chilensis* (Chow and de Oliveira 2008), the diatom *Thalassiosira pseudonana* (Berges *et al.*, 1995), brown macroalgae *Fucus serratus* and *Fucus vesiculosus* (Young *et al.*, 2009), and *Arabidopsis* plants (Tschoep *et al.*, 2009). Oxidative pathways in the cell can provide nitrate in the absence of a nitrogen supply (Berges *et al.*, 1995) and may account for NR activity in the absence of external nitrogen. However, it is generally accepted that many algae cannot re-oxidise organic nitrogen back to nitrate (Giordano *et al.*, 2005). Other possible inputs of nitrate may arise from trace amounts of nitrate contamination in ASW (Berges, *et al*, 1995). In studies with *T. pseudonana*, at least 1 μM nitrate was introduced from trace contamination of reagent grade salts, particularly sodium chloride. Furthermore, in the present study nitrate could have been released from bacteria in the growth media as *G. gracilis* cultures were non-axenic. However, according to Gebrekiros (2003) who compared external nitrate concentrations of axenic and xenic *G. gracilis* cultures, bacterial contribution to the external nitrate concentration is negligible. In addition, Gebrekiros (2003) found that extracellular nitrate was consumed within ten days after transfer to nitrogen free medium. Internal stores represent another source of nitrate for NR of nitrogen starved *G. gracilis* cells. Macroalgae are able to take up and store nitrate in large vacuoles (Smit *et al.*, 1997, Chow, 2012). Internal nitrogen stores of *Gracilaria* species can maintain growth for up to three weeks in nitrogen limiting conditions. Thus, once external nitrate was depleted, internal stores of nitrate may be

assimilated by the *G. gracilis* thalli. After fourteen days NR activity was reduced to almost undetectable levels suggesting that internal nitrate may be depleted.

NR regulation is mediated via transcription, translation and post-translational modifications that are initiated in response to changes in the external environment (Klein *et al.*, 2000). In the unicellular red alga *C. merolae*, nitrogen deprivation caused an increase in the nuclear located transcription factor CmMYB1 that binds to the promoter regions of nitrogen transport genes (*NRT*) and the nitrogen assimilation genes *CmNR* and *CmGS* (Imamura *et al.*, 2010). Unlike *C. merolae*, *G. gracilis* *GgNR* was not induced but down-regulated in nitrogen deplete conditions. This corresponded with the rapid reduction in NR protein and may indicate that the enzyme is regulated via transcription in addition to post-translational mechanisms that target the protein for degradation as documented for *G. chilensis* (Chow and de Oliveira, 2008). However, NR activity was still detected throughout the starvation period, despite the reduction in NR protein. This may be a consequence of the limitation of the commercial NR antibody used as it may not be sensitive enough to detect minimal amounts of NR. Conversely, similar to findings by Parker and Armbrust (2005), *G. gracilis* NR protein may be uncoupled from *GgNR* expression and NR activity because of changing environmental conditions. Another possibility is that like *Fucus* species, two forms of NR, namely an inducible and constitutive NR isoform (as found in higher plants), may exist in *G. gracilis* (Young *et al.*, 2009). The constitutive form may be responsible for the NR activity detected in nitrogen starved conditions whilst qPCR and western hybridisation analysis may have detected the inducible form. Two NR isoforms may be an adaptive advantage as it allows immediate assimilation when nitrogen becomes available. However, further investigation of all these possibilities is required. Nevertheless, findings in the present study suggest that *G. gracilis* NR protein synthesis was sometimes uncoupled from NR transcription and NR activity, and NR was likely to be regulated by transcriptional, translational and post-translational modifications that included protein turnover. In addition, similar to *G. chilensis*, nitrate may be required to prevent *G. gracilis* NR degradation (Chow and de Oliveira, 2008). However unlike *G. chilensis*, nitrate may not be required for *G. gracilis* NR biosynthesis. This theory was further supported by the reduction of NR protein over fourteen days of culture in nitrogen replete medium. Although nitrate was available, NR protein levels eventually decreased. Western

hybridisation of total protein extracts from nitrogen replete *G. gracilis* indicated that another metabolite of nitrogen assimilation or factor other than/in addition to nitrate may regulate NR translation/biosynthesis. During nitrogen deprivation, high affinity nitrate transport (HANT) systems of *C. reinhardtii* and *E. huxleyi* accumulated enough intracellular nitrate to maintain the constitutive expression of nitrate assimilation genes even though NR activity was reduced (Bruhn *et al.*, 2010). Therefore, it may be possible that *G. gracilis* also possesses HANT systems that allow the accumulation of enough intracellular nitrate to maintain a continuous positive signal for the expression of *GgNR* and possibly other assimilatory genes. When conditions become favourable, the protein can be translated and nitrogen assimilated. This may explain why after an initial reduction, *GgNR* transcripts remained unchanged in nitrogen starved *G. gracilis*. If *GgNR* transcription is induced by nitrate but the protein translated when conditions become favourable, it supports the hypothesis that although nitrate may not be necessary for NR translation/biosynthesis, it is necessary for the induction of *GgNR* transcription, further emphasising the numerous levels of NR regulation.

Similar to NR, an evaluation of GS expression revealed numerous mechanisms of gene regulation and their implications in allowing *G. gracilis* to acclimatise to different environmental conditions. Low *G. gracilis* GS activity in cultures supplied with nitrogen and high activity in thalli starved of nitrogen are congruent with findings in *Arabidopsis* plants cultured in high and low nitrogen conditions, respectively (Tschöep *et al.*, 2009). In *Chaetoceros affinis*, GS is responsible for nitrogen assimilation when cells are ammonium deficient while the alternate GDH pathway is the preferred route of assimilation when nitrogen is sufficient (Maurin and Gal, 1997). The role of the GDH pathway must be kept in mind when examining the response of *G. gracilis* to an input of nitrogen. For example, low GS activity in nitrogen replete conditions may mean that the GDH pathway is in operation in *G. gracilis*. However, it is generally accepted that the GS-GOGAT pathway is the established route of ammonia assimilation in macroalgae (Lobban and Harrison, 1994). In nitrogen replete conditions, carbon skeletons and nitrogen metabolites are high and GS activity is low (Mérida *et al.*, 1991). In addition, increased amino acid concentrations, particularly glutamine, can cause a reduction in GS activity. Therefore, low *G. gracilis* GS activity in nitrogen replete conditions is likely attributable to the fact that the thalli were nitrogen replete and a switch in metabolism

from nitrogen assimilation to nitrogen storage had occurred. This switch in metabolism is supported by a low C:N ratio and reduced SGR. Conditions like nitrogen starvation diminish the C:N ratio, resulting in increased GS activity in order to restore homeostasis (Mérida *et al.*, 1991). This response is typically documented in cyanobacteria and is in agreement with findings in *G. gracilis*. In general, *G. gracilis* GS activity of thalli starved of nitrogen was far greater than thalli supplied with nitrogen replete. Differences in GS activity of *G. gracilis* cultured in nitrogen deplete and replete media may be attributed to differences in intracellular concentrations of nitrogen, carbon and amino acids. However, GS activity of *E. huxleyi* cells exposed to nitrogen starved conditions was stimulated no matter the physiological state of the cells (Maurin and Gal, 1997). This type of response has been observed in *G. gracilis* thalli cultured in nitrogen starved conditions, even though thalli were not nitrogen limited (data not shown). Furthermore, Paone and Stevens (1981) observed that the type of nitrogen source affected the increase in GS activity of nitrogen starved *Agmenellum quadruplicatum*. Highest *A. quadruplicatum* GS activity, following transfer to nitrogen free medium, was measured in cells pre-cultured in nitrate followed by urea and then ammonium. Therefore, one needs to consider whether the marked increase in GS activity in thalli starved of nitrogen was a consequence of pre-culturing *G. gracilis* in PES supplemented ASW that contained predominantly nitrate.

The induction of GS activity by nitrogen starvation in *C. reinhardtii* and *Chlorella sorokiniana* was suggested to be a result of increased protein synthesis. Western hybridisation techniques using the commercial antibody GLN1-2 rarely detected *G. gracilis* GS₁. The absence of GS₁ may be explained by the observation that GLN1-2 has a greater affinity for the GS₂ isoform or GS₁ may be a minor isoform in *G. gracilis* thalli and present in lower amounts, making its detection more difficult. However, despite the possible limitation of antibody sensitivity, findings in the current study suggested that GS activity was not regulated through increased protein expression, but rather post-translational activation of an already present protein. For example, stimulation of GS activity in nitrogen starved *G. gracilis* occurred without a corresponding increase in GS₂ protein. Furthermore, the 2-fold increase in GS₂ protein after six days of culture in nitrogen deplete conditions did not correspond with increased total GS activity whereas GS₁ protein was only detected after fourteen days of culture. The mechanism of post-

translational control of *G. gracilis* GS was further substantiated by nitrogen replete studies where a decrease in enzyme activity did not correspond to a decrease in GS₂ protein. In addition, there seems to be a critical concentration of total intracellular nitrogen where GS₂ expression is induced, but the protein is not active. Synthesis of inactive GS was reported in legume roots grown in nitrogen limited conditions (Peat and Tobin, 1996). It may be possible that increased GS₂ protein expression may be an adaptive response initiated when thalli reach their critical nitrogen concentration and may serve to ready the alga for rapid assimilation when ammonium becomes available. However, further investigation is required. In order to elucidate the regulatory mechanisms controlling the expression and activity of *G. gracilis* GS isoforms, investigations to determine the isoform that accounts for the majority of enzyme activity in nitrogen replete and deplete conditions must be completed.

The findings in this study do however show strong support for post-translational regulation of *G. gracilis* GS isoforms. GS is post-translationally regulated by a variety of cellular mechanisms, one of which is oxidative modification which results in degradation of the protein (Temple *et al.*, 1996). Oxidative modification is mediated by increased reactive oxygen species (ROS) and it is possible that *G. gracilis* GS₂ may undergo oxidative modification which ultimately targets the enzyme for degradation. ROS are known to participate in the oxidative modification and degradation of GS the unicellular green alga *C. braunii* (Humanes *et al.*, 1995), the cyanobacterium *Prochlorococcus marinus* strain PCC 9511 (Gómez-Baena, 2006) and in wheat GS₂ (Palatnik *et al.*, 1999). In addition, reduced activity with no reduction in GS₂ protein in nitrogen replete thalli, suggested that other mechanisms are also responsible for post-translational modification of GS. This discussion will focus on the proposed hypothesis for the oxidative degradation of *G. gracilis* GS₂. Photosynthetic processes can be modified when nutrients are low (Grossman, 2000). The result is reduced photosynthetic electron transport (PET) that causes the plastoquinone pool to become reduced, resulting in the phosphorylation of the light harvesting complex (LHC) PSII. Energy is then directed to PSI, a process that is advantageous to the plant because energy production (ATP) and reductant (NADPH) is favoured. However, nutrient deprivation and low light can cause the PET system to be fully reduced, resulting in an increased redox potential and the accumulation of ROS. Moreover, GS activity was

linked with accessory pigment and nitrogen concentrations in the nitrogen starved cyanobacterium *A. quadruplicatum*, suggesting that a relationship exists between energy yielding processes of photosynthesis and nitrogen metabolism (Paone and Stevens, 1981). Phycobiliproteins have a dual role, namely nitrogen storage and photosynthesis in red algae (Lignell and Pedersén, 1987). In *Gracilaria lemaneiformis*, it is reported that increases in the photosynthetic pigment phycoerythrin could be partly responsible for increases in the rate of photosynthesis (Yu and Yang, 2008). Thus, it is possible that decreases in phycoerythrin could partly reduce the rate of photosynthesis. *G. gracilis* phycoerythrin concentrations were nearly depleted after fourteen days of nitrogen starvation, indicating that photosynthesis may have been reduced resulting in a fully reduced PET system and in turn an increase in ROS that resulted in the oxidative modification and degradation of GS₂. In order to confirm this hypothesis, further investigation of the described processes is required.

The degradation of GS₂ may have implications for GS₁ regulation. The proposed role for GS₁ is the assimilation of ammonia from protein degradation and the reassimilation of glutamine during senescence (Kamachi *et al.*, 1991). Reduction in *G. gracilis* GS₂ activity/protein would cause a reduction in the cellular glutamine/glutamate (Gln:Glu) ratio. Reduced Gln:Glu concentrations could serve as a positive signal for *GgGS₁* expression and may explain the sudden increase in *GgGS₁* transcripts, protein and possibly activity after fourteen days of nitrogen starvation. However, many other factors may explain or affect *GgGS₁* transcription in the present study. *GgGS₁* transcript levels seemed to peak after prolonged culture in nitrogen replete conditions without a corresponding increase or detection of GS₁ protein or GS activity. The limitation of the commercial GLN1-2 antibody has already been discussed. However, other possibilities may explain differences between *GgGS₁* gene and GS₁ protein expression. For example, *Arabidopsis* has five different GS₁ genes that are differentially regulated in response to different conditions (Ishiyama *et al.*, 2004). Thus, in order to fully understand GS regulation in *G. gracilis*, and in particular *GgGS₁*, it is important to establish whether *G. gracilis* has more than one GS₁ gene. *GgGS₁* was originally identified from a *G. gracilis* disease stressed cDNA library (Ealand, 2011). Increased bacterial pathogenicity when algae are exposed to nitrogen starvation has been documented (Cren and Hirel, 1999). Therefore, increased *GgGS₁* could be a defence response in *G. gracilis* starved of

nitrogen, and possibly thalli supplied with nitrogen. Furthermore, studies performed on cyanobacteria indicated that a correlation between mRNA and GS activity was not always observed (Muro-Pastor *et al.*, 2001). Moreover, an accumulation of GS₁ and GS₂ mRNA did not determine protein abundance in senescing rice leaves (Kamachi *et al.*, 1991). Senescence is induced by nutrient deprivation, age and darkness (Park *et al.*, 1998). During early senescence, rice GS₁ mRNA remained constant but transcript levels increased by 3-fold in the middle phase of senescence, followed by greater increases during late senescence (Kamachi *et al.*, 1991). Therefore, nutrient deprivation induced senescence in *G. gracilis* thalli could have contributed to increased GS₁ mRNA transcripts. In addition, similar to rice leaves, increases in *GgGS₁* may indicate the stage of senescence in *G. gracilis* thalli. The onset of senescence is supported by a reduction of GS₂ protein in nitrogen starved thalli after fourteen days of culture in nitrogen deplete medium. GS₂ protein levels of rice plants decreased and was almost lost at late senescence (Kamachi *et al.*, 1991). However, to confirm this hypothesis later time points would need to be sampled to determine whether *GgGS₁* increases further and GS₂ is no longer detected. Conversely, increased *GgGS₁* in nitrogen replete thalli after fourteen days of culture may indicate the onset of senescence as a consequence of thallus age. Thus, *GgGS₁* may not be responsive or may have multiple roles that include the regulation of nitrogen nutrition, senescence and disease stress responses in *G. gracilis*. All of these factors warrant further investigation of factors controlling *GgGS₁* expression.

The present study has emphasised that there are many factors at play in the regulation of *G. gracilis* NR and GS. In summary, these findings have highlighted the complexity of the regulation of nitrogen metabolism and its enzymes in *G. gracilis* cells. Metabolic shifts are impacted by nitrogen availability in the external environment and nitrogen metabolic enzymes are responsible for co-ordinating these shifts. Novel findings include the identification of possible mechanisms regulating NR and GS expression, the possibility of inducible and constitutive NR isoforms, and the presence of GS₁ and GS₂ *G. gracilis* isoforms. In addition, there is evidence to suggest that *GgGS₁* may have multiple roles in *G. gracilis* cellular metabolism and that interplay between photosynthesis and nitrogen metabolism is likely to regulate the GS isoforms of *G. gracilis*. Finally, NR and GS seem to be key enzymes in regulating/initiating metabolic shifts in nitrogen deplete and replete cells, respectively. This study provides a good

starting point for further investigations of nitrogen stress mechanisms in *G. gracilis* that include distinguishing the specific roles of NR and GS isoforms and investigating how different nitrogen sources effect NR and GS expression.

CHAPTER 5

The effect of different nitrogen sources on nitrate reductase and glutamine synthetase expression in nitrogen starved *Gracilaria gracilis*

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5.1 INTRODUCTION

Transcription, translation and enzyme activity of plant, green algal and cyanobacterial NR and GS are regulated by external cues that include inorganic nitrogen sources (Takabayashi *et al.*, 2005). Plant species differ in their response to nutrients and generally grow better when they have access to nitrate and ammonium (Claussen and Lenz, 1999). However, certain species may prefer one form of nitrogen over another (Nicodemus *et al.*, 2008). Furthermore, although extracellular nitrogen regulates the assimilatory genes NR, GS₁ and GS₂, the extent of the regulation is dependent on the plant species, nitrogen source and plant organ or tissue (Zozaya-Hinchliffe *et al.*, 2005).

Investigations examining the response of macroalgal nitrogen metabolic enzymes to different nitrogen sources are useful as nutrient limitation is overcome when nitrate or ammonium is introduced into the natural environment (Lartigue and Sherman, 2005). However, the time taken to recover from nitrogen limitation is dependent on the nitrogen storage capacity of the alga and the rate of nitrate assimilation. Thus, understanding how macroalgae respond to the supply of different nitrogen sources can aid in the development of nitrogen pulse or “renewal” strategies that minimise algal energy costs associated with nitrogen uptake and assimilation (Lartigue and Sherman, 2005; Nicodemus *et al.*, 2008).

Therefore, the aim of the current study was to investigate the effect of different nitrogen sources on NR and GS expression in nitrogen starved *G. gracilis*. A molecular evaluation of mRNA, protein and enzyme activity was performed as described (Chapter 4) to achieve the aims of this study. However, in addition to total GS activity, GS₁ and GS₂ activity were measured in this study.

5.2 MATERIALS AND METHODS

All media and solutions in this study are listed in Appendix A. *G. gracilis* was sourced and pre-cultured as described (3.2.1).

5.2.1 Experimental design

G. gracilis cultures were initiated with a 4 g FW inoculum of alga per 0.8 L culture medium. Prior to the start of the experiments, the alga was acclimated to the photoperiod of 16:8 (L:D) by pre-culture, for seven days, in PES-N ASW.

Following the acclimation period, fresh PES-N ASW was added to the flasks, followed by the exogenous addition of nitrogen, followed by a second addition 48 h later. In total, three nitrogen treatments were performed: nitrate (100 μM NaNO_3), ammonium (100 μM NH_4Cl) and nitrate+ammonium (100 μM each of NaNO_3 and NH_4Cl). Three biological repeats were set-up for each nitrogen treatment.

Cultures grown in nitrogen free media (for 7 d) represented the control (designated Time 0). Thalli were sampled 0.5, 2 and 96 h after the addition of nitrogen. The entire algal sample was homogenised in a mortar and pestle using liquid nitrogen and stored at -80°C prior to being used for enzyme assays, RNA isolations (2.2.1.1) and protein isolations (4.2.5). The complete thallus was sampled to prevent any bias when weighing separate pieces of a single thallus. When required, frozen samples for enzymes assays, RNA and protein isolations were removed from -80°C and immediately re-suspended in the appropriate extraction buffers.

5.2.2 Isolation and measurement of GS₁ and GS₂ activity

5.2.2.1 Isolation of cytosol and chloroplast fractions

Intact *G. gracilis* chloroplasts were isolated according to the differential centrifugation method described by Hapgonian *et al.* (2002) with modifications.

Frozen algal material (2 g) was removed from -80°C and immediately re-suspended in 4 mL extraction buffer (Appendix 6.1). The homogenate was filtered through cheesecloth and centrifuged at 3000 x *g* for 1 min to pellet unbroken cells and cell fragments. This step was repeated until the cell suspension was free of cell debris. Following centrifugation, 1 mL of the supernatant fraction was removed, transferred to a fresh microfuge tube and flash frozen in liquid nitrogen to be used in total GS activity measurements. The remaining supernatant was decanted into a fresh JA20 tube and centrifuged at 6000 x *g* for 10 min to pellet the chloroplasts. The supernatant was decanted into a fresh JA20 tube, flash frozen with liquid nitrogen and retained for measurement of GS₁ activity. The pelleted chloroplasts were re-suspended in 500 µl extraction buffer, transferred to a fresh microfuge tube and flash frozen in liquid nitrogen.

5.2.2.2 Preparation of crude extracts for measurement of total GS, GS₁ and GS₂ activity

All enzyme assays were completed 24 h after sampling. Total GS, GS₁ and GS₂ fractions (5.2.2.1) were thawed at 37°C and the suspension was centrifuged at 10 000 x *g* for 5 min. The supernatant fraction was retained for measurement of enzyme activity.

5.2.2.3 Testing the purity of cytosol fractions

The purity of cytosol fractions was tested by performing western blots using the antibody PsbA (Agrisera). Since PsbA cross reacts with the chloroplast located D1 protein, an important component of PSII, it is a useful marker for testing the contribution of chloroplast 'contamination' to isolated cytosol fractions.

Following enzyme assays, the remaining cytosolic and chloroplastic protein fractions were precipitated (4.2.5) and SDS-PAGE and western hybridisation was performed as described (4.2.6). Fifteen micrograms of protein from each fraction was separated on 10% resolving gels by SDS-PAGE (4.2.6.1). Following SDS-PAGE, proteins were transferred to nitrocellulose membranes and western hybridisation was completed as described (4.2.6.2). Membranes were incubated with a 1:3000 and 1:5000 dilution of

the primary antibody, PsbA, and secondary antibody (peroxidase labelled goat anti-rabbit IgG (H+L) liquid conjugate), respectively.

5.2.3 *In vitro* enzyme assays

5.2.3.1 NR assay

NR extraction and assays were performed as described (3.2.3 and 3.2.4).

5.2.3.2 GS transferase assay

The GS transferase assay was used to measure GS activity as the extraction buffer used to isolate the cytosol and chloroplast protein fractions was not compatible with the forward GS assay reaction mix. A reaction occurred when the stop reagent was added to the forward reaction assay mix, thereby producing a false positive measurement for enzyme activity. However, this reaction did not occur when the GS transferase assay mix was used.

Total GS, GS₁ and GS₂ activity was determined according to Thomas *et al.* (1984) with modifications. The assay reaction mixture consisted of 5 µg total protein for GS₂ activity and 10 µg total protein for the GS₁ and total GS enzyme assays. Each protein sample was added to 0.23 mL GS transferase assay mix (Appendix 4.4) and adjusted to a final volume of 0.5 mL by the addition of 0.1 M Tris pH7.5. The reaction was initiated by the addition of the total protein extract and the enzymatic reaction was allowed to proceed for 30 min at 25°C. Controls that lacked glutamine were prepared. The reaction was stopped by the addition of 0.5 mL of GS assay stop reagent (Appendix A 4.7). The assay mixture was centrifuged at 10 000 x *g* for 5 min at 20°C to remove precipitates. The appearance of γ-glutamyl hydroxamate (GGH) was determined by measuring the absorbance at 540 nm. GGH (Sigma) was used to generate a standard curve. One unit of GS was defined as the amount of enzyme required to produce 1 µmol of GGH per min at 25°C.

5.2.4 Evaluation of NR and GS expression

GgNR, *GgGS₁* (4.2.3, 4.2.4) and protein expression (4.2.5, 4.2.6) were evaluated as described.

5.2.5 Statistical analysis

Statistical analysis was performed using SigmaStat version 3.1 (Systat Software™ Inc. GmbH, 2004). One way Anova analysis was used to determine statistical differences in gene expression data. Log10 transformations were conducted on data that was not normally distributed. $P < 0.05$ was used to define statistical significance. Descriptive statistics (mean, standard deviation and standard error) were determined using Microsoft Excel (Microsoft Office 2007, Microsoft Corporation).

5.3 RESULTS

Previous studies have reported that *G. gracilis* subjected to eighteen days of culture in nitrogen lacking media recovered within four days following the resupply of nitrogen (Gebrekiros, 2003). Therefore, this recovery time was used as a guideline for the sampling timescale employed in the current investigation. In addition, to gain a greater understanding of the role of GS in nitrogen metabolism into *G. gracilis*, GS₁ and GS₂ activity was assayed in the three nitrogen treatments.

5.3.1 Assessment of the purity of cytosol fractions

The approximately 32 kDa D1 chloroplast protein was detected in both chloroplast and cytosol fractions (Figure 1A). A higher molecular weight polypeptide was detected and may represent either non-specific binding or a complex of PsbA and another protein. According to the manufacturer's (Agrisera) guidelines, the detection of higher molecular weight polypeptides is not uncommon with this antibody. Even though PsbA was detected in both fractions, the amount of PsbA in the cytosol fractions was minimal, while the majority of PsbA was detected in the chloroplast fractions. Ponceau S staining of the membrane indicated that protein samples from both the chloroplast and cytosol fractions were generally equally loaded and transferred, thereby confirming the western hybridisation findings (Figure 1B). Thus, the contribution of GS₂ activity to that of GS₁ in the cytosol fractions was deemed to be minimal.

5.3.2 The effect of the resupply of nitrogen on NR mRNA, protein and activity in nitrogen starved *G. gracilis*

Changes in NR mRNA (*GgNR*), protein and activity in the three nitrogen treatments were compared to NR expression of the control (Time 0), which represented nitrogen starved *G. gracilis*. While significant differences in *GgNR*, NR protein and NR activity was not detected when either nitrate, ammonium or nitrate+ammonium was supplied to nitrogen starved *G. gracilis*, distinct trends were observed.

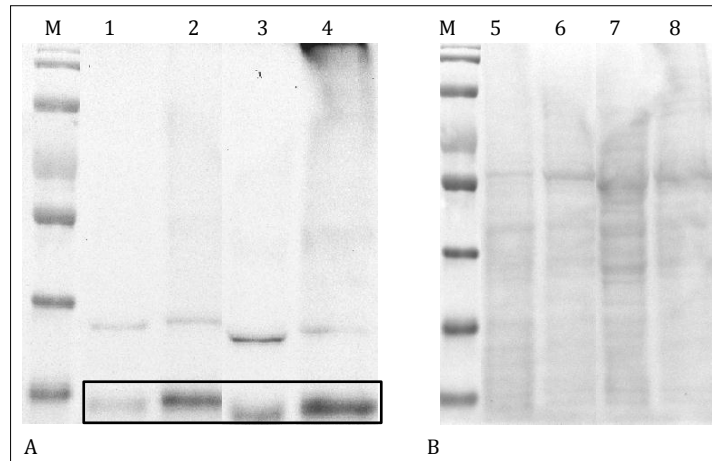


Figure 1. Evaluation of the purity of the cytosol fractions. **(A)** Fifteen micrograms of protein was separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with a 1:3000 dilution of PsbA primary antibody and a 1:5000 dilution of the secondary antibody (alkaline phosphatase goat anti-rabbit-IgG conjugate). **(B)** Ponceau S staining of the membrane was carried out to ensure that the proteins were equally loaded and transferred. Lanes 1 & 5: nitrate treatment, 0.5 h cytosolic fraction; lanes 2 & 6: nitrate treatment, 0.5 h chloroplast fraction; lanes 3 & 7: ammonium treatment, 2 h cytosolic fraction; lanes 4 & 8: ammonium treatment, 2 h chloroplast fraction. A pre-stained molecular weight marker (M) was used to size the detected proteins. The 32 kDa D1 protein is outlined by the black box.

GgNR induction (Figure 2A), increased NR protein (Figure 2B) and increased NR activity (Figure 2C) occurred 30 min (0.5 h) after the addition of nitrate to nitrogen-lacking medium. NR protein levels remained unchanged two hours after nitrate had been added to the nitrogen limited culture (Figure 2B), while NR activity decreased and later increased again at 96 h (Figure 2C). Similarly, *GgNR* was induced (1.5-fold increase) and NR protein increased 30 min after ammonium had been added to the nitrogen-lacking medium. However, the increased NR protein levels in the ammonium treated culture (0.5 and 2 h) did not coincide with increased NR activity in *G. gracilis* sampled at these time points. Instead, NR activity of the ammonium treated cultures remained unchanged, while prolonged culture (96 h) resulted in a reduction in NR protein (Figure 2B) that may be due to protein degradation (Figure 2B & 2D). Interestingly, increased NR activity was detected in the ammonium treated culture after 96 h despite, the reduced NR protein levels detected at this time point.

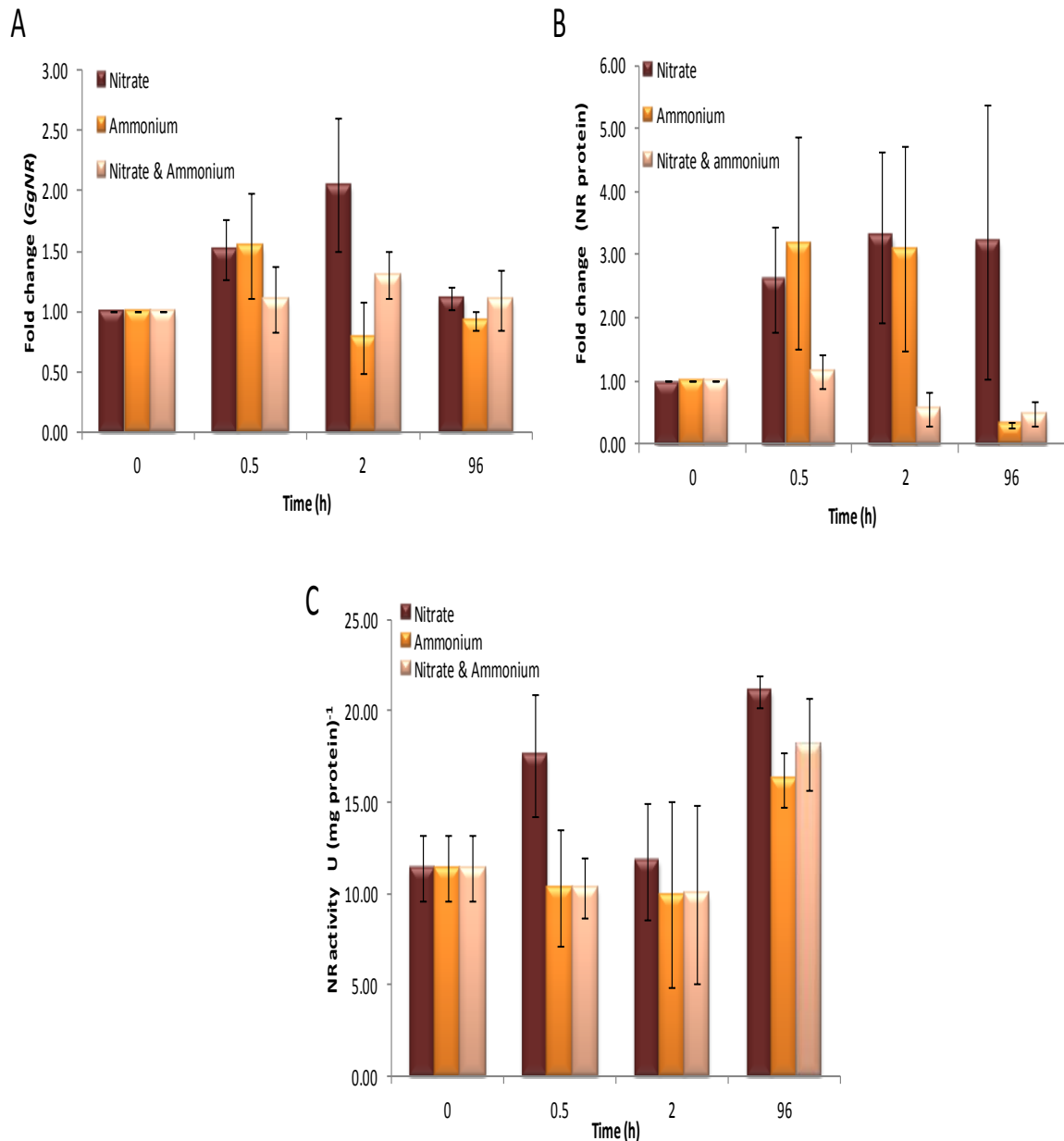


Figure 2. The effect of the addition of nitrate, ammonium or nitrate+ammonium on (A) NR mRNA (*GgNR*), (B) NR protein and (C) NR activity of *G. gracilis* cultured in nitrogen deplete (PES-N) ASW. The control (Time 0) represents thalli cultured in PES-N ASW before the addition of nitrogen. Thalli were sampled 0.5, 2 and 96 h after the addition of nitrogen. (A): *GgNR* transcript abundance is represented as the fold change in expression compared to the control \pm SE ($n = 3$). (B): fold change increase/decrease of NR protein at each time point relative to Time 0 \pm SE ($n = 3$). The density (intensity. mm^{-2}) of each positive NR signal was calculated using the Image Lab Software (version 2.01, BIORAD). Densities of positive NR signals at 0.5, 2 and 96 h were calibrated to the density of NR at Time 0. (C): mean NR activity \pm SE ($n = 3$). One unit of NR is defined as the amount of enzyme required to reduce 1 μmol of nitrate to nitrite in 1 min at 37°C.

In contrast, *GgNR* remained unchanged in the nitrate+ammonium treatment (Figure 2A) while a gradual reduction in NR protein was observed over the course of the experiment (Figure 2B). Once again, the reduced NR protein levels did not correspond with NR activity which remained unchanged for the first 2 h after nitrate and ammonium had been added to the culture medium, and then increased by approximately 2-fold after 96 h (Figure 2C).

5.3.3 The effect of the resupply of nitrogen on GS mRNA, protein and activity of nitrogen starved *G. gracilis*

Changes in GS mRNA (*GgGS₁*), GS₁ and GS₂ protein, and total GS, GS₁ and GS₂ activity in the three nitrogen treatments were compared to the control (Time 0), which represented nitrogen starved *G. gracilis*. Similar to NR, even though significant differences in *GgGS₁*, GS₁ and GS₂ protein and total GS, GS₁ and GS₂ activity were not always detected when nitrate, ammonium and nitrate+ammonium was supplied to nitrogen starved *G. gracilis*, distinct trends were observed.

5.3.3.1 The effect of the resupply of nitrogen on *GgGS₁* mRNA, GS₁ protein and GS₁ activity in nitrogen starved *G. gracilis*

A 1.5-2 fold increase in *GgGS₁* transcripts occurred after 0.5 h and was maintained for the duration of the experimental period in the nitrate treatment (Figure 3A). Similar increases in *GgGS₁* after 0.5 and 2 h were observed in the ammonium and the nitrate+ammonium treatment, followed by a reduction after 96 h. Increased *GgGS₁* did not correspond to increased GS₁ protein. Instead, GS₁ expression was not reproducible between biological repeats (Figure 3C-E). Instead, multiple GS₁ proteins that did not exhibit a distinct pattern of expression were detected at certain sampling points. However, compared to the control, the re-supply of nitrogen to nitrogen starved *G. gracilis* induced GS₁ activity in the three nitrogen treatments. GS₁ activity in the nitrate+ammonium treatment was statistically higher (Holm-Sidak, $P < 0.05$) than the control at 0.5 and 2 h (Figure 3B). Although not statistically significant, an approximately 2-fold increase in GS₁ activity occurred 30 min after the addition of ammonium and was maintained for the duration of the experimental period (Figure

3B). The induction of GS₁ activity by exogenous nitrate was slower, with an approximately 1.5-fold increase occurring after 2 h.

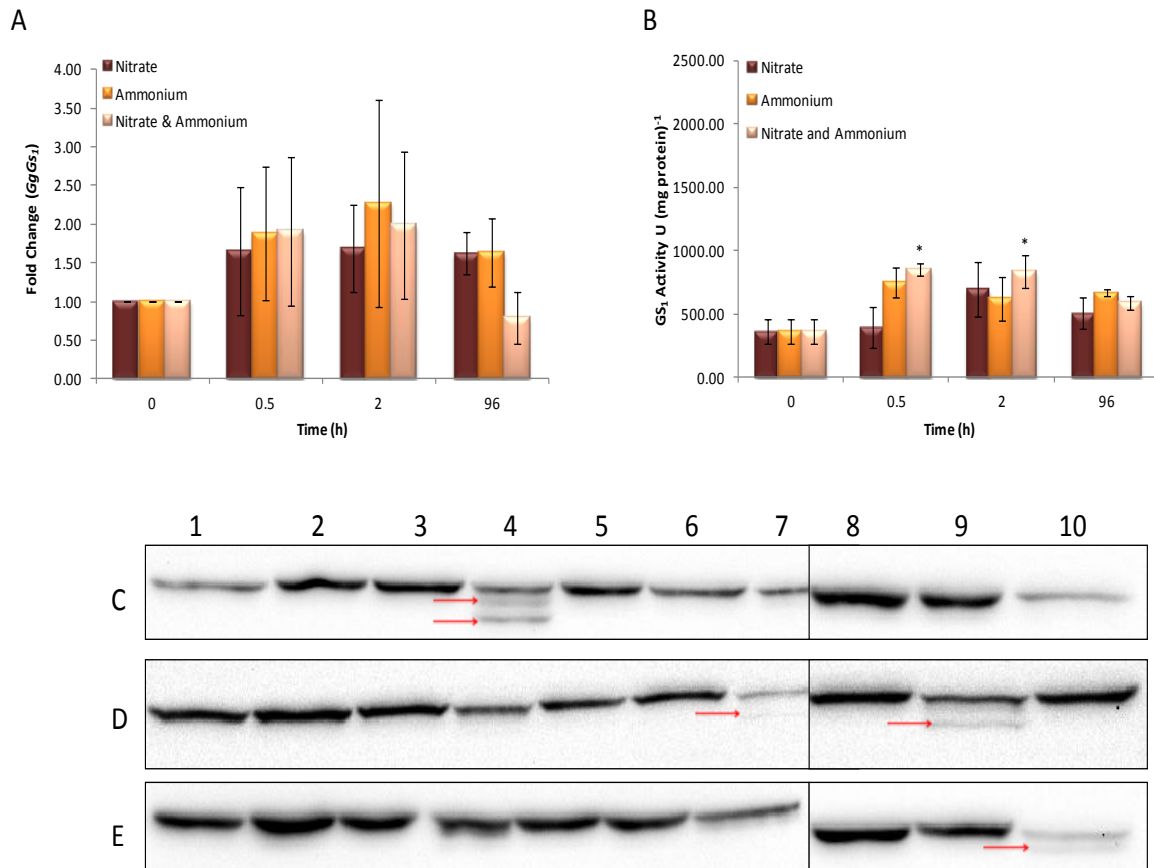


Figure 3. The effect of the addition of nitrate, ammonium or nitrate+ammonium on (A) *GgGS1* (B) GS₁ activity and (C, D, E) GS₁ and GS₂ protein expression in *G. gracilis* cultured in nitrogen deplete (PES-N) ASW. The control (Time 0) represents thalli cultured in PES-N ASW before the addition of nitrogen. Thalli were sampled 0.5, 2 and 96 h after the addition of nitrogen. (A): *GgGS1* transcript abundance is represented as the fold change in expression compared to the control \pm SE ($n = 3$). (B): mean GS₁ activity \pm SE ($n = 3$). One unit of GS₁ is defined as the amount of enzyme required to produce 1 μ mol of γ -glutamyl hydroxamate (GGH) per min at 25°C. (C,D,E): Thirty micrograms of protein was separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with a 1:10 000 dilution of the GLN1-2 GS primary antibody and a 1:20 000 dilution of the secondary antibody (alkaline phosphatase goat anti-rabbit IgG conjugate). Western hybridisation results for biological repeats 1, 2 and 3 are represented by C, D and E, respectively. GS₁ proteins are indicated by the red arrows. Lane 1: Time 0; lanes 2-4: 0.5, 2 and 96 h following nitrate treatment; lanes 5-7: 0.5, 2 and 96 h following ammonium treatment and lanes 8-10: 0.5, 2 and 96 h following nitrate+ammonium treatment. Asterisk (*) represents data that is statistically significant in comparison to the control (One-way ANOVA, $P < 0.05$).

5.3.3.2 The effect of the resupply of nitrogen on GS₂ protein expression in nitrogen starved *G. gracilis*

In general, exogenous nitrate, ammonium and nitrate+ammonium induced a 1.5-2 fold increase in *G. gracilis* GS₂ protein at 0.5 h (Figure 4A). Thereafter, a reduction in GS₂ protein occurred in the ammonium and the nitrate+ammonium treatments. In contrast, statistically significant increases in GS₂ protein expression occurred 0.5, 2 and 96 h (Holm-Sidak, $P \leq 0.05$) following nitrate addition. GS₂ protein expression did not correspond with GS₂ activity in the three nitrogen treatments (*cf.* Figure 4A and 4B). Instead, despite increased synthesis of GS₂ protein, GS₂ activity was not affected by the re-supply of nitrate, ammonium or nitrate and ammonium to nitrogen starved *G. gracilis* (Figure 4B).

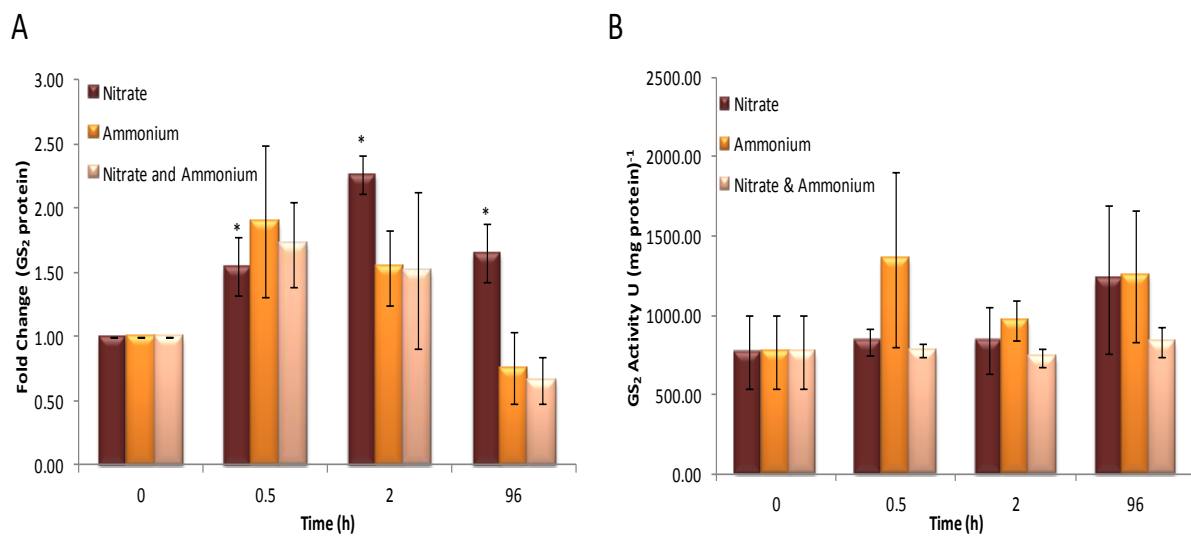


Figure 4. The effect of the addition of nitrate, ammonium or nitrate+ammonium on (A) GS₂ protein (B) GS₂ activity of *G. gracilis* cultured in nitrogen deplete (PES-N) ASW. The control (Time 0) represents thalli cultured in PES-N ASW before the addition of nitrogen. Thalli were sampled 0.5, 2 and 96 h after the addition of nitrogen. (A): fold increase/decrease of GS₂ protein at each time point relative to the control, Time 0 \pm SE ($n = 3$). A density (intensity.mm⁻²) of each positive GS₂ signal was calculated using the Image Lab Software (version 2.0.1, BIORAD). Densities of the positive GS₂ signals at 0.5, 2 and 96 h were calibrated to the density of the positive GS₂ signal at Time 0. (B): mean GS₂ activity \pm SE ($n = 3$). One unit of GS₂ is defined as the amount of enzyme required to produce 1 μ mol of γ -glutamyl hydroxymate (GGH) per min at 25°C. Asterisk (*) represents data that is statistically significant in comparison to the control (One-way ANOVA, $P < 0.05$).

5.3.3.3 The effect of the resupply of nitrogen on total GS activity in nitrogen starved *G. gracilis*

Although a statistically significant difference in total GS activity was not detected when nitrate was supplied to nitrogen starved *G. gracilis*, a sustained increase, approximately 1.5-fold after 0.5 h to 3-fold after 96 h, was observed (Figure 5A). Total GS activity increased significantly 0.5 and 96 h (Holm-Sidak, $P < 0.05$) after the addition of ammonium and 96 h (Holm-Sidak, $P < 0.05$) following nitrate+ammonium treatment (Figure 5A). The majority of total GS activity was attributed to GS₂ in the nitrate and the ammonium treatments. GS₁ activity accounted for less than 40% of total GS activity when nitrogen was supplied as either nitrate or ammonium (Table 1; Figure 5B & 5C). However, GS₁ represented an average of approximately 40-45% of total GS activity in the nitrate+ammonium treatment over the 96 h time course of the experiment (Table 1; Figure 5D).

Table 1. Percentage GS₁ and GS₂ activity in nitrate (NO₃⁻), ammonium (NH₄⁺) and nitrate+ammonium (NO₃⁻+NH₄⁺) treatments.

Time (h)	% GS ₁ activity			% GS ₂ activity		
	NO ₃ ⁻	NH ₄ ⁺	NO ₃ ⁻ +NH ₄ ⁺	NO ₃ ⁻	NH ₄ ⁺	NO ₃ ⁻ +NH ₄ ⁺
0	33.87	33.87	33.87	66.13	66.13	66.13
0.5	29.44	39.84	52.13	70.56	60.16	47.87
2	46.84	37.77	52.63	53.16	62.23	47.37
96	31.57	37.88	41.63	68.43	62.12	58.37

GS₁ and GS₂ activity is expressed as a percentage of the total GS activity for nitrate, ammonium and nitrate+ammonium treatments ($n = 3$).

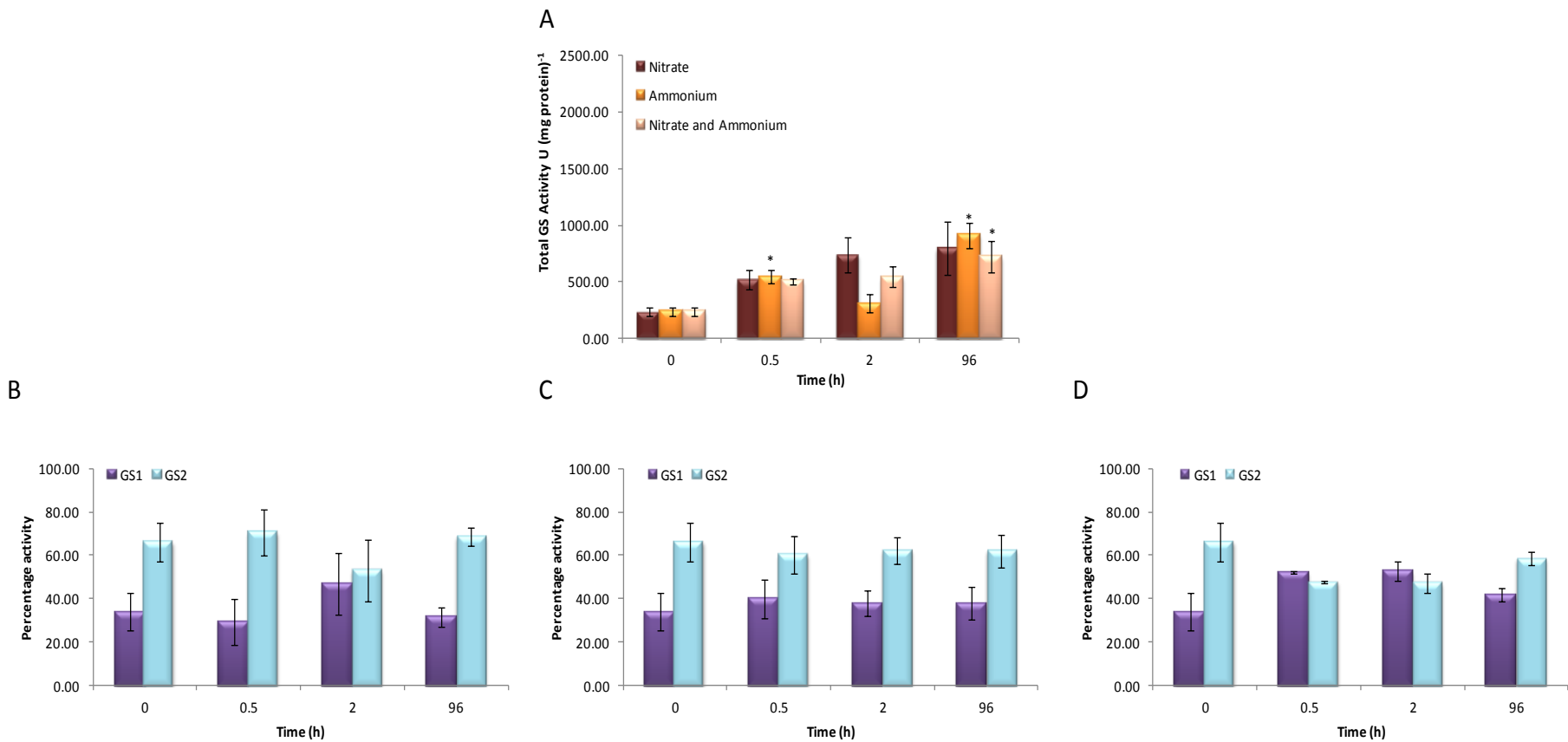


Figure 5. The effect of the addition of nitrate, ammonium and nitrate+ammonium on (A) total GS activity of *G. gracilis* cultured in nitrogen deplete (PES-N) ASW. The control (Time 0) represents thalli cultured in PES-N ASW before the addition of nitrogen. Thalli were sampled 0.5, 2 and 96 h after the addition of nitrogen. Bars represent the mean activity \pm SE ($n = 3$). Asterisk (*) represents statistically significant differences (One-Way ANOVA, $P < 0.05$) from the control (Time 0). GS₁ and GS₂ activity is expressed as a percentage of the total GS activity for (B) nitrate, (C) ammonium and (D) nitrate+ammonium treatments. Bars represent the mean percentage \pm SE ($n = 3$).

5.4 DISCUSSION

5.4.1 Effect of different nitrogen sources on NR expression in nitrogen starved *G. gracilis*

Plants, algae and fungi induce the expression of assimilation genes in response to nutrient signals within minutes of nitrogen becoming available (Llamas *et al.*, 2002). The rapid induction of plant NR in response to the addition of nitrate is a feature of the primary (early/rapid) response of plants to nutrient signals. Nitrate induction is known to increase NR mRNA and subsequently NR activity within a matter of hours (Chow, 2012). *GgNR* mRNA levels of *G. gracilis* cultured with nitrate as the sole nitrogen source, is congruent with findings in maize roots (Redinbaugh and Campbell, 1993) and alfalfa (Zozaya-Hinchliffe *et al.*, 2005) that were supplied with nitrate. In the present study, the approximately 1.5-2 fold increase in *G. gracilis GgNR*, NR protein and NR activity after 0.5 h may represent a primary response to nitrate addition. After the initial induction, *G. gracilis* NR activity returned to its original level. The rapid assimilation of nitrate is congruent with a study conducted by Smit (2002) who observed that when 50 μM nitrate was supplied to nitrogen starved *G. gracilis*, the external nitrate was completely taken up by the alga after approximately 2 h (Smit, 2002). A 2.5-fold increase in *G. chilensis* NR activity occurred within two minutes after the addition of nitrate and decreased to initial values after fifteen minutes (Chow and de Oliveira, 2008), whereas an increase in NR activity occurred between 4-7 h after the addition of nitrate to nitrogen starved *F. serratus* and *F. vesiculosus* (Young *et al.*, 2009). The increase in the NR activity of *F. serratus* and *F. vesiculosus* was consistent with *de novo* protein synthesis of NR. When considering the rate of nitrate uptake in *G. gracilis* and the induction of NR activity in *G. chilensis*, *F. serratus* and *F. vesiculosus*, the findings suggest that future studies should include more sampling points over a 96 h time course as it is possible that insufficient sampling points in the current study may have missed earlier or later responses to nitrogen addition.

The addition of ammonium to nitrogen starved *G. gracilis* resulted in an initial transient increase in *GgNR* followed by a reduction, while the addition of nitrate+ammonium did not affect *GgNR* transcript levels. These findings contrast with studies in

Chlamydomonas (Llamas, 2002) and *C. merolae* (Imamura *et al.*, 2010). *CmNR* transcripts in the red microalga *C. merolae* were not detected when cells were grown with nitrate and ammonium, and only detected when nitrate was the sole nitrogen source (Imamura *et al.*, 2010). Ammonium addition did not induce the *Chlamydomonas Nia1* promoter (induces NR gene expression), while an increased supply of ammonium to nitrate grown cultures reduced NR transcripts (Llamas *et al.*, 2002). The authors suggested that expression of the *Nia1* promoter was dependent on competition between positive nitrate signals and negative ammonium/derivatives of ammonium signals. Similar regulatory effects exerted by competing nitrate and ammonium signals may explain why no induction of *GgNR* occurred when both nitrogen sources were supplied to nitrogen starved *G. gracilis*.

Initial increases in *GgNR* levels in the ammonium treatment corresponded with an increase in NR protein whereas NR protein levels decreased in the nitrate+ammonium treatment. It must be emphasised that during the 96 h time course, either an increase or decrease in NR protein levels did not necessarily mirror NR activity levels in the three nitrogen treatments. Transcription and/or activity of proteins can be up-regulated, provided that the genes are inducible (Takabayashi *et al.*, 2005). Thus, differences in *GgNR*, NR protein and NR activity allude to the existence of inducible and constitutive *G. gracilis* NR isoforms. Similar to ammonium cultured *T. pseudonana* and *D. tertiolecta*, continued *G. gracilis* NR activity in the ammonium treatment could be attributed to a constitutive (non-ammonium repressible) isoform of NR (Berges *et al.*, 1995). Conversely, increased *GgNR*, NR protein and NR activity after 0.5 h in the nitrate treatment may be attributed to a nitrate inducible isoform. In the nitrate+ammonium treatment, competition between nitrate and ammonium signals may be influencing the activity of inducible and constitutive *G. gracilis* NR isoforms and may explain the discrepancies between the enzyme assays and western hybridisation. It is possible that the enzyme assays measured total NR activity whereas western hybridisation may be evaluating the expression of only one of the NR isoforms. Differences in NR protein and activity may also be explained by differential post-translational regulation of inducible and constitutive isoforms, as NR can be regulated post-translationally by degradation processes or phosphorylation and dephosphorylation of the enzyme (Chow, 2012).

Other factors may also be responsible for the differences detected between protein expression and enzyme activity. Firstly, increased *G. gracilis* NR activity after 96 h in the nitrate+ammonium treatment may be due to decreased extracellular ammonium levels that resulted in the release of nitrate uptake inhibition and consequently, an increase in nitrate assimilation. Smit (2002) observed that ammonium concentrations greater than 5 μM reduced the uptake rate of nitrate in *G. gracilis*, but once extracellular ammonium levels fell below 5 μM , inhibition of nitrate uptake was no longer present. In contrast, NR activity increased 96 h after the addition of ammonium to nitrogen starved *G. gracilis*. This increase in NR activity may be a consequence of increased growth of *G. gracilis*. In comparison to the control, nitrogen starved *G. gracilis* displayed greater increases in specific growth (SGR) rate 96 h after the addition of ammonium (data not shown). Suspension cultures of the free flowering plant Paul's scarlet rose showed increased NR activity, which was related to growth rate, when cells were supplied with ammonium (Mohanty and Fletcher, 1976). Increased cell number of Paul's scarlet rose cultures was observed in ammonium supplemented cultures compared to those without ammonium and is congruent with the SGR data obtained for *G. gracilis* cultured in ammonium supplemented media. However, the relationship between NR activity and growth rate in ammonium cultured *G. gracilis* would need to be investigated further. A third possibility is that extra- and intracellular concentrations of nitrogen may or may not influence gene expression and enzyme activity. Nitrate concentration in the medium is known to modify the biochemical composition (concentration of different metabolites) and metabolic processes of plants and macroalgae (Cabello-Pasini *et al.*, 2011). For example, the response of NR activity to the input of nitrogen was slower in nitrogen starved *Fucus* species compared to fresh/nitrogen replete *Fucus* species, even though internal nitrate and ammonium concentrations increased more considerably in nitrogen starved *Fucus* (Young *et al.*, 2009). Conversely, the addition of nitrate directly enhanced NR activity in some Arctic species of macroalgae and was independent of the nitrogen status of the cell (Gordillo *et al.*, 2006). Fourthly, studies in *Fucus* species suggested that factors other than extra- and intracellular nitrogen concentration may influence enzyme activity (Young *et al.*, 2009). NR response to the input of nitrogen in nitrogen starved *Fucus* species was found to be dependent on the availability of carbon skeletons and photosynthetic enzyme pools. Therefore, similar to *Fucus*, pre-culture of *G. gracilis* in nitrogen lacking media may have reduced carbon skeletons from

photosynthesis and photosynthetic enzyme pools like Rubisco (Andria *et al.*, 1999; Young *et al.*, 2009). A reduction in these pools could have attenuated the induction of *G. gracilis* NR activity and may explain the 1.5-2-fold increase in NR activity after 96 h in the three nitrogen treatments. Since *G. gracilis* recovered from nitrogen starvation 96 h after the re-supply of nitrogen (Gebrekiros, 2003), it is likely that carbon stores and photosynthetic enzyme pools are replenished after 96 h. However, further investigation of all these hypotheses is required.

The data obtained from the ammonium and nitrate+ammonium treatments highlighted an important difference between *G. gracilis* and other algae. Even though ammonium addition may have attenuated the induction of *G. gracilis* NR, ammonium did not completely reduce NR mRNA and activity. This contrasts with studies with unicellular algae, the brown macroalga *Giffordia mitchellae*, green macroalgae, plants and diatoms (Young *et al.*, 2007). Ammonium insensitivity of *G. gracilis* NR activity is congruent with studies conducted on *Fucus* species, (Young *et al.*, 2007), *G. pusillum* (Lartigue and Sherman, 2006), *T. pseudonana* (Berges, 1997) and dinoflagellates. Ammonium inhibition of NR activity in the red macroalga *G. chilensis* was attributed to ammonium toxicity (Chow and de Oliveira, 2008). Therefore, it seems that *G. gracilis* has a larger nitrogen storage capacity and is thus able to withstand higher ammonium concentrations. Tolerance for high ammonium concentrations has been further validated by other studies that showed that maximal *G. gracilis* growth was achieved when the alga was supplied with nitrogen pulses of 1200 μM ammonium (Smit *et al.*, 1997). Increased NR protein in the presence of ammonium further emphasises the ammonium insensitivity of *G. gracilis* NR. These findings extend the argument that nitrate may not be necessary to induce the biosynthesis (referring to synthesis of NR protein) of NR (Berges *et al.*, 1995) as an approximately 3-fold increase in NR protein occurred between 0.5–2 h following the re-supply of ammonium to nitrogen starved *G. gracilis*. The increase in *G. gracilis* NR protein in the ammonium treatment may have been a response to pre-culture in nitrogen deplete conditions, and may position the alga to assimilate nitrate once it becomes available. Since nitrate is the most abundant form of inorganic nitrogen in marine environments (Valiela, 1984; Chow and de Oliveira, 2008), this may be an adaptive response to growth in an environment where ammonium is less abundant. The reduction in NR protein at 96 h in the ammonium

treatment suggests that although nitrate may not be necessary for the biosynthesis of NR, it may be necessary for protecting the enzyme from degradation (Berges, 1997). However, this hypothesis is not supported by findings in the nitrate+ammonium treatment where a reduction in NR protein occurred even though nitrate was present. Since ammonium uptake is energetically more favourable (Chow *et al.*, 2012) and high ammonium concentrations (greater than 5 μM for *G. gracilis*) are able to saturate nitrate uptake (Smit, 2002), the concentration of ammonium in the present study (100 μM) may have saturated nitrate uptake thereby inhibiting NR synthesis, as a consequence of the accumulation of ammonium and its derivatives.

Therefore, besides the effect of nitrate, *GgNR* mRNA levels, NR biosynthesis and NR activity may be regulated by the energetic cost incurred by the uptake and assimilation of nitrate as opposed to ammonium, other metabolites of nitrogen assimilation, carbon skeletons and photosynthesis. Moreover, these factors may differentially regulate inducible and constitutive NR isoforms.

5.4.2 Effect of the addition of different nitrogen sources on GS expression in nitrogen starved *G. gracilis*

Before addressing GS expression, the purity of the *G. gracilis* chloroplast fractions will be discussed. While the cytosolic enzyme, sucrose phosphate synthase (SPS), is employed as a marker for testing the contribution of cytosol 'contamination' to chloroplast fractions (Agrisera), sucrose has not been detected in red algae (Eaton-Rye *et al.*, 2012) and SPS is not likely to occur in red algal cells. This is supported by the results of western hybridisation studies that failed to detect SPS when an anti-SPS antibody (Agrisera) was used to probe total, cytosol and chloroplast *G. gracilis* protein extracts (data not shown). Therefore, SPS could not be used in this study to test for cytosolic contamination of the chloroplast fractions. In addition, it is not certain whether other available cytosol compartment markers only occur in the cytosol of *G. gracilis* and thus, may not be suitable for testing the purity of chloroplast fractions. Future studies should identify suitable chloroplast, cytosol and other subcellular compartment markers for *G. gracilis*. When purifying subcellular fractions, researchers routinely assay the activity of enzymes found exclusively in the cytosol and chloroplast

as well as other subcellular compartments (Padh, 1992). These marker enzymes, as they are known, assess the level of contamination by other organelles such as mitochondria, and indicate the degree of enrichment of the desired organelle. Therefore, identifying suitable marker enzymes for *G. gracilis* would aid future work that involves the isolation of enzymes from different subcellular organelles. This approach was not feasible in the current study as chloroplast yield and therefore, total protein yield of the chloroplast fractions, was too low to allow the assaying of multiple enzymes or detection of multiple proteins using western hybridisation. Although it would be possible to overcome this problem by increasing the mass of *G. gracilis* used to extract chloroplasts, the amount of alga that can be inoculated into flask cultures is limited and the nature of the available culturing facilities made it impossible to scale up the *G. gracilis* cultures. For example, while Hapgonian *et al.* (2002) were able to isolate 30-50 µg of plastid DNA from 100 g of *G. tenuistipitata*, this approach is neither practical nor realistic when performing experiments over a time course. Since the differential centrifugation method employed in this study resulted in dense sediments of intact chloroplasts (Appendix B3), it is likely that the presence of *G. gracilis* cytosolic proteins in the chloroplast fraction was negligible. Thus, GS₂ activities were assayed from chloroplast fractions. However, GS₂ activity data should be viewed as preliminary, and require further validation as the assessment of the purity of chloroplast fractions is necessary.

GgGS₁ transcription was induced within 0.5 h in the three nitrogen treatments. Induction of *GgGS₁* following the addition of nitrate is in agreement with findings of nitrate treated alfalfa roots (Zozaya-Hinchliffe *et al.*, 2005) and the maize GS₁ genes *pGS1c* and *pGS1d*, whereas ammonium induction of *GgGS₁* is congruent with findings of maize *pGS1a* and *pGS1b* transcripts (Sakakibara *et al.*, 1996). The differential regulation of maize GS₁ transcripts in response to the addition of different nitrogen sources emphasises the need to determine whether *G. gracilis* has more than one GS₁ gene. Data obtained from the western hybridisation experiments suggest that at least two GS₁ isoforms are present in *G. gracilis*. In addition, *G. gracilis* GS₁ protein was not consistently detected between biological repeats, and protein levels did not correlate with changes in *GgGS₁* mRNA levels. Lack of congruency between *GgGS₁* mRNA and GS₁ protein levels is in agreement with studies that have shown that GS₁ transcript increases can significantly exceed the corresponding protein level as a result of regulatory processes

downstream of transcription (Temple *et al.*, 1996). The sporadic detection of *G. gracilis* GS₁ isoforms may be explained by the assembly and stability of the oligomeric protein. The migration pattern of nine different GS₁ isoenzymes from *P. vulgaris*, each containing different proportions of γ and β subunits, was dependent on which subunits were associated with one another while the stability of the *P. vulgaris* isoforms determined whether they were detectable (Temple *et al.*, 1996). Therefore, since *G. gracilis* may contain more than one GS₁ gene, each encoding a particular subunit, association of different subunits to form different hetero-oligomers may explain the sporadic pattern of lower molecular weight GS₁ proteins.

Even though GS₁ protein was not consistently detected, GS₁ activity was induced in the three nitrogen treatments. This finding is congruent with those reported for French bean, pea and soybean roots (Temple *et al.*, 1996). In addition, concomitant increases in GS₁ activity and protein levels were not detected in French bean, pea and soybean. The induction of *G. gracilis* GS₁ activity by the addition of ammonium following nitrogen starvation is in agreement with studies conducted on nitrogen starved *E. huxleyi* (Maurin and Gal, 1997). In contrast, re-supply of nitrogen to nitrogen starved *G. gracilis* did not induce GS₂ activity, no matter the nitrogen source. However, biosynthesis of *G. gracilis* GS₂ protein was induced in the three nitrogen treatments. Increases in *G. gracilis* GS₂ protein without a corresponding increase in GS₂ activity was unexpected as GS₂ is responsible for primary nitrogen assimilation in plants, bacteria, cyanobacteria (Maurin and Gal, 1997; Gómez-Baena *et al.*, 2006), micro- and macroalgae (Taylor *et al.*, 2006). Since nitrogen has been re-supplied to nitrogen starved *G. gracilis*, it is expected that the GS₂-GOGAT cycle would be induced. Failure to detect an increase in GS₂ activity may be explained by insufficient sampling time points (the initial induction may have been missed and activity may represent basal levels) or as a consequence of pre-culture in nitrogen starved conditions. Since nitrogen starvation induces starch synthesis (Nyvall *et al.*, 1999), the addition of nitrogen would require starch to be mobilised to soluble carbohydrate stores before nitrogen could be assimilated. Moreover, GOGAT requires 2-oxoglutarate and reductant from the TCA cycle and photosynthesis, respectively. Thus, GS₂ activity may increase once starch reserves are mobilised and these metabolites are available for GOGAT. In contrast, since GS₁ is responsible for nitrogen translocation (Bruhn *et al.*, 2010), induction of GS₁ activity in response to the

re-supply of nitrogen may be to provide nitrogen metabolites for the synthesis of proteins that were depleted due to culture in nitrogen lacking media. Another explanation may be that in contrast to GS₁, *G. gracilis* GS₂ activity may not be substrate inducible. For example, GS₁ transcripts in *C. reinhardtii* were repressed by ammonium and induced by nitrate, whereas GS₂ transcripts were not affected by either nitrogen source (Chen and Silflow, 1996). The same regulatory controls may apply to *G. gracilis* GS₁ and GS₂ activity. These hypotheses would need to be investigated further.

Total GS activity of nitrogen starved *G. gracilis* was induced independently of the nitrogen source and is congruent with findings with maize roots exposed to ammonium (Sakakibara *et al.*, 1996). Conversely, total GS activity of *C. sorokiniana* was found to be dependent on the type of nitrogen source supplied and was suppressed by ammonium (Tischner and Hüttermann, 1980) whereas total GS activity remained unchanged no matter the nitrogen source supplied to blueberry, raspberry and strawberry plants (Claussen and Lenz, 1999). Nitrogen starvation was shown to attenuate the effect of ammonium on GS activity in the cyanobacterium *Synechocystis* species strain PCC 6803 (Mérida *et al.*, 1991). When exposed to longer periods of nitrogen starvation, there was a slower inactivation of GS upon transfer to ammonium containing media. Similarly, insensitivity of *G. gracilis* GS activity to ammonium repression may be a consequence of pre-culture in nitrogen lacking media. The insensitivity of *G. gracilis* nitrogen metabolic enzymes to ammonium inhibition may be an adaptive response to growth in an environment where nitrogen availability fluctuates. Therefore, the ability to take up and assimilate either nitrogen source when it becomes available ensures the survival of the alga.

Insight into the role and adaptive advantages multiple GS isoforms confer to plants and algae can be obtained by examining the ratios and relative activities of GS isoforms (McKnally *et al.*, 1983; Casselton *et al.*, 1986). Similar to sunflower cotyledons (Cabello *et al.*, 1998), GS₂ activity of *G. gracilis* was generally always higher than GS₁ activity no matter the nitrogen source, with the exception of algae simultaneously supplied with both nitrogen sources. The proportion of GS₁:GS₂ varies within different organs of the same plant, between different plant species and is dependent on the photosynthetic biochemistry of the plant (Harrison *et al.*, 2003). Algal species that possess both GS₁ and

GS₂ are likely to exhibit the group C pattern of expression documented in plants (Casselton *et al.*, 1986). Group C plants consist of C₃ grasses, temperate legumes and halophytic plant species in which photorespiratory rates exceed nitrate reduction rates by ten-fold, and GS₁ is the minor isoform in that it contributes 30% or less of the total leaf GS activity (McNally *et al.*, 1983). GS₁ activity was $\leq 30\%$ of total GS activity of *G. gracilis* cultured in either nitrate or ammonium, indicating high photorespiratory compared to nitrate reduction rates. Since Rhodophyta species are biochemically C₃ plants (Cole and Sheath, 1990) higher *G. gracilis* GS₂ activity in the nitrate and the ammonium treatment may be attributed to increased photorespiration rates. However, when *G. gracilis* was cultured in media containing both nitrate and ammonium, *G. gracilis* exhibited the group D pattern. Group D plants include C₄, CAM plants and some tropical legumes where photorespiration is very low or absent, and plants possess both GS isoforms, but GS₁ activity accounts for 45% or more of total GS activity (McNally *et al.*, 1983). Therefore, photorespiration rates may have been low in nitrogen starved *G. gracilis* re-supplied with both nitrate and ammonium. Similar to the unicellular green alga *C. kessleri*, *G. gracilis* is able to change its pattern from that of group C to group D depending on the nitrogen source, light and carbon dioxide status (Casselton *et al.*, 1986). In addition, the identification of the Group D pattern suggests that *G. gracilis* may have strategies to deal with changes in carbon dioxide status. For example, the C₄-pathway is transcribed in the red macroalga *Porphyra haitanensis*, and enzymes required for the C₄-pathway are found in plants that have established C₃ photosynthetic biochemistry (Raven, 2010). Thus, future investigations should examine carbon metabolism (specifically characterising Rubisco and mechanisms that regulate carbon dioxide status in *G. gracilis*) and the interaction between carbon and nitrogen metabolism in *G. gracilis*. In addition, it must be established whether photorespiration in *G. gracilis* may be a consequence of culturing, as few algae photorespire *in vivo* (Casselton *et al.*, 1986). However, *G. gracilis*'s ability to change the proportion of GS₁:GS₂ activity is in agreement with studies in other algae that exist in aquatic environments where nitrogen starvation occurs. Therefore, it is likely that similar to *C. kessleri* the regulation of *G. gracilis* GS and the ability to interchange proportions of GS₁:GS₂ activity, may be an adaptation to growth in a nitrogen limited environment.

In summary, findings regarding the regulation of NR and GS in the present study are in agreement with those of Chapter 4. It is evident that both NR and GS are subject to transcriptional, post-transcriptional, translational and post-translational regulation, and changes in internal metabolites are likely to play a vital role in orchestrating regulation at all these levels. Findings in the present study indicated that in certain instances nitrate and ammonium induced NR and GS and in others it did not. Therefore, it must be determined whether the nitrogen source or a metabolite downstream of nitrogen assimilation (e.g. amino acid concentrations, energy status, carbon skeletons) is responsible for the induction (Zozaya-Hinchliffe *et al.*, 2005). Future studies should include shorter sampling times to examine whether early/initial induction responses were indeed missed in this study and the sampling interval across the 96 h time course should be increased, to test whether a later induction effect is initiated once starch has been mobilised and photosynthetic enzyme pools are restored. The effect of tissue nitrogen and/or extracellular nitrogen concentrations on NR and GS should also be examined. This study has presented evidence for the existence of constitutive and inducible *G. gracilis* NR isoforms and multiple GS₁ isoforms. Furthermore, the insensitivity of these enzymes to ammonium inhibition and the ability of *G. gracilis* to change the proportion of GS₁:GS₂ activity further establishes that the alga has adapted to a constantly changing nitrogen environment. However, further investigation is required to identify and characterise these isoforms as well as to establish what adaptive advantage multiple isoforms confer on *G. gracilis*

CHAPTER 6

Localisation of nitrate reductase and glutamine synthetase isoforms in nitrogen replete and deplete *Gracilaria gracilis* cells

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6.1 INTRODUCTION

It is not possible to understand the complexity of metabolic networks and their regulation without knowing the location of different isoenzymes (Plaxton and McManus, 2006). Some proteins are encoded by small gene families where each gene encodes a specific isoform. The long standing idea that different isoenzymes represent functional redundancy has been opposed by many findings which indicate that more often than not, isoenzymes have non-overlapping specific roles that are dependent on their location in the organism. For example, the localisation of GS and GOGAT to plant chloroplasts aided researchers in identifying the GS₂-GOGAT pathway as the primary route for nitrogen assimilation in plants (Weber and Flugge, 2002). Therefore, protein localisation is invaluable as it provides a foundation that will aid the understanding of metabolic control within the cell (Plaxton and McManus, 2006).

Studies have begun documenting lists of proteins that are localised in certain tissues, cell types and organelles (Plaxton and McManus, 2006). However, more success has been achieved with model plant species such as *Arabidopsis* and rice as the complete genomes are available. *In silico* determination of the protein sequences would allow transit peptide sequences to be detected thereby identifying the subcellular location of NR and GS isoenzymes. Genes encoding a cytosolic GS and NR in the red macroalgae *Gelidium crinale* (Freshwater *et al.*, 2002) and *G. tenuistpitata* (Falcão *et al.*, 2010), respectively, have been identified. In addition, genes encoding a chloroplast and cytosolic GS were identified in the red microalga *Dixoniella. grisea* (Ghoshroy and Robertson, 2012). Partial GS sequences have also been identified in the red macroalgae, *Chondrus crispus*, *Gracilaria changii*, *Bangia atropurpurea*, *P. yezoensis*, and *P. haitanensis* (Ghoshroy and Robertson, 2012). Conversely, other approaches can be employed to identify the cellular location of proteins. The two most common methods employed to determine the cellular localisation of NR and GS include biochemical and immunocytochemical approaches (Peat and Tobin, 1996; Tischner *et al.*, 1989). Biochemical studies employ an array of methods that include: the isolation of enzymes from crude cell extracts through aqueous polymer two phase systems (biphasic systems where a polyethylene glycol (PEG)-dextran system is most commonly used) (Gustaffsson and Wennerström, 1986; Ward *et al.*, 1989), isolation of organelles using

sucrose or percoll density gradients (Fischer and Klein, 1988) or the separation of isoforms through the use of ion-exchange chromatography (Lopez-Ruiz *et al.*, 1985; Robertson and Alberte, 1996). When applying these techniques, it is important to isolate pure organelles that have minimal contamination from other subcellular compartments (Plaxton and McManus, 2006). In addition, enzymes specific to each organelle must still be active. However, this is quite difficult and not always possible. In contrast, cellular localisation of enzymes is more successful when immunocytochemical methods using gold or fluorescent labelling are employed, provided that adequate controls are included (Kamachi *et al.*, 1987). However, the success of immunocytochemical methods is dependent on the antibody specifically recognising the antigen of interest and not cross-reacting with proteins sharing similar antigenic properties (Stringher and Colepicolo, 1996). Thus, antibody characterisation is necessary prior to use in immunocytochemical studies.

The majority of biochemical and immunocytochemical investigations of NR and GS localisation have focussed on higher plants (Peat and Tobin, 1996; Botella *et al.*, 1988; Brugiere *et al.*, 2000; Brangeon *et al.*, 1989, Ward *et al.*, 1989; and Kamachi *et al.*, 1987), unicellular green algae (Garcia-Fernandez *et al.*, 1994, Lopez-Ruiz, 1985) and cyanobacteria (Bergman *et al.*, 1985). However, little attention has been given to the localisation and regulation of these enzymes in macroalgae. The limited biochemical and immunocytochemical localisation studies investigating NR and GS localisation in red algae have identified a single cytosolic GS and multiple GS isoenzymes in the unicellular red algae, *C. merolae* (Terashita *et al.*, 2006) and *Porphyridium cruentum* (Casselton, 1986), respectively. Findings in Chapters 4 and 5 examining protein, activity and mRNA expression profiles indicated that nutritional status regulated the expression of at least one NR isoform and two GS isoforms in *G. gracilis*. Since immunocytochemical investigations of red macroalgal NR and GS are limited, investigations in this study would be invaluable to identifying red macroalgal NR and GS isoenzymes, as well as providing information regarding the metabolic control of nitrogen metabolism in *G. gracilis*.

Biochemical methods (Chapter 5) suggested that active *G. gracilis* GS₁ and GS₂ enzymes exist in *G. gracilis* cells. In addition, the possibility of inducible and constitutive NR

isoforms was also established. Since biochemical purification of enzymes can be difficult (Stringher and Colepicolo, 1996), using an immunocytochemical approach, the aim of this study was to establish (i) whether a single NR, GS₁ and GS₂ isoform is present in *G. gracilis*, (ii) to gain more insight into how these isoforms may be regulated when exposed to nitrogen replete and nitrogen deplete culture conditions and (iii) to evaluate changes in the cellular ultrastructure of *G. gracilis* cultured in nitrogen replete and nitrogen deplete conditions.

6.2 MATERIALS AND METHODS

Solutions used in this study and supplementary information relating to experiments completed in Chapter 6 can be found in Appendix A and B.

6.2.1 Growth Experiments

G. gracilis samples used in immuno-gold localisation experiments were cultured as described (4.2.1.2). *G. gracilis* thalli were sampled after 18 days of culturing in either PES (nitrogen replete) or PES-N (nitrogen deplete/free) ASW. Thalli for immuno-gold localisation studies were immediately fixed upon harvesting. The remaining thalli were homogenised in a mortar and pestle using liquid nitrogen and stored at -80°C until required for enzyme assays (5.2.3.2).

6.2.2 Testing the effect of fixative on the antigenic properties of *G. gracilis* tissue

Before commencing immuno-gold localisation studies, the effect of different concentrations and combinations of fixative on the antigenic properties of *G. gracilis* tissue were tested (Appendix B 4.1).

6.2.3 Sample fixation and embedding

Fresh *G. gracilis* thalli (6 mm thalli) were washed in base buffer (BB) (Appendix A 7.2). Thereafter, thalli required for the examination of cell ultrastructure were fixed in 2.5% gluteraldehyde (Ga) in BB (Appendix A 7.3) whereas thalli required for immuno-gold localisation of NR and GS were fixed in 4% paraformaldehyde (Pfa) and 0.1% Ga in BB (Appendix A 7.4). All samples were fixed overnight at 4°C and subsequently rinsed twice (5 min each) in BB. However, thalli required for cell ultrastructure observations were post-fixed in 1% osmium tetroxide (Sigma) for 1 h at 22°C, prior to rinsing in water. Following washing, thalli were dehydrated by completing a series of ethanol (30–100%) washes and infiltrated with LR White resin (London Resin Company) according to the method described by Schroeder *et al.* 2003. Individual thalli pieces were then placed in Beem capsules and covered with LR White resin. The capsules were placed in

a 60°C oven for two days to allow polymerisation of the resin blocks. Once polymerised, the resin blocks were stored at room temperature.

6.2.4 Post-embedding for transmission electron microscopy (TEM)

Ultra thin sections (120 nm) were cut with a diamond knife using a Reichert Ultracut S Ultramicrotome (Leica, Vienna, Austria) and collected on formvar-coated nickel grids. Sections were stained for 5 min with 2% uranyl acetate (Appendix A 7.5) and washed 5 times (1 min each) with ultrapure water. The sections were then stained with Reynolds lead citrate (Appendix A 7.6) for 5 min followed by a final 2 min wash with ultrapure water. Sections were viewed with a LEO-912 Omega transmission electron microscope (Zeiss, Oberkochen, Germany).

6.2.5 Immunohistochemistry

For immunohistochemical observation by TEM, sections were prepared as described (6.2.3 and 6.2.4). Immunostaining was performed as described by Schroeder *et al.* (2003) with modifications. The grids were floated (section down) on phosphate buffered saline (PBS) (Appendix A 7.1) containing 1% BSA (PBS BSA) (Appendix A 7.7) for 5 min and subsequently transferred to PBS BSA containing 0.1% fish skin gelatine (FSG) (Sigma), 20 mM glycine (Merck), 2% goat serum (Sigma) and 0.01% Tween-20 (Merck) for 10 min. Thereafter, the grids were washed twice (1 minute each) in PBS BSA and floated overnight on primary antibody that was diluted in PBS containing 0.5% BSA. A range of primary antibody dilutions were tested and the optimal primary antibody dilution of 1:200 and 1:100 for AS08 210 NR and GLN1-2, respectively, was used. A series of washes in PBS BSA containing 0.1% Tween-20 (5 times for 1 min each) followed by three washes (1 min each) in PBS BSA was completed thereafter. The grids were then floated on a 1:50 dilution of 10 nm gold anti-rabbit probe (Sigma) in PBS containing 0.5% BSA for 2 h and subsequently rinsed five times (1 min each) in PBS BSA containing 0.1% Tween-20, followed by three washes (1 min each) with PBS BSA. The conjugant label complexes were then fixed for 3 min in PBS containing 1% glutaraldehyde (Appendix A 7.8) at 22°C. Excess glutaraldehyde was removed by completing five washes (1 min each) in ultrapure water and the grids were stained as

described (6.2.4) using 2% uranyl acetate and Reynolds lead citrate and visualised with a LEO-912 Omega transmission electron microscope.

6.2.5.1 Negative controls

Non-immune serum (NIS) was purchased from Agrisera to serve as a negative control. However, western hybridisation analysis using NIS as the primary antibody revealed an approximately 70 kDa polypeptide (Appendix B 4.2) indicating that NIS cross-reacts to *G. gracilis* proteins. Therefore, the NIS was pre-absorbed against *G. gracilis* protein extracts before use as a negative control in immuno-gold localisation studies. In addition, GLN1-2 antibodies (Agrisera) were pre-absorbed against *G. gracilis* and *A. thaliana* protein extracts to remove all GS antigen and used as an additional negative control. A third control was included where primary antibodies were omitted from the protocol and the grids were incubated with gold alone. Unlike GLN1-2 antibodies, AS08 210 NR antibodies were supplied as affinity purified (using a column containing a NR peptide) antibody. Therefore, it was not necessary to pre-absorb anti-NR antibodies against *G. gracilis* and *A. thaliana* total protein extracts.

6.3 RESULTS

6.3.1 GS assays

Enzyme activity is a direct measure of whether a protein is active, as proteins can be synthesised but remain inactive (Peat and Tobin, 1996). Since western hybridisation analysis routinely detected GS₂ and not GS₁ (Chapters 4 & 5), *G. gracilis* GS₁ and GS₂ activity was evaluated to confirm that both isoforms were indeed present in algal thalli used in immuno-localisation studies. Chloroplast and cytosol fractions of nitrogen replete and nitrogen starved thalli were prepared as described (5.2.2) and GS transferase activity was assayed thereafter.

The increase in total GS activity of nitrogen starved algae was in agreement with findings in Chapter 4 (Figure 1). Following eighteen days of cultivation, GS₁ activity represented the majority of total GS activity in nitrogen replete and nitrogen deplete treatments. Furthermore, culture in nitrogen free media resulted in an approximately 20% increase in GS₁ activity and a 50% reduction in GS₂ activity.

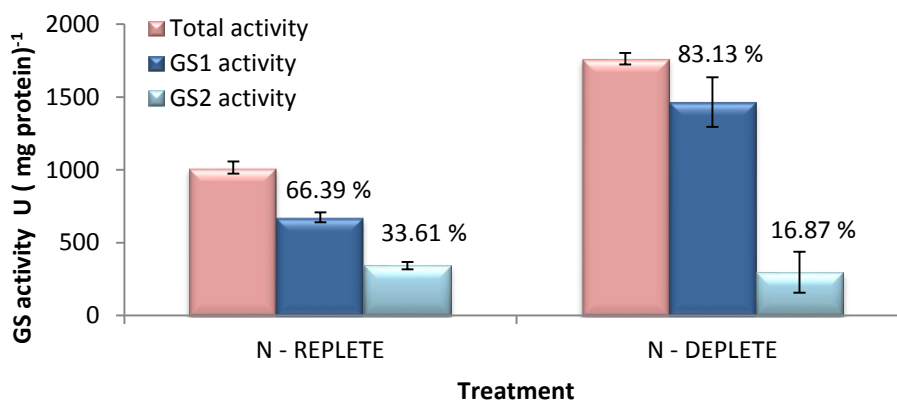


Figure 1. *G. gracilis* GS activity after 18 days of culture in nitrogen replete (PES) and nitrogen deplete (PES-N) ASW. Total GS, GS₁ and GS₂ activity was measured using the transferase GS assay. Bars represent mean GS activity \pm SD ($n = 1$). One unit of GS is defined as the amount of enzyme required to produce 1 μ mol of γ -glutamyl hydroxymate (GGH) per minute at 25°C. The percent contribution of GS₁ and GS₂ activity to total GS activity is indicated above the bars.

6.3.2 Changes in *G. gracilis* ultrastructure following culture in nitrogen replete and nitrogen deplete media

The effect of the absence or presence of nitrogen on the ultrastructure of *G. gracilis* was determined. Thalli, 6 mm in diameter, were excised and fixed using the traditional fixation conditions and conditions routinely used in immuno-gold localisation studies. Traditional fixation conditions employ high concentrations of glutaraldehyde and osmium tetroxide that generally result in greater preservation of ultrastructural features whereas immunolabelling fixation conditions favour antibody-antigen recognition reactions, which may not preserve ultrastructural features.

A comparison of traditional (Figure 2) and immunocytochemical (Figure 3) fixation conditions indicated that both methods preserved cell morphology, as different organelles were easily distinguishable. In general, outer cortical cells were smaller than the inner subcortical and larger medulla cells of nitrogen replete and nitrogen deplete *G. gracilis*.

The cortical cells of nitrogen replete thalli contained numerous chloroplasts in addition to other organelles (Figure 2A). Chloroplasts were associated with the cell wall and appeared elongated to ovoid in shape with parallel thylakoid membranes that were unstacked and not associated with one another. Fat droplets, known as plastoglobuli, were also detected in the chloroplasts. Few starch granules were visible in the cortical cells. In general, nitrogen replete cortical and medulla cells contained few to no starch granules. The larger medulla cells (Figure 3A) appeared almost empty and chloroplasts, connected by stromules, lined the periphery. Cell walls of nitrogen replete *G. gracilis* were distinct, structured and the amorphous matrix containing fibrillar regions, components that make up the cell wall, were clearly visible.

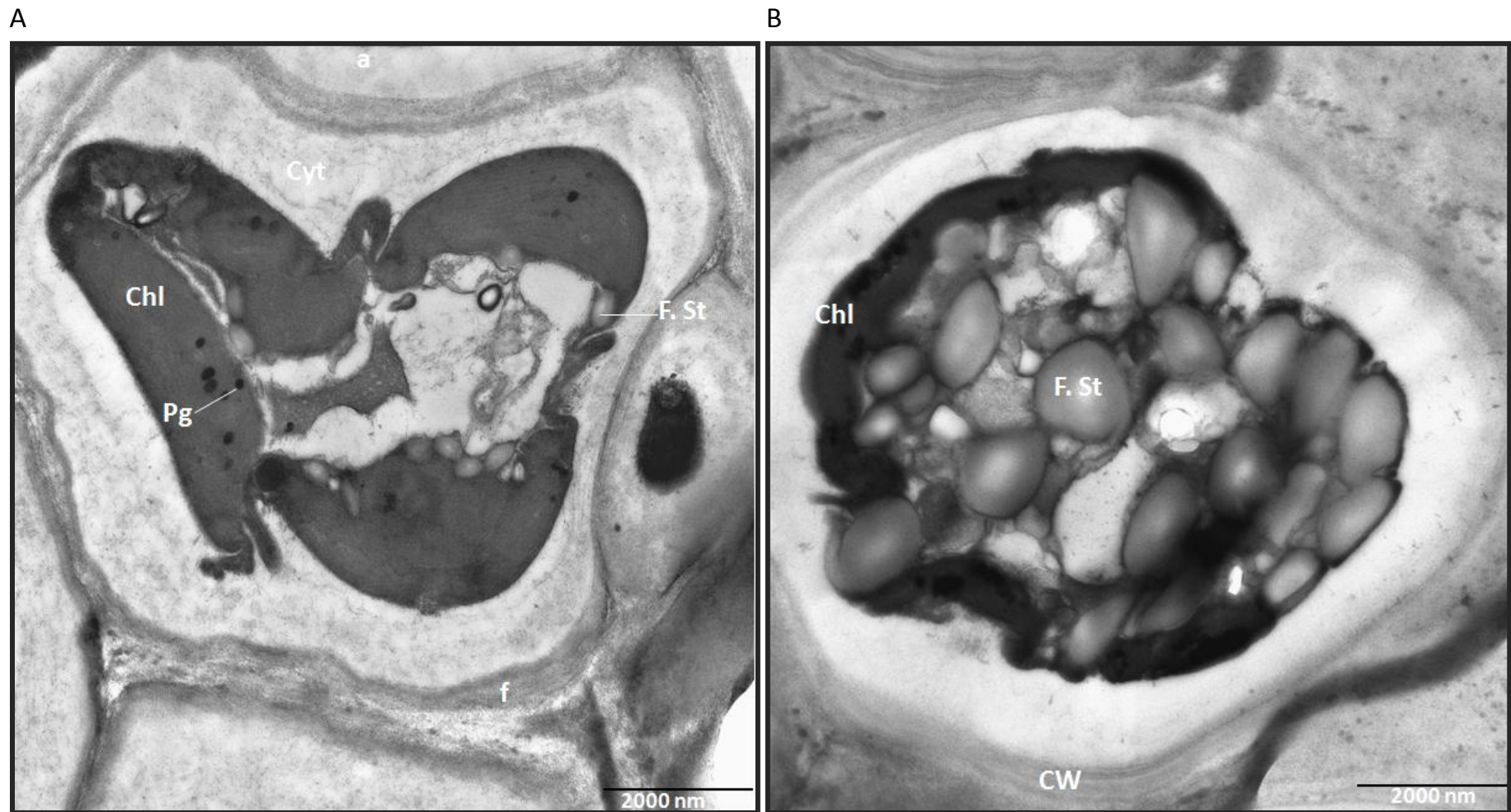


Figure 2. Traditional fixation of *G. gracilis* cortical cells after 18 days of culture in (A) nitrogen replete (PES) and (B) nitrogen deplete (PES-N) ASW. Thalli were fixed with 2.5% glutaraldehyde and post fixed with osmium tetroxide. Letters denote: a, amorphous matrix; Cyt, cytosol; Chl, chloroplast; Pg, plastoglobuli; f, fibrillar region; F.St, starch granules (floridean starch) and CW, cell wall.

The overall cell shape of nitrogen deplete *G. gracilis* was maintained (Figure 2B). The most striking feature of nitrogen starved cells was the dramatic increase in starch granules that varied in size and shape (Figure 2B and Figure 3B). Smaller cortical cells were completely filled with starch granules and very little cytosolic space was visible (Figure 2B). Similar to nitrogen replete thalli, medulla cells of nitrogen starved thalli appeared emptier compared to the cortical cells (*cf.* Figure 2B & 3B). However, a marked increase in starch granules within these cells was observed. In general, chloroplasts of cortical, subcortical and medulla cells of nitrogen starved thalli, were reduced in size and volume (Figure 3B). Chloroplasts located along the periphery of the cell wall appeared longer, thinner, almost stretched and seemed to contour and conform themselves to the shape of the cell and starch granules (Figure 2B & 3B).

6.3.3 Immuno-gold localisation

Immuno-gold studies were performed to determine the subcellular localisation of NR and GS, and the effect of nitrogen on the expression of these isoforms. When control sections were probed with pre-absorbed NIS, negligible to no gold labelling was detected in cellular organelles and cell walls of nitrogen replete (Figure 4A) and nitrogen deplete (Figure 4 B) cells. In addition, no gold labelling was detected when sections were probed with the pre-absorbed GLN1-2 primary antibody and gold alone (data not shown).

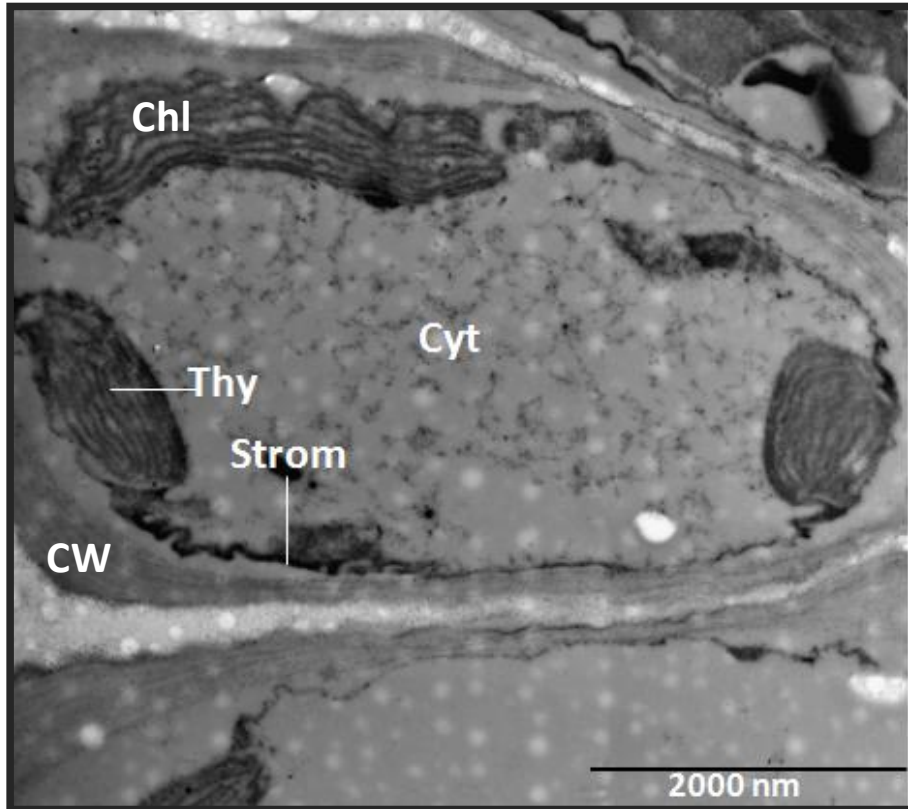
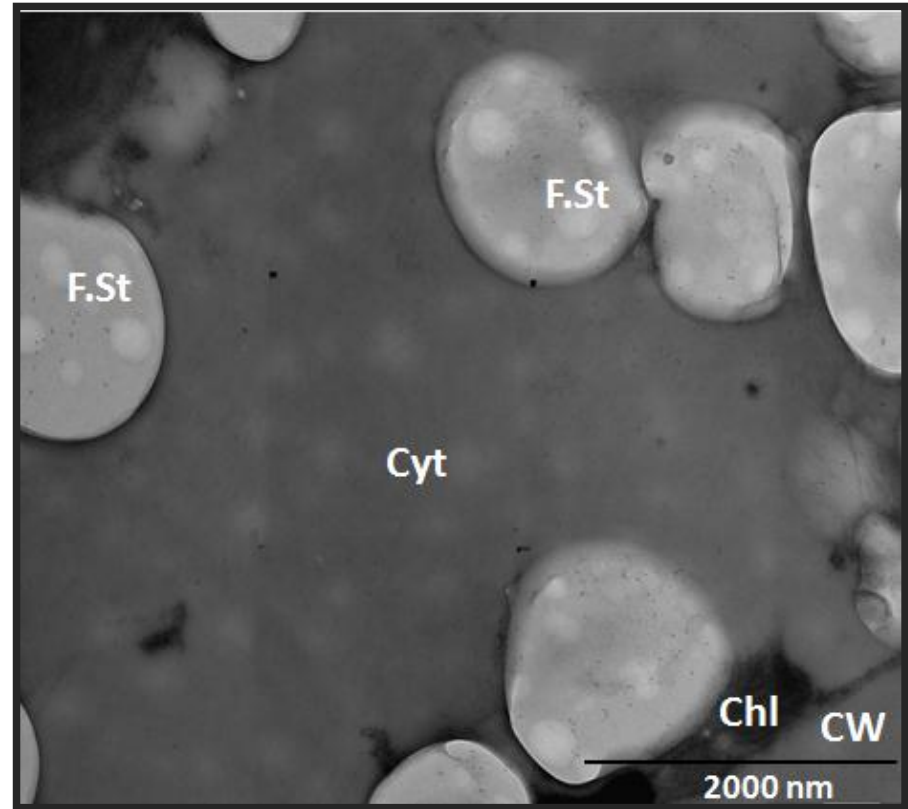
A**B**

Figure 3. *G. gracilis* medulla cells after 18 days of culture in (A) nitrogen replete (PES) and (B) nitrogen deplete (PES-N) ASW. Thalli were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde. Chloroplasts (Chl) line the periphery of nitrogen replete cells and are connected by stromules (Strom). Parallel thylakoids (Thy) are visible. Nitrogen starvation causes the chloroplasts to shrink and induces starch (F.St) synthesis which reduces cytosol (Cyt) space.

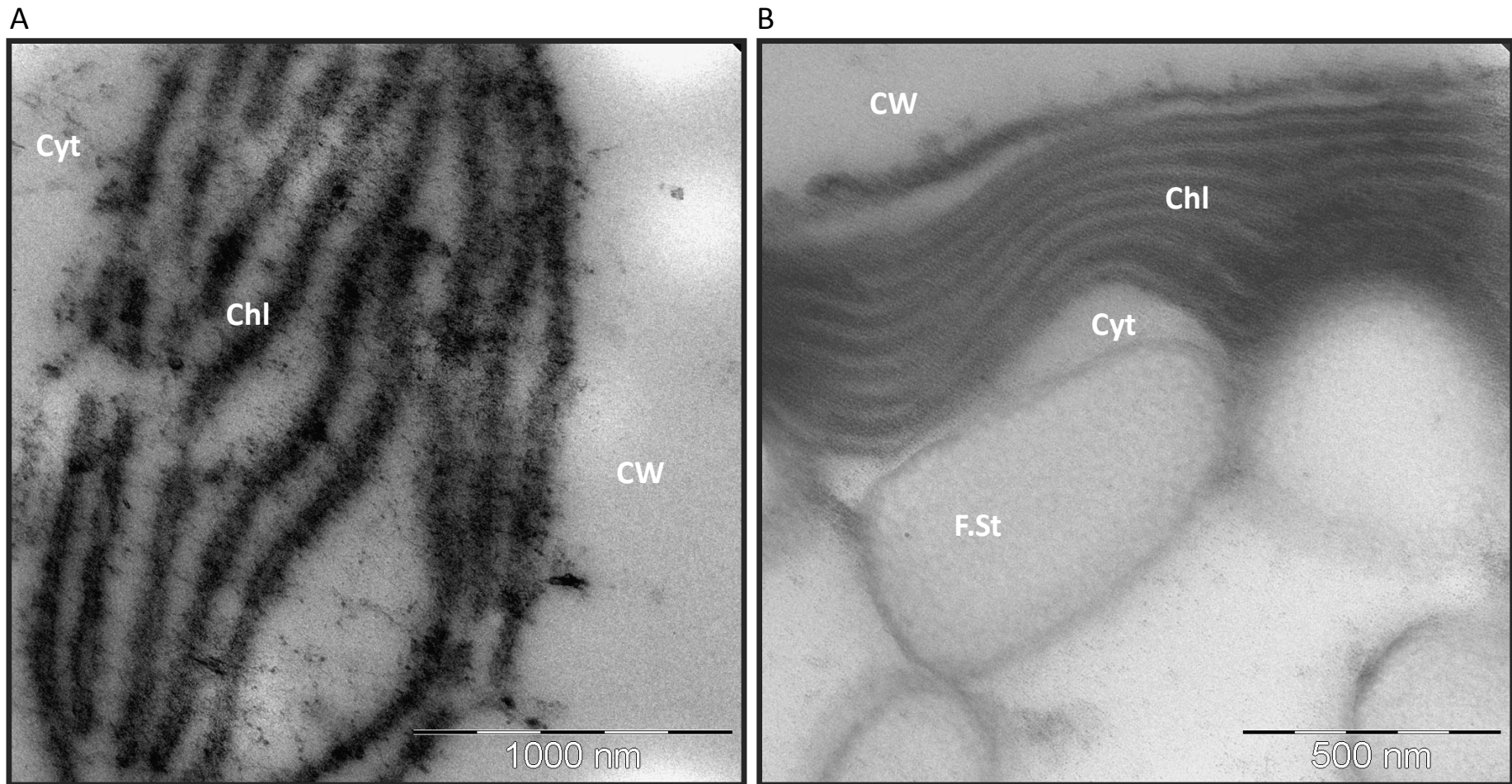


Figure 4. Control sections representing *G. gracilis* cells after 18 days of culture in (A) nitrogen replete (PES) and (B) nitrogen deplete (PES-N) ASW. The control sections were probed with NIS pre-absorbed against *G. gracilis* total protein extract. No gold labelling was observed in the chloroplasts (chl), cytosol (Cyt) and cell wall (CW) of nitrogen replete or deplete *G. gracilis*. No gold labelling was observed in the starch granules (F.St) of nitrogen deplete *G. gracilis*.

6.3.3.1. Immuno-gold localisation of NR

Gold labelled NR was detected in the chloroplasts, cytoplasm, and cell walls of nitrogen replete and nitrogen starved *G. gracilis* cells (Table 1). Gold labelled NR was located in the stroma of the chloroplasts and not associated with the thylakoid membranes (Figure 5A & 5B).

Quantification of gold labelling was difficult due to differences in the morphology of the outer and inner cells as well as differences in the ultrastructure of nitrogen replete and nitrogen starved cells. However, a qualitative measure of the degree of labelling between organelles was determined by examining numerous tissue sections. The degree of gold labelled NR did not differ between nitrogen replete and nitrogen starved chloroplasts. However, fewer gold labelled cytosolic NR was detected in nitrogen deplete *G. gracilis* (*cf.* Figure 5C and 5D). In comparison to the chloroplasts and cytosol, a low level of gold labelled NR was detected in the cell walls of nitrogen replete and nitrogen starved algae (Figure 5A and 5B). However, less gold labelled NR was detected in the cell walls of nitrogen starved algae. In general, the amount of cell wall labelled NR was far reduced in comparison to gold labelling of chloroplastic and cytosolic NR. Gold labelling was not detected in starch granules (Figure 5C).

Table 1. Cellular location of gold labelled NR and GS in nitrogen replete and nitrogen starved *G. gracilis*.

	NR		GS	
	NE	NL	NE	NL
Organelle				
Chloroplast	D	D	D	D ^L
Cytosol	D	D ^L	D	D
Cell wall	D	negligible	D	negligible
Starch granule		ND		D

NE: *G. gracilis* cultured in nitrogen replete (PES) ASW

NL: *G. gracilis* cultured in nitrogen deplete (PES-N) ASW

D: gold labelled NR or GS was detected

ND: gold labelled NR or GS was not detected

^L: refers to a reduction (low) of gold labelled NR or GS in organelles of nitrogen deplete *G. gracilis*, compared to nitrogen replete *G. gracilis*.

Where too few gold labelled NR and GS was detected, a score of negligible is given.

Starch granules were not detected in sections from *G. gracilis* cultured in nitrogen replete ASW and a score cannot be given.

6.3.3.2 Immuno-gold localisation of GS isoforms

Similar to NR, gold labelled GS was detected in the chloroplast, cytosol and cell walls of *G. gracilis* cells (Table 1). The majority of GS was detected in the chloroplasts and cytosol. A closer examination of the chloroplasts indicated that GS was located in the stromal space and not associated with thylakoid membranes (Figure 6 & Figure 7A). In comparison to nitrogen replete cells, gold labelled GS₂ was reduced in nitrogen starved cells whereas the amount of gold labelled GS₁ remained unchanged (Table 1). The most striking observation was the detection of gold labelled GS in the inner surface and not the periphery of starch granules (Figure 7B). Moreover, the degree of labelling of GS varied between starch granules, with some being more labelled than others. In general, more gold labelled GS was detected in the starch granules of nitrogen starved cells compared to the chloroplasts and cytosol. A low level of gold labelled GS was detected in the cell walls with fewer gold-GS complexes detected in the cell walls of nitrogen starved *G. gracilis*. Furthermore, gold labelled GS in cell walls was negligible in comparison to the chloroplasts, cytoplasm and starch granules.

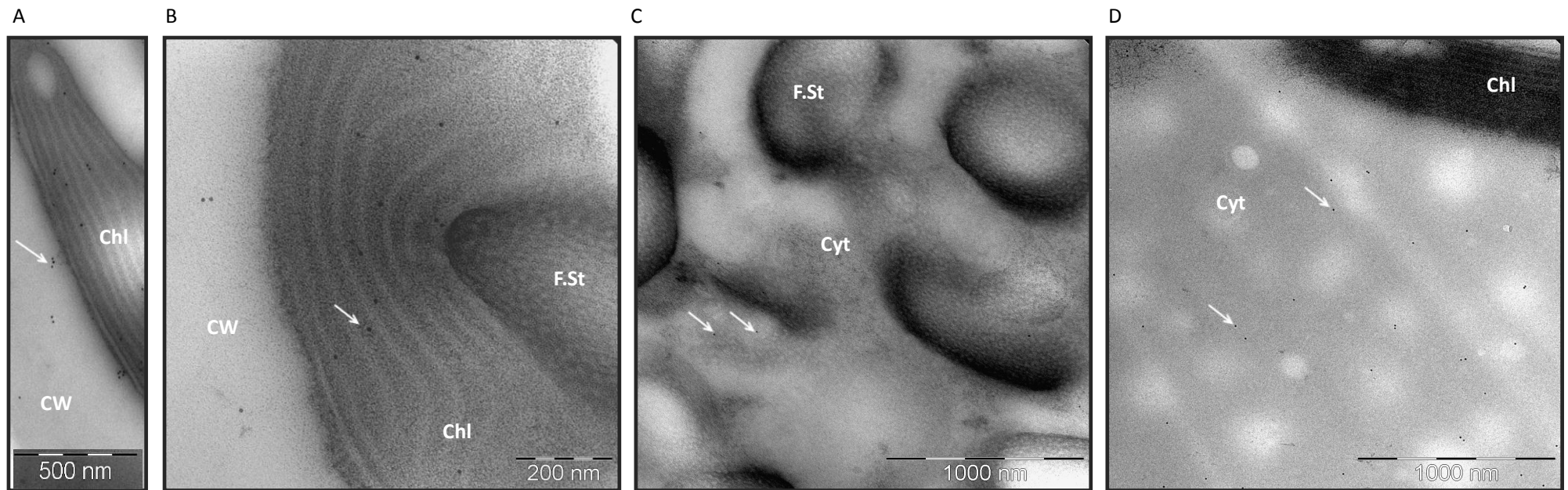


Figure 5. Immunogold localisation of *G. gracilis* NR. (A), (B) and (C) represent sections that were prepared from *G. gracilis* cultured in nitrogen deplete (PES-N) and (D) nitrogen replete (PES) ASW. The chloroplast (Chl) in Panel B has contoured itself around a starch granule (F.St) and gold labelled NR is visible in the chloroplasts (Chl) and the cell wall (CW) of nitrogen deplete *G. gracilis*. Fewer gold labelled NR was detected in the cytosol (Cyt) of nitrogen deplete compared to nitrogen replete *G. gracilis*. Gold labelled NR was not detected in the starch grains (F.St). Gold labelled NR (black dots) is indicated by white arrows.

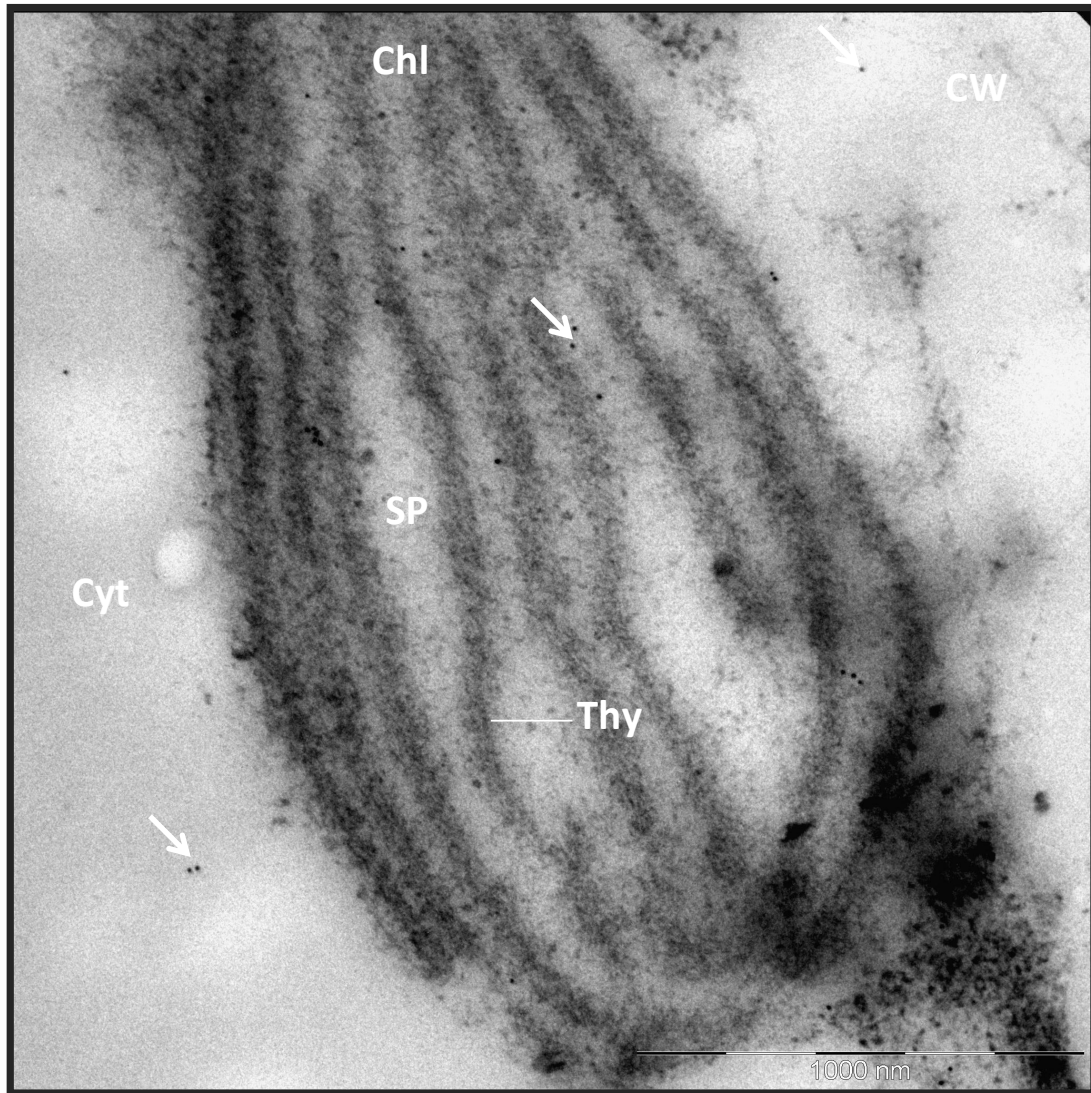


Figure 6. Immuno-gold localisation of *G. gracilis* GS after 18 days of culture in nitrogen replete (PES) ASW. Gold labelled GS was detected in the chloroplast (Chl), cytosol (Cyt) and cell wall (CW). Gold labelled GS in the chloroplasts was not associated with the thylakoids (Thy) but appeared in the stromal space (SP). Gold labelled GS (black dots) is indicated by white arrows.

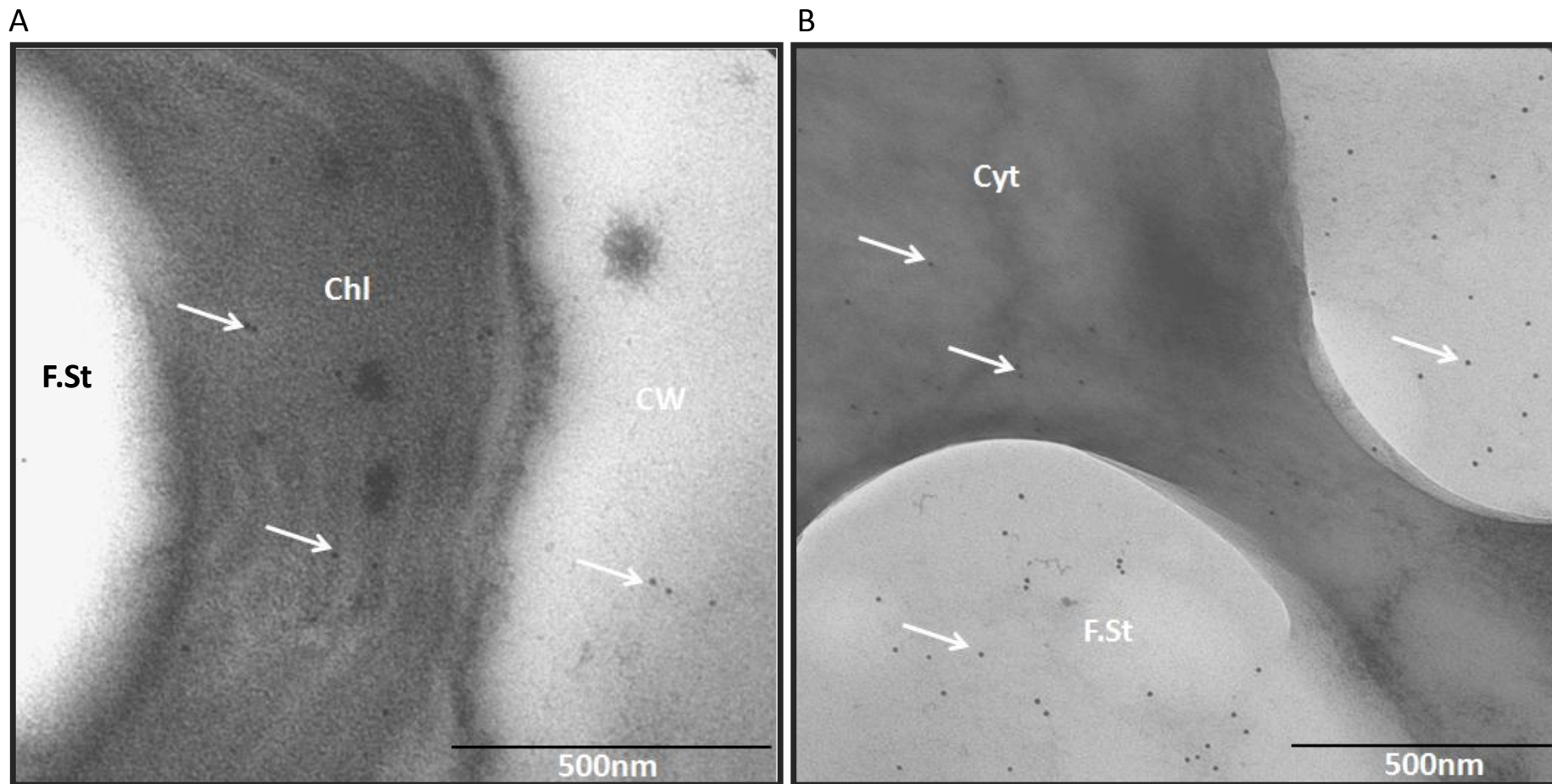


Figure 7. Immuno-gold localisation of *G. gracilis* GS after 18 days of culture in nitrogen deplete (PES-N) ASW. (A): an elongated chloroplast (Chl) has contoured itself around a starch grain (F.St) and lines the periphery of the cell wall (CW). Gold labelled GS was detected in the chloroplast and cell wall. (B): greater amounts of gold labelled GS were detected in the starch granules (F.St) and cytosol (Cyt) of nitrogen deplete *G. gracilis*. Gold labelled GS (black dots) is indicated by the white arrows.

6.4 DISCUSSION

6.4.1 Changes in the ultrastructure of nitrogen replete and nitrogen starved *G. gracilis* cells

The general cellular structure of *G. gracilis* is in agreement with the morphological features reported for other red algae. Thick cell walls containing fibrillar components embedded in an amorphous matrix are congruent with observations of *Gymnogonrus torulosus* (Estevez and Cáceres, 2003), *Gracilaria domingensis* (Schmidt *et al.*, 2009) and *G. tenuistipitata* (Nyvall *et al.*, 1999). Cell ultrastructure of *G. gracilis* supplied with nitrogen was in agreement with studies completed by Tripodi and Beth (1976), who examined the cell structure of nitrogen replete *G. gracilis* thalli harvested from the sea. Furthermore, similar to nitrogen replete *G. domingensis* and *G. torulosus*, *G. gracilis* cells contained a large quantity of chloroplasts that occupied the entire cytoplasm of cortical cells and lined the periphery of the larger medullary cells. Red algal chloroplasts are termed rhodoplasts as they appear red to purple in colour (Wise and Hooper, 2006). As observed in *G. domingensis* and *G. secundata* (Eckman *et al.*, 1989), *G. gracilis* rhodoplasts were elongated. The thylakoid structure of *G. gracilis* rhodoplasts had a parallel distribution and were not associated with one another, a feature characteristic of red algae (Cole and Sheath, 1990; Schmidt *et al.*, 2009). A considerable change in cell morphology was observed in nitrogen starved *G. gracilis*. Structural changes were similar to changes observed in the unicellular red microalga *Galdieria sulphuraria* (Sinetova *et al.*, 2006) and macroalga *G. secundata* (Ekman *et al.*, 1989) cultured under similar conditions. *G. gracilis* and *G. sulphuraria* maintained cell shape, exhibited a reduction in the average area of rhodoplasts and the cell walls became more homogenous. The most striking observation was the marked increase in the number of starch granules present in nitrogen starved *G. gracilis* cells, a feature shared with nitrogen starved *G. secundata* and *G. tenuistipitata* cells (Nyvall *et al.*, 1999). Nitrogen starvation causes a reduction in nitrogen components required for metabolic processes, with a subsequent increase in carbohydrate accumulation (Viola, *et al.*, 2001). In red algae, this major carbon store is floridean starch, which under certain growth conditions such as nitrogen starvation, can account for almost 80% of the total cell

volume. In addition, starch assays performed by Naidoo (2012) confirmed that culturing *G. gracilis* in nitrogen deplete conditions resulted in the accumulation of starch.

The changes in cell ultrastructure indicate that different metabolic shifts are initiated in nitrogen replete and deplete conditions and set the foundation for immunocytochemical investigations.

6.4.2 Immuno-localisation of *G. gracilis* NR

According to the literature, NR is a cytosolic enzyme (Vaughn and Campbell, 1988). However, immunocytochemical studies have localised NR to the chloroplasts of the unicellular green alga *C. reinhardtii* (Fischer and Klein, 1988), the marine dinoflagellate *Gonyaulax polyedra* (Fritz *et al.*, 1996) and spinach leaves (Kamachi *et al.*, 1987). The present study localised NR to the cytosol and rhodoplasts of *G. gracilis* cells. The localisation of NR to *G. gracilis* rhodoplasts warranted further investigation. This was achieved by biochemical separation of NR from chloroplast and cytosol fractions from nitrogen replete *G. gracilis* as described (Chapter 5) (data not shown). Findings from the biochemical approach were not congruent with immuno-localisation observations. Even though immuno-localisation methods detected NR in chloroplasts of *G. gracilis* cultured in nitrogen replete conditions, NR activity was detected in the cytosol and not the chloroplast fractions in thalli supplied with nitrogen. Similar inconsistencies between enzyme assays and immuno-gold studies were reported in *C. reinhardtii* (Fischer and Klein, 1988) and maize roots (Peat and Tobin, 1996). The differences between biochemical and immunocytochemical studies can be explained by the fact that different plants have different NR isoforms with differing electron donor and/or tissue specificities (Fritz *et al.*, 1996). Moreover, plant chloroplasts are known to vary in number, activity and enzymatic composition depending on the type of tissue, stage of development, genetic and environmental factors (Kamachi *et al.*, 1987). All of these may contribute to the conflicting results observed in this study. Research has shown that enzymes can be synthesised without being active *in vivo* (Peat and Tobin, 1996). Synthesis of these proteins enables the plant to respond quickly to the continually changing external environment. It was suggested that the chloroplast located NR of *C. reinhardtii* which was not active when cells were nitrogen replete, may either serve as

a protein pool that could be degraded and rapidly mobilised when needed or the enzyme could be activated when nitrogen becomes limiting (Fischer and Klein, 1988). In addition, immuno-gold localisation studies that compared day-night variations in *G. polyedra* NR, revealed that the chloroplast isoform was reduced during the dark phase whereas no change in cytosol NR protein levels were detected (Fritz *et al.*, 1996). Further support for the localisation of *G. gracilis* NR to chloroplasts is provided by the NR sequence of the red macroalga *G. tenuistipitata*. The programme ChloroP (Emanuelsson *et al.*, 1999) predicted that the *G. tenuistipitata* NR sequence possessed a chloroplast transit peptide sequence. Thus, it is not unlikely that *G. gracilis* may possess a chloroplast located NR. When considering immunocytochemical findings in *C. reinhardtii*, *G. polyedra* and *G. gracilis*, it seems that *G. gracilis* NR isoforms may have differing and non-overlapping roles. The possible roles of these isoforms are discussed below.

NR protein decreased in *G. gracilis* starved of nitrogen as shown by western hybridisation (Chapter 4). This correlated with immuno-gold localisation findings that detected a marked reduction in cytosolic NR and suggests that western hybridisation may have detected expression of the cytosolic NR isoform. This is further validated by the observation that the amount of gold labelled chloroplast NR remain unchanged in *G. gracilis* supplied and starved of nitrogen. In addition, the partial gene sequence of *G. gracilis* NR may represent the cytosolic NR isoform as *GgNR* transcript levels decreased dramatically upon transfer of *G. gracilis* to nitrogen free media. Therefore, immuno-gold localisation and western hybridisation suggest that cytosolic NR may be the active isoform required for nitrogen assimilation in *G. gracilis*. Conversely, similar to *C. reinhardtii*, *G. gracilis* chloroplastic NR may represent either a nitrogen pool that could be degraded when cells are nitrogen limited or the enzyme may be activated in nitrogen limiting conditions. Future studies should evaluate the activity of NR in cytosol and chloroplast fractions of thalli starved of nitrogen as this was not evaluated in the present study. In addition, a role for *G. gracilis* chloroplastic NR in the dark assimilation of nitrogen cannot be excluded. Samples for immuno-localisation observation were taken during the light phase. However, high *G. gracilis* NR activities were measured in the dark (Chapter 3) and allude to the fact that, like *G. polyedra*, *G. gracilis* may be able to assimilate nitrogen in the dark (Stringher and Colepicolo, 1996).

6.4.3 Immuno-localisation of *G. gracilis* GS

Before addressing the immuno-localisation findings for GS, the differences between immuno-gold localisation and western hybridisation results will be discussed. Immuno-localisation studies detected both GS₁ and GS₂ isoforms whereas GS₂ was the only isoform consistently detected in western hybridisation analysis (Chapter 4). Similar observations were reported in whole-root protein extracts of young barley plants (Bernard and Habash, 2009). Since GS₂ is the minor isoform in barley roots, it was not easily detected by western hybridisation techniques. Conversely, *G. gracilis* cell ultrastructure is typical of the Florideophyceae, which are known to contain large quantities of chloroplasts (Schmidt *et al.*, 2009). This suggests that GS₂ may be the major isoform in *G. gracilis* cells. Thus, detection of GS₁ by the commercial antibody GLN1-2 may be limited when performing western hybridisation analysis as it is the minor GS isoform in *G. gracilis*. Western hybridisation is limited by the amount of target protein required for visualisation, a problem not associated with immuno-gold localisation techniques. Regardless of the limitations of detecting GS isoforms when employing western hybridisation, this study has confirmed that the GLN1-2 antibody has affinity for both *G. gracilis* GS isoforms. Thus, the antibody was suitable for use in immuno-localisation studies.

Immuno-localisation of GS yielded results that confirmed the hypothesis that both GS₂ and GS₁ isoforms are found in *G. gracilis* cells. The most striking observation was the localisation of GS to starch granules. Moreover, the amount of gold labelled GS detected differed between starch granules. Similar studies conducted on soybean (Brangeon *et al.*, 1989) and barley roots (Peat and Tobin, 1996) detected labelled GS within plant amyloplasts (starch storage plastids). The amount of gold labelled GS within different amyloplasts differed and was dependant on the phase of starch grain filling. A reduction in gold labelled GS was detected as the amyloplasts were filled with starch, due to a reduction in stromal space. In addition, studies have confirmed that GS₂ is the isoform detected in amyloplasts (Wise and Hooper, 2006). However, unlike plants, red algae do not exchange insoluble or soluble products of carbon assimilation across a plastidic membrane (Viola *et al.*, 2001). Instead, synthesis of starch occurs in the cytoplasm. A

starch branching enzyme cloned from *G. gracilis* that lacks a transit peptide sequence supports the evidence for the cytosolic synthesis of starch (Nyvall *et al.*, 1999). To date there is very little information regarding the regulation of carbon allocation and the starch biosynthetic pathway in red algae (Viola *et al.*, 2001). The only enzymes immunolocalised to red algal starch granules are granule bound starch synthases and an α -glucan phosphorylase (Nyvall *et al.*, 1999). Therefore, further studies need to be completed in order to determine which isoform of GS is bound to starch grains and subsequently, the role of this isoform in starch synthesis in *G. gracilis* cells.

When comparing GS₁ and GS₂ isoforms, gold labelling of these isoforms differed between *G. gracilis* cells starved of nitrogen and those supplied with nitrogen. The levels of gold labelled GS₁ remained unchanged regardless of the nitrogen content of the growth medium used to culture *G. gracilis* prior to sampling. Although no noticeable increase in GS₁ protein was observed, GS₁ activity in nitrogen starved *G. gracilis* cells increased. In contrast to GS₁, gold labelled GS₂ decreased when *G. gracilis* was cultured in nitrogen deplete media and is in agreement with findings observed in tobacco leaves (Brugière *et al.*, 2000). Mature green tobacco leaves contained GS₂ while yellow senescent leaves showed a reduction in GS₂ and an increase in GS₁ protein. In higher plants senescence causes a decrease in GS₂ gene and/or protein expression whereas GS₁ gene and protein expression are found to increase or stay the same, but the activity of GS₁ increases. In addition, a reduction in NR is observed in early senescence, becoming almost undetectable in later stages. Essentially, senescence causes a switch in plants from “nitrogen assimilation” to “nitrogen remobilisation”. Therefore, considering enzyme activity (Chapter 4) and the immuno-localisation of GS₁ and GS₂ data presented in the current study, it can be surmised that culturing *G. gracilis* in nitrogen deplete conditions for long periods of time induces senescence. Protein translocation and remobilisation to metabolically more important processes within the cell are features of senescence (Brugière *et al.*, 2000). Therefore, considering the immunocytochemical, enzymatic assays and western hybridisation findings thus far, the following can be concluded: *G. gracilis* GS₂ may be responsible for primary nitrogen assimilation when nitrogen is sufficient whereas GS₁ may be responsible for the translocation and remobilisation of nitrogen during senescence. Nitrogen starvation results in protein catabolism within the cell cytosol and these breakdown products may serve as substrates for GS₁ (Brugière *et*

al., 2000). Furthermore, since red algae synthesise starch in the cytoplasm, it may be possible that gold labelled GS in starch grains represents the cytosolic isoform and thus, could account for the observed increase in *G. gracilis* GS₁ activity. If this is true, a role for *G. gracilis* GS₁ in starch grain synthesis is possible. Studies with GS₁ knockout mutants of rice plants indicated that GS₁ in rice is associated with starch grain filling (Bernard and Habash, 2009). Researchers propose that in plants, GS₁ may be responsible for the translocation of metabolites required for starch synthesis to amyloplasts whilst GS₂ in amyloplasts may be responsible for re-mobilisation of starch when nitrogen becomes available. Since starch is synthesised in the cytoplasm of red algae, it is possible that *G. gracilis* GS₁ location in starch granules may be energetically more favourable. Cytosolic synthesis and degradation of starch may circumvent the energetic cost incurred from transporting substrates across a plastidic membrane, allowing the alga to respond more rapidly to the resupply of nitrogen. These hypotheses need to be tested and explored further before any conclusions can be made. Further investigations into the processes involved in starch synthesis, carbon allocation and regulation of metabolic shifts in response to nitrogen in red algae are essential. Increasing our knowledge of these processes and the enzymes involved in these pathways is key in determining the role of GS in regulating nitrogen metabolism in *G. gracilis*.

6.4.4 Role of cell wall located *G. gracilis* NR and GS isoforms

The association of enzymes with the cell wall of plants is not uncommon. Under certain nutritional conditions, hydrolytic enzymes are transported across the plasma membrane and associated with the cell wall (Grossman, 2000). The localisation of NR and GS to *G. gracilis* cell walls is noteworthy and should be discussed as no labelling of NR and GS was detected in the cell walls of control sections. NR has been detected in the plasma membrane of *Neurospora crassa*, barley roots, corn roots and the unicellular green alga *C. sorokiniana* (Ward *et al.*, 1988; Tischner *et al.*, 1989; Ward *et al.*, 1989). In the present study, it was difficult to determine whether gold labelled NR was associated with the plasma membrane as the tissue used for immuno-localisation was not post fixed with osmium tetroxide, a chemical that stains the plasma membrane. It is suggested that NR in the plasma membrane may be part of an enzyme complex that transports and reduces nitrate (Ward *et al.*, 1988; Tischner *et al.*, 1989). In addition, low

levels of gold labelled GS were detected in the cell walls of the nitrogen fixing cyanobacterium *Anabaena cylindrica* (Bergman *et al.*, 1985). The cell wall of this cyanobacterium changes under different growth conditions and it is suggested that GS located in the cell wall may play some role in cell wall biosynthesis. Investigations in the bacterium *Mycobacterium tuberculosis* established that aside from playing a role in nitrogen metabolism, GS has an important role in cell wall biosynthesis (Harth and Horwitz, 2003). Furthermore, cell wall composition of red algae is complex (composed of cellulose, mannans, xylans, sulphated galactans which include agars and carrageenans, proteins and glycoproteins) and is known to change in response to differing environmental conditions (Cole and Sheath, 1990). Possible roles for red algal cell wall proteins include ion transport, movement of organic molecules, biosynthesis of cell wall polymers, cell adhesion, detoxification, physical protection of the thallus and serving as structural materials. *G. gracilis* cell wall NR and GS may perform similar functions. In addition, algal cell wall synthesis is directly proportional to growth rate (Raven, 1982) and may explain the reduction in gold labelling of cell wall NR and GS in nitrogen starved cells. The substrates for cell wall synthesis are generated from different metabolic pathways that include carbon dioxide fixation by the light and dark reactions of photosynthesis and nitrogen assimilation (Raven, 1982). Since NR and GS have an important role in regulating these processes, localisation of NR and GS to algal cell walls may be advantageous for the synthesis of metabolites required for cell biosynthesis. This is further supported by the detection of low levels of Rubisco in the cell walls of *G. secundata* (Ekman *et al.*, 1989). Moreover, localisation of *G. gracilis* NR and GS to the cell wall may prevent energetic costs incurred from transporting metabolites from other cellular compartments to the cell wall.

In summary, this study is the first to successfully localise NR and GS to the chloroplasts as well as the cytosol of red macroalgal cells. The localisation of GS to starch granules, and NR and GS to the cell wall is novel and warrants further investigation. Immunolocalisation of NR and GS suggests that these enzymes may have multiple roles in *G. gracilis* that include nitrogen assimilation/transport, senescence and cell wall biosynthesis.

CHAPTER 7

General discussion and future work

The study of the genetic response to nitrogen and nitrogen stress in the agarophyte *G. gracilis* has great ecological and commercial importance. Since the *Gracilaria* industry in South Africa was largely dependent on the natural *G. gracilis* populations in Saldanha Bay, knowledge gained from this study may enable the re-establishment of the alga by improving rope rafting (suspended culture) practices. Furthermore, the development of methods/assays for determining when *G. gracilis* becomes stressed may enable farmers to employ mitigating strategies. Conversely, genetically engineered nitrogen stress tolerant strains of *G. gracilis* could improve existing land based aquaculture operations. Land based culturing is particularly attractive as it allows all year culturing of *G. gracilis*, standardises the quality of seaweed cultured as conditions are controlled, and circumvents the issue of introducing genetically modified algae into aquatic systems. Since growth conditions affect biochemical processes and the accumulation of different metabolites, an understanding of these metabolic processes will enable operators of land based aquaculture systems to modify growth conditions to suit their specific needs. For example, integrated abalone-seaweed aquaculture practices use seaweed as biofilters to reduce the nitrogen load of the effluent from abalone raceways, as feed for farmed abalone and depending on the seaweed species, as a source of commercially important phycocolloids (Jonell, 2008). Therefore, an understanding of how algal physiology responds to nutrient stress is required to achieve these goals. In order to generate a model of the algal physiological response to nutrient input and deprivation, an understanding of the underlying metabolic processes is required (Flynn, 1991). This study embarked on elucidating nitrogen stress response mechanisms of *G. gracilis* by employing a molecular approach to investigate the role and regulation of two important nitrogen metabolic enzymes, NR and GS.

Before addressing the important findings of this study, biological variation and the sampling timescale will be discussed. First, in many studies, including this study, experimental data exhibits a large degree of variance due to biological variability. For example a large degree of variance was detected in monthly measurements of NR

activity in *L. digitata* over a growing season (a period of 12 months) (Young *et al.*, 2007). This trend is seen in many field and laboratory studies (Lartigue and Sherman, 2006; Young *et al.*, 2007). Inherent biological variation between thalli is a consequence of changing environmental conditions that result in the accumulation of differing concentrations of intracellular nitrate, ammonium, amino acids, proteins, carbohydrates and other metabolites. Algae used in the present study do not represent a clonal population as facilities for long term culture of *G. gracilis* are not available. Moreover, *G. gracilis* cultures used in the present study were sourced throughout the year from Gaansbaai where *G. gracilis* is maintained in a flow through tank system that is supplied by local seawater. Thus, seasonal changes in nitrate and ammonium concentrations ultimately increases variance between biological samples, and thereby affects statistical analysis by masking significant differences that may indeed exist. In the present study, statistical analyses were often below the desired power (0.8) which meant that significant differences were likely to be missed. The power of statistical tests can be increased by reducing the biological variance. In order to accomplish this, more biological repeats would need to be included in each experiment. However, this was not always possible due to cost considerations. Therefore, although certain differences did not appear to be statistically significant, distinct patterns of expression were evident and consequently discussed. Some researchers employ chemostats (continuous flow through system) to overcome variations in culture conditions, thereby limiting biological differences between flask cultures (Andria *et al.*, 1999). Chemostat culturing is routinely used in microalgal studies (Vergara *et al.*, 1993). Since culture conditions can be controlled and easily manipulated in chemostat systems, gradual changes in properties of the culture medium, generally observed with batch systems, are overcome and the effect of any variable on macroalgal physiology can be examined. Thus, it is suggested that future investigations in macroalgae be performed in chemostats. Continuous flow through systems have been used to culture the red macroalga *Gelidium sesquipedale* (Vergara *et al.*, 1993) and *Gracilaria* species (Andria *et al.*, 1999). Second, findings in Chapter 5 illustrated the importance of the sampling interval. For example, when cyanobacterial cells were transferred from one growth condition to another, changes in metabolism occurred within seconds to minutes with steady state levels being completely restored within thirty minutes (Muro-Pastor *et al.*, 2001). Therefore, sampling interval/number of sampling points over the duration of the nutrient replete,

deplete and nitrogen sources experimental period in the present study must be considered. The sampling interval is important as initial/late 'repression' and 'derepression' (referring to transcriptional changes) of genes or 'inhibition' and 'activation' (referring to post-translational mechanisms) of enzymes (Flynn, 1991) may have been missed. For example, initial induction of transcription and protein synthesis or later enzyme inactivation may not have been detected. However, despite the limitation of the power of statistical tests, variation due to batch culturing and insufficient sampling intervals, data obtained from the present study highlighted (i) the complexity of nitrogen metabolism, (ii) the impact of changing environmental conditions on metabolism, (iii) the interaction of nitrogen metabolism with other metabolic pathways and (iv) elucidation of the mechanisms responsible for the regulation of nitrogen metabolism and the nitrogen stress response in *G. gracilis*. Using the data from the current study together with the findings of Lebi (2006), Naidoo (2012) and the existing literature, a putative model of nitrogen metabolism/nitrogen stress responses in *G. gracilis* has been developed.

Nitrogen metabolism and nitrogen stress responses of G. gracilis

Nitrogen replete conditions

In the presence of nitrogen, the GS₂-GOGAT pathway is likely to be responsible for primary nitrogen assimilation in *G. gracilis* (Figure 1). When nitrogen is sufficient, chloroplasts are enlarged, elongated, have a parallel thylakoid structure and phycobilisomes consisting of the pigment proteins phycoerythrin and phycocyanin are intact. Once the saturation concentration of nitrogen is reached, a shift in metabolism from nitrogen assimilation to 'nitrogen storage' may occur. Findings in Chapter 4 indicated that when nitrogen was sufficient, total GS activity initially decreased before steady state levels were maintained. The reduction in total GS activity may represent the change from active primary nitrogen assimilation to nitrogen storage. In addition, once the intracellular nitrogen concentration in *G. gracilis* became saturated, decreases in specific growth rate occurred even though biomass continued to increase.

NITROGEN REPLETE CELL

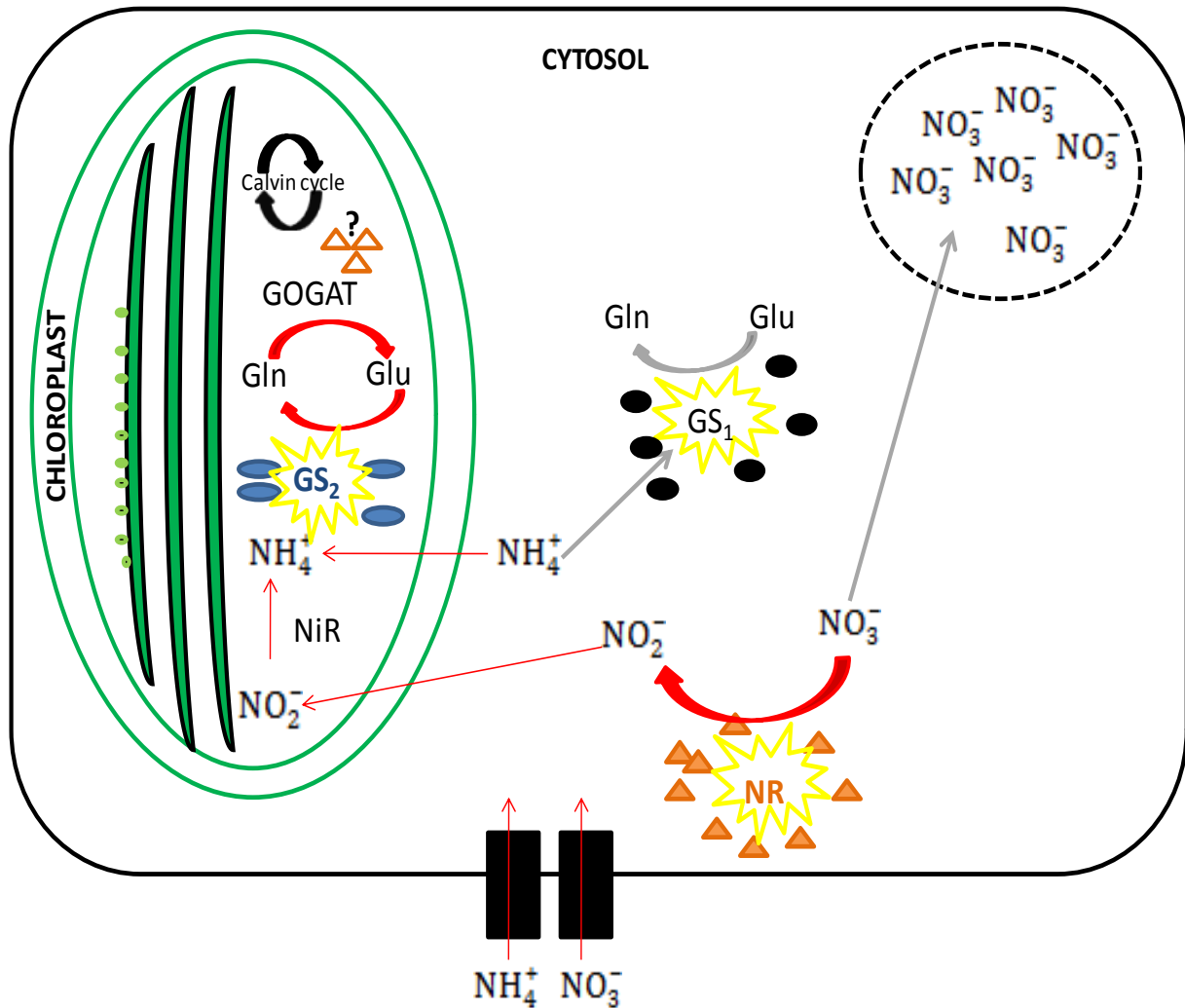


Figure 1. Simplified schematic representing the metabolic changes that occur when *G. gracilis* is growing in nitrogen replete conditions. The route of nitrogen assimilation in *G. gracilis* cells is indicated by the solid red arrows. Nitrate (NO_3^-) and ammonium (NH_4^+) are likely to be transported into the cell by nitrogen transporters. Once in the cytosol NO_3^- is reduced to nitrite (NO_2^-) by nitrate reductase (NR, \blacktriangle) which is transported into the chloroplasts where it is reduced to NH_4^+ by nitrite reductase (NiR). Further investigations into the role of chloroplastic NR (\blacktriangle) in nitrogen replete conditions must be completed. The NH_4^+ serves as a substrate for chloroplastic glutamine synthetase (GS₂, \bullet) to produce glutamine (Gln) which is subsequently converted to glutamate (Glu) by glutamate synthase (GOGAT) in the GS₂-GOGAT pathway. Once *G. gracilis* reaches the saturation nitrogen concentration, a shift from nitrogen assimilation to nitrogen storage may occur, as indicated by the solid grey arrows. NH_4^+ is then reduced to Gln by the cytosolic glutamine synthetase (GS₁, \bullet) and serves as a building block for the synthesis of other storage proteins. In addition, metabolites from nitrogen assimilation in chloroplasts may be used to synthesise pigment protein stores. Carbon skeletons provided by the Calvin cycle (solid black arrow) are used in the synthesis of storage proteins while NO_3^- may be transported to storage vacuoles where it is accumulated until required. During nitrogen replete conditions chloroplasts are elongated and thylakoids have a parallel distribution with many phycobilisomes (\bullet).

Nitrate taken up from the external environment may be stored in vacuoles (Chow, 2012) whereas ammonium may have been incorporated and stored as pigment proteins, other macromolecules or amino acids. Increases in storage metabolites would increase the biomass of the alga.

Nitrogen deplete conditions

When nitrogen levels are low or no external nitrogen is available, initial acclimation responses to nitrogen deprivation are induced in *G. gracilis* (Naidoo, 2012). Thus, expression of ATP synthase, actin, chaperonin GroEL, galactose-1-phosphate-uridylyl-transferase (GALT) and the glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and enolase, are up-regulated after 6 h in nitrogen deplete media. The metabolite UDP-galactose is one of the products of the enzyme reaction catalysed by GALT (key enzyme in galactose metabolism) and serves as a substrate for agar synthesis (Naidoo, 2012). Increased agar content during nitrogen limitation has been reported for *G. gracilis* (Smit *et al.*, 1996). In addition, D-galactose is an important building block for the synthesis of red algal cell wall components that include carrageenans and agarans (Naidoo, 2012) whereas actin is responsible for initiating changes in cytoskeletal structure within cells. GAPDH and enolase are important glycolytic enzymes as they catalyse reactions of glycolysis that produce the energy rich compounds 1,3 bisphosphoglycerate and phosphoenolpyruvate, respectively, from which ATP and reductant is ultimately generated (Mathews and van Holde, 1990). ATP is also generated by the action of the enzyme ATP synthase. Energy generated could be used in the synthesis of other macromolecules required to ensure algal survival in nitrogen deprived conditions and to initiate changes in cell ultrastructure whereas reductant is important for the continued activity of various enzymes (e.g. the GS₂-GOGAT cycle). The up-regulation of GroEL (assists in the refolding of proteins under stress conditions) may be correlated to increases in actin and may serve to protect or maintain cellular structure and the function of important proteins during nitrogen stress conditions (Naidoo, 2012). Thus, early responses to nitrogen deprivation involve acclimation processes that include changes in cell structure and an induction of metabolic processes that increase the demand for energy and reductant.

As nitrogen deprivation progresses, the alga is able to utilise nitrate stored that may be stored in vacuoles as well as ammonium released from the degradation of phycobilisomes to ensure that nitrogen assimilation and cellular metabolism can continue uninterrupted (Figure 2). The present study suggests that cytosolic NR may be involved in primary nitrate assimilation in *G. gracilis*. However, cytosolic NR was found to rapidly degrade during nitrogen starvation (Chapter 4) whereas chloroplast NR protein levels remained unchanged (Chapter 6). Therefore, continued NR activity detected in nitrogen deplete alga may be attributed to chloroplast NR isoforms. In nitrogen deprived conditions, it may be energetically more favourable to transport nitrate directly to chloroplasts where it is reduced to nitrite by chloroplastic NR thereby ensuring continued functioning of the GS₂-GOGAT pathway. Further investigation of this hypothesis is however required. As growth in nitrogen deprivation conditions continue, GS₁ activity may increase while GS₂ activity decreases as nitrate and pigment protein stores are depleted. Findings in Chapter 4 indicated that total GS activity increased considerably during nitrogen deprivation. Since *G. gracilis* cultured for eighteen days in nitrogen starved conditions showed an increase in GS₁ and a reduction in GS₂ activity (Chapter 6), increases in total GS activity of nitrogen starved *G. gracilis* may likely be attributed to increased GS₁ activity. This is further supported by the observation that GS₁ is responsible for the translocation of nitrogen (Bruhn *et al.*, 2010), an important process during nitrogen starvation. In addition, nitrogen and carbon metabolism are closely linked. When intracellular nitrogen is reduced and/or insufficient nitrogen is present, red algae divert the products of photosynthesis to the synthesis of starch, lipids or soluble compounds such as floridoside (Cole and Sheath, 1990). Triose phosphates from the Calvin cycle may be transported to the cytosol to serve as the initial substrate for starch synthesis (Viola *et al.*, 2001). Thus, up-regulation of energy producing pathways, accumulation of starch, re-organisation of cell structure and utilisation of nitrate and pigment protein stores, may represent strategies implemented to allow acclimation to and survival during the early phase of nitrogen starvation.

However, when *G. gracilis* reached the critical intracellular nitrogen concentration of 1.7% and was termed nitrogen limited, interesting metabolic changes occurred.

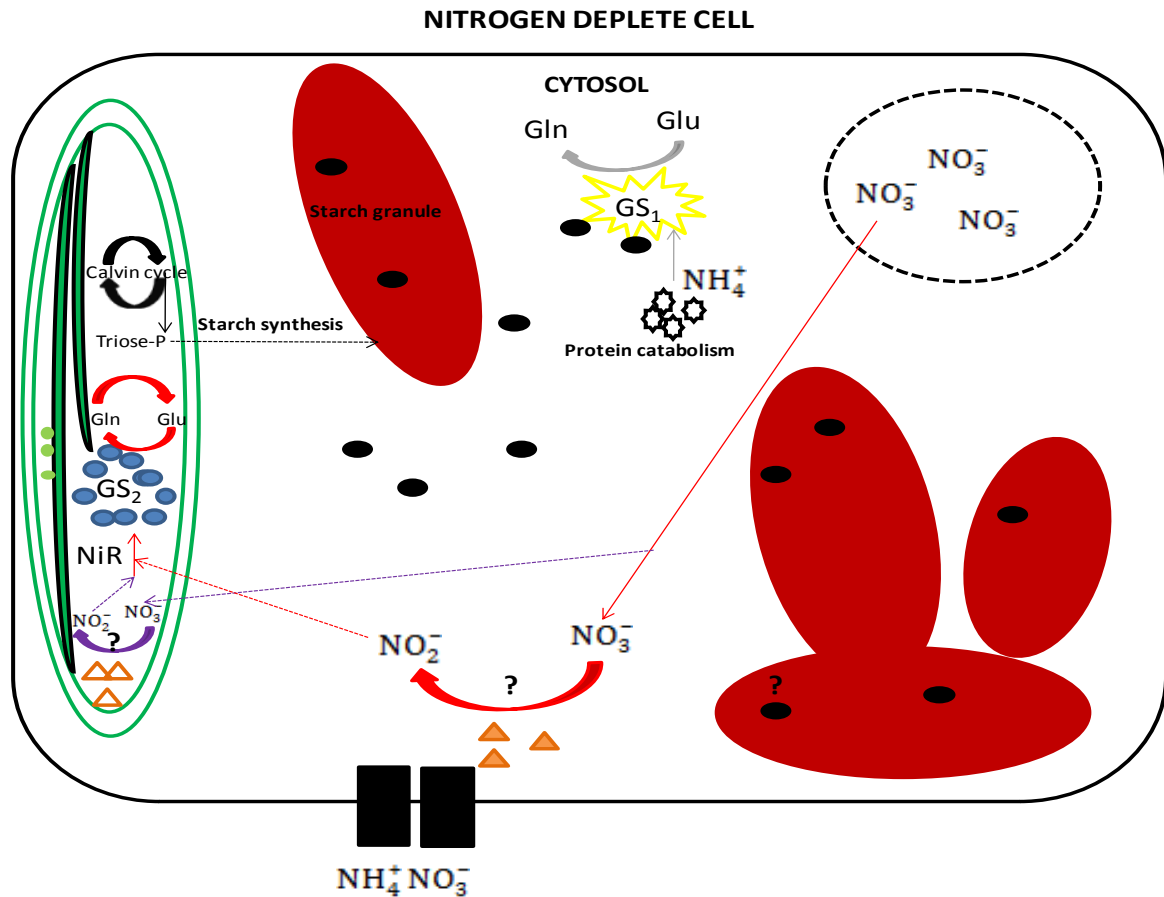


Figure 2. Simplified schematic representing metabolic changes that occur when *G. gracilis* is growing in nitrogen deplete conditions. Nitrate (NO_3^-) may be transported from vacuoles to the cytosol where it may be reduced to nitrite (NO_2^-) by cytosolic nitrate reductase (NR, \blacktriangle). Thus, the accepted route of nitrogen assimilation as indicated by the solid & dotted red lines may proceed. However, nitrogen starvation caused a reduction in cytosolic NR protein and/or activity levels. Therefore, it may be possible that NO_3^- is transported from the vacuoles directly into the chloroplasts where it is reduced to NO_2^- by chloroplast NR (\blacktriangle) as indicated by the dotted & solid purple arrows. Primary nitrogen assimilation via chloroplastic glutamine synthetase (GS_2 , \bullet) and glutamate synthase (GOGAT), indicated by the solid red line, is maintained until NO_3^- and protein stores are depleted. Degradation of phycobilisomes (\bullet) may also provide the GS_2 -GOGAT pathway with NH_4^+ in the early phase of nitrogen limitation. Prolonged growth in nitrogen starved conditions results in a reduction in GS_2 activity. NH_4^+ generated by protein catabolism in the cytosol is then reduced to glutamine (Gln) by cytosolic glutamine synthetase (GS_1 , \bullet) as indicated by the solid grey arrows. This nitrogen store can then be translocated to other organelles and used to synthesise proteins and metabolites necessary for algal survival. Nitrogen starvation also induces the synthesis of starch. Even though GS_1 was localised to starch grains, the involvement of GS_1 in starch grain synthesis must still be elucidated. Triose phosphates from the Calvin cycle, indicated by the solid black arrow, may be transported to the cytosol as indicated by the dotted black line and used in the synthesis of starch. Once *G. gracilis* cells are nitrogen limited, 'priming' responses that induce synthesis of proteins required for the re-supply of nitrogen/recovery from nitrogen starvation (such as an increase in GS_2) may be initiated.

For example, GS₂ protein increased without a concomitant increase in total GS activity (Chapter 4). It may be that once *G. gracilis* reaches the critical intracellular nitrogen concentration, in addition to 'acclimation' responses, a 'priming' response may be initiated that essentially positions the alga for the re-supply of nitrogen. Since cytosolic NR may be responsible for the primary reduction of nitrate, 'priming responses' should include increased synthesis of cytosolic NR protein. However, critical nitrogen concentrations limits growth and proliferation of the alga (Hanisak, 1979) and thus, it may not be energetically favourable to synthesise cytosolic NR in the 'priming' response. Conversely, GS₂ is important as it assimilates ammonia released from photorespiration. Indeed, barley GS₂ mutants did not survive when the plants were exposed to photorespiratory growth conditions (Baron *et al.*, 1994; Kamachi *et al.*, 1992). Data obtained in Chapter 5 suggest that re-supply of nitrate or ammonium to nitrogen starved *G. gracilis* may induce increased photorespiration. Thus, increased translation of GS₂ may be a 'priming' response to ensure algal recovery and survival following nitrogen starvation.

Microarray analysis indicated that mRNA transcripts encoding proteins for chromodomain helicase, elongation factor 3 homolog, elongation factor 1 α , gamma-glutamyl transferase, aspartate aminotransferase, the enzyme ribulose-phosphate-3-epimerase (RPEase) and cyclase dehydrase were up-regulated when *G. gracilis* was nitrogen limited while transcription of a chloroplast protein was down-regulated (Lebi, 2006). A reduction in transcripts encoding a chloroplast protein is in agreement with changes typical of nitrogen limitation. Since photosynthesis is reduced during nitrogen limitation (Berges *et al.*, 1996), there is no need to synthesise proteins that may be involved in photosynthetic reactions. Chromodomain helicase, elongation factor 3 and elongation factor 1 α are responsible for protein synthesis in cells (Lebi, 2006). Up-regulation of these transcripts supports the idea that important nitrogen metabolic enzymes, required to prime the cell for the re-supply of nitrogen as well as enzymes required for general maintenance of homeostasis, may be synthesised. Gamma glutamyl transferase is known to play a role in the redox control of enzymes and protection against oxidative stress (Lebi, 2006). Nutrient limitation is known to increase reactive oxygen species (ROS) (Palatnik *et al.*, 1999) and up-regulation of these transcripts may result in the synthesis of proteins required to protect important metabolic enzymes

against the deleterious effects of ROS. Aspartate aminotransferase catalyses the reversible reaction where glutamate and oxaloacetate are converted to aspartate and 2-oxoglutarate. Since 2-oxoglutarate is required by the GS₂-GOGAT cycle, increases in aspartate aminotransferase may supply 2-oxoglutarate to ensure continued activity of the GS₂-GOGAT cycle or it may be diverted from primary nitrogen assimilation to the TCA cycle which is responsible for producing energy and reductant. Energy and reductant are important as they are required to maintain metabolic functions and thus homeostasis during nitrogen limitation. In addition, these metabolites may be required in anabolic metabolic processes that prime the cell for the re-supply of nitrogen. The enzyme RPEase plays a role in both the Calvin cycle and the oxidative pentose phosphate (OPP) pathway and serves to partition these two pathways according to the metabolic needs and redox status of the cell (Chen *et al.*, 1998). The OPP pathway produces reducing equivalents and metabolites required for synthesis of nucleotides, nucleic acids and aromatic amino acids (Kruger and von Schaewen, 2003). Aromatic amino acids are important because they are precursors of many biosynthetic pathways that include the synthesis of secondary metabolites (Tzin and Galili, 2010). Since transcription of cyclase dehydrase, which is responsible for synthesis of secondary metabolites was up-regulated in nitrogen starved *G. gracilis* (Lebi, 2006), it may mean that RPEase is functioning in the OPP pathway to generate the precursors required for the synthesis of secondary metabolites. Secondary metabolites such as lipids are common in red algae (Cole and Sheath, 1990). Some red algae are able to store carbon as inclusion bodies of triacylglyceride during nitrate limitation and upon re-supply of nitrogen, the lipids are used to regenerate the plastid and serves as a store for membrane components. Up-regulation of RPEase and cyclase dehydrase transcription further supports the hypothesis that the critical nitrogen concentration may induce 'priming' responses or changes, where genes required to co-ordinate recovery responses when conditions become favourable are transcribed and/or their protein products translated. In addition to initiating a 'priming' response, metabolic shifts that maintain homeostasis are implemented. Therefore, this study together with data obtained by Lebi (2006) and Naidoo (2012) indicates that during nitrogen deprivation *G. gracilis* cells translocate energy, metabolites and reductant to the most necessary metabolic processes that maintain homeostasis as well as to processes that ready the alga for the re-supply of nitrogen.

However, *G.gracilis* is only able to sustain survival in a nitrogen limited condition for a finite period. As intracellular nitrogen percentages reach 1% or less, metabolic processes change once again (Figure 3). At these percentages, the alga may be termed severely nitrogen limited.

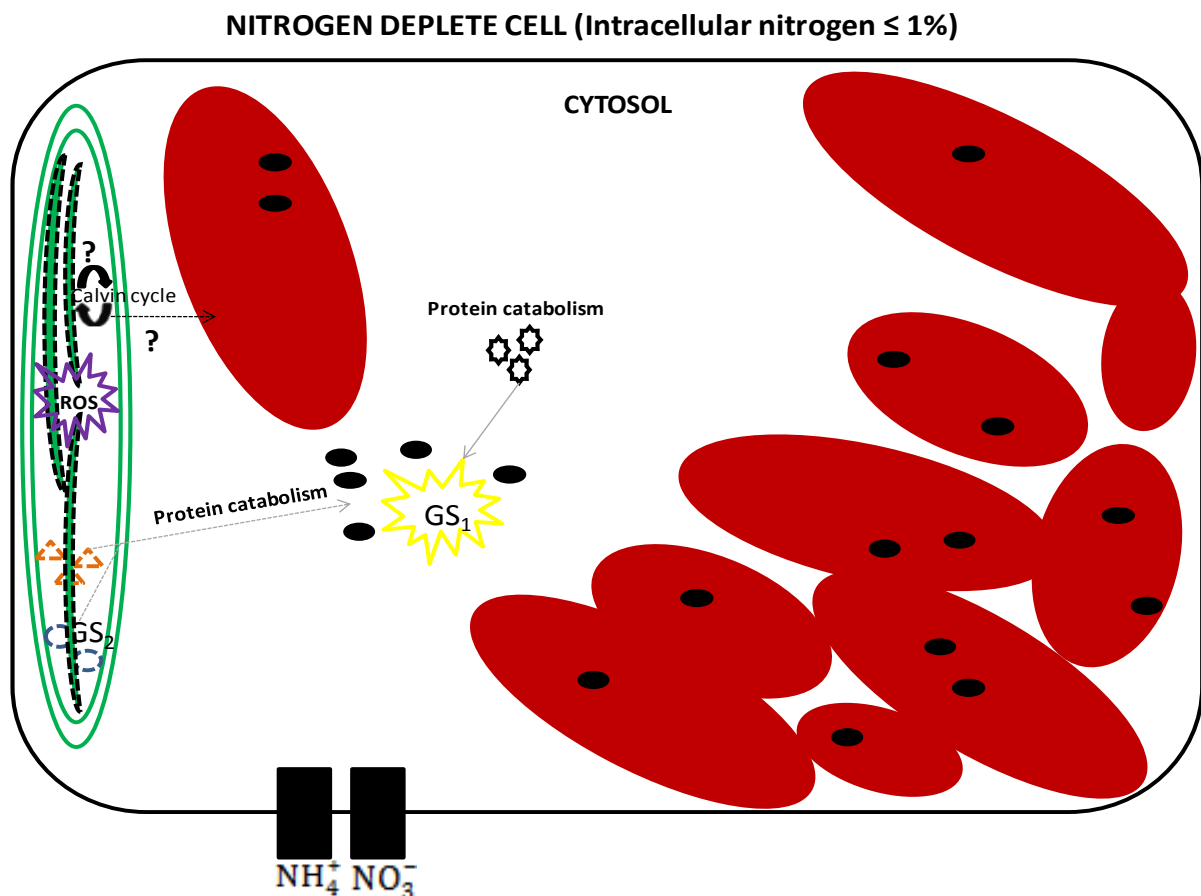





Figure 3. Simplified schematic representing metabolic changes that occur when *G. gracilis* is severely nitrogen limited. Chloroplasts are reduced in volume, thylakoids are dismantled and chloroplast proteins are degraded. An accumulation of ROS in the chloroplasts may lead to the oxidative degradation of chloroplastic glutamine synthetase (GS₂, ) . Degradation of chloroplast NR () may also represent a protein store that enables continued viability of algal cells during dormancy. Ammonium (NH₄⁺) from chloroplast protein degradation may be transported to the cytosol (dotted grey line), where it is reduced to glutamine (Gln) by cytosolic glutamine synthetase (GS₁, ), to be used in processes that maintain algal viability during dormancy. In addition, cytosolic protein catabolism (solid grey arrow) may also provide GS₁ with NH₄⁺. Prolonged culture in nitrogen starvation also leads to increased starch synthesis. However, the enzymatic reactions of the Calvin cycle may be reduced in severe nitrogen limitation as light reactions of photosynthesis are reduced and Rubisco is degraded. The effect of nitrogen limitation on carbon metabolism of *G. gracilis* must remain to be investigated.

GS₂ protein decreases to almost undetectable levels and little to no NR activity can be detected (Chapter 4). The marked reduction in phycoerythrin as well as decreases in chloroplast volume and breakdown of thylakoids, reduces photosynthesis. The reduction in photosynthesis may result in the accumulation of ROS which in turn could oxidise GS₂, thereby targeting it for degradation (Chapter 4). The degradation of chloroplast proteins such as Rubisco is not uncommon during nitrogen starvation as chloroplast proteins are more affected by nitrogen limitation (Vergara *et al.*, 1995; Andria *et al.*, 1999). Since NR activity was almost undetectable and *G. gracilis* was nearly depleted of all nitrogen stores during severe nitrogen limitation (Chapter 4), similar to *C. reinhardtii* (Fischer and Klein, 1988), chloroplast NR may be degraded and mobilised as an additional protein pool. The reduction in GS₂ protein, increased mRNA and activity of GS₁, dismantling of the thylakoids and degradation of chloroplast located proteins indicates that the cell may be entering the late phase of senescence. Consequently, *G. gracilis* cells may terminate the 'acclimation' and 'priming' responses and instead, enter a semi-dormant to dormant state in order to maintain cell viability. At approximately 1% intracellular nitrogen, a reduction in the glycolytic proteins GAPDH, phosphoglycerate kinase and fructose 1,6, bisphosphate aldolase, the photosynthetic protein ferredoxin NADPH reductase and ATP synthase occurred in nitrogen starved *G. gracilis* (Naidoo, 2012). Data obtained in Chapter 4 indicated that biomass accumulation ceased when *G. gracilis* intracellular nitrogen percentages were 1% or less. Biomass (proteins, nucleotides and amino acids) accumulation requires that metabolic processes producing energy, reductant, carbon skeletons and other necessary macromolecules are functioning (Plaxton and Mcmanus, 2006). Therefore, the cessation in biomass accumulation in severely nitrogen limited *G. gracilis* suggests that these processes are down-regulated, which is congruent with data obtained by Naidoo (2012). Thus, the hypothesis that intracellular nitrogen percentages of 1% and less may cause the alga may to enter a state of dormancy, a common response to stress (Grossman, 2002), is further supported.

In summary, nitrogen deprivation induces initial acclimation processes that allows algal survival in nitrogen deprived conditions. Once *G. gracilis* reaches the critical nitrogen concentration, 'priming' responses may be initiated in addition to responses required to maintain algal growth in nitrogen deprived conditions. However, prolonged nitrogen

deprivation causes the alga to become severely nitrogen limited and metabolic enzymes are down-regulated, chloroplast protein pools are degraded and the alga enters a semi-dormant to dormant phase that is likely to ensure continued survival. Once nitrogen is re-supplied to nitrogen starved *G. gracilis*, the alga is able to recover within four days (Gebrekiros, 2003). Data presented in Chapter 5 and 6 indicate that in general, cytosolic NR and GS₂ are transcribed and translated, but the induction of enzyme activity is dependent on whether carbohydrate reserves have been mobilised, as well as on the external nitrogen concentration and intracellular nitrogen status of the alga. Increased transcription, translation and activation of nitrogen metabolic enzymes, photosynthetic systems as well as other important metabolic enzymes, is congruent with data obtained by Naidoo (2012). The re-supply of nitrogen to starved *G. gracilis* up-regulated translation of GS, tRNA-dihydrouridine synthase, fructose 1.6. bisphosphate aldolase, thioredoxin, ATP synthase and CbbX protein. These proteins are involved in nitrogen metabolism, ATP synthesis, transcriptional regulation and glycolysis. Therefore, the presented model adequately describes/emphasises some of the important findings of the present study and sets a firm foundation for future studies.

Summary of the important discoveries in the present study and future work

One of the important discoveries was the identification of multiple NR and GS isoforms that illustrated the complexity of the regulation of nitrogen metabolism in *G. gracilis*. Immunocytochemical investigations localised NR and GS to the cell wall, cytosol and chloroplasts of the alga. The most striking observation was the localisation of GS to intracellular starch granules. In addition, it would seem that *G. gracilis* may contain inducible and constitutive NR isoforms. Cellular localisation studies complemented nutrient studies and were successful in establishing putative roles for NR and GS, in addition to their involvement in nitrogen assimilation. These roles include, nitrate transport, cell wall biosynthesis and senescence. Similar to other plants and algae, *G. gracilis* NR and GS isoforms have non-overlapping functions that are independently regulated by transcriptional, post-transcriptional, translational and post-translational mechanisms. The induction of NR and GS isoforms seemed to be dependent on the external nitrogen environment, intracellular nitrogen status, intracellular concentrations of metabolites, and other metabolic processes such as carbon

metabolism and photosynthesis. The observation that NR and GS may not be circadian regulated nor repressed/inhibited by ammonium alluded to possible adaptive strategies that have allowed *G. gracilis* to survive in an environment where nitrogen is availability fluctuates.

In attempting to elucidate the nitrogen metabolic processes and nitrogen stress responses of *G. gracilis*, the current study has given emphasis to many other avenues that require exploration if a clearer understanding of these responses in *G. gracilis* is to be gained. Future studies must further characterise the function, regulation and location of NR and GS isoforms. First, it is important to identify all the genes encoding NR and GS proteins. For example, GS is an oligomeric enzyme that exists as a homo- or hetero-oligomer (Temple *et al.*, 1996). Regulation of an oligomeric enzyme such as GS is complex as the assembly of different subunits requires that the expression of genes encoding each subunit be co-ordinated (Brechlin *et al.*, 2000). This means that all genes required for haloenzyme assembly must be identified and their expression evaluated. Identifying and evaluating the expression all GS genes in an organism is a complex task which has not been solved in the majority of genetic studies to date. Second, immunocytochemical localisation of chloroplast located NR, starch granule GS and cell wall NR and GS must be further investigated. Identifying gene sequences would confirm immuno-localisation findings as transit peptide sequences would allow subcellular targets to be identified. Conversely, NR and GS isoenzymes can be purified from various subcellular compartments using biochemical methods described in Chapter 6. Third, regulatory mechanisms controlling NR and GS expression and activity warrant further investigation. *G. gracilis* NR and GS are likely to be regulated transcriptionally, post-transcriptionally and post-translationally via mechanisms that involve allosteric modulation or degradation of the enzyme. Regulatory mechanisms could be assessed by completing studies using inhibitors of transcription and enzyme activity, or assessing GS and NR interactions with regulatory proteins such as 14-3-3 proteins, similar to studies described by Chow and de Oliveira (2008), Pozuelo *et al.* (2001) and Humanes *et al.* (1995), to name a few. In addition, purification of NR and GS would allow the specific biochemical properties of the different isoenzymes to be investigated and may aid in understanding how they are regulated. Fourth, in order to gain a more complete understanding of the role of these enzymes in cellular metabolism, additional growth

studies employing the same techniques used in the present study can be performed. For example, the present study indicated that NR and GS may be regulated by photosynthesis, carbon metabolism, light-dark transitions and pathogen attack. In addition, further investigation of the effect of irradiance on circadian control may give greater insight into the function and regulation of NR and GS. The impact or interaction of nitrogen metabolism with other metabolic processes emphasised that intracellular tissue nitrogen concentrations and metabolite pools (amino acids, carbon skeletons, energy and reductant) are key in directing and regulating expression of NR and GS. Thus, findings in the present study would be complemented by completing metabolomic studies that evaluate changes in metabolites on a large scale. Our research group has already employed microarray (Lebi, 2006) and two dimensional SDS-PAGE (Naidoo, 2012) techniques to address global changes in mRNA and protein abundance, respectively, in nitrogen replete and deplete *G. gracilis*. A global understanding of the processes occurring in nitrogen replete/deplete *G. gracilis* would allow studies like the present study (that focus on the specific role of key enzymes) to elucidate the finer details of nitrogen metabolism and mechanisms of the stress response in *G. gracilis*.

In conclusion, in attempting to elucidate nitrogen metabolism and stress responses in *G. gracilis*, this study has (i) opened the door to many interesting avenues that can be explored to explain *G. gracilis*'s ability to survive in a nitrogen limited environment, (ii) successfully established a putative model for nitrogen and other metabolic responses that occur when *G. gracilis* is nitrogen starved and nitrogen replete and (iii) elucidated the possible roles and regulation of the nitrogen metabolic enzymes NR and GS, their isoforms and the physiological advantage they confer to *G. gracilis*.

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APPENDIX A

MEDIA, BUFFERS AND SOLUTIONS

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A.1 GENERAL MEDIA

A.1.1 Luria Agar

Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g
Agar	15 g
Water to	1000 mL

Autoclave

When making luria broth (LB), omit the agar.

A.2 CULTURE MEDIA

A.2.1 ARTIFICIAL SEAWATER (ASW)

NaCl	24.7 g
MgCl ₂ .6H ₂ O	4.7 g
CaCl ₂ .2H ₂ O	1.9 g
MgSO ₄ .7H ₂ O	6.3 g
KCl	0.66 g
NaHCO ₃	0.18 g
Water to	100 mL

Autoclave

A.2.2 PES-N (Nitrogen - free/deplete) MEDIA

Na ₂ glycerophosphate	50 mg
Fe - solution	25 mL
PII metal solution	25 mL
Vitamin B12	10 µg
Thiamine	0.5 mg
Biotin	5 µg
Tris	500 mg
Water to	1000 mL

Adjust to pH 7.8, prior to autoclaving

Store at 4°C

Add 6.6 mL per 1 L ASW

A.2.3 PES (nitrogen replete) media (1/3 strength) (Provasoli, 1968)

Made according to method described for PES-N with the exception that nitrate in the form of NaNO₃ is added. Additionally, the Fe - solutions differ.

A.2.4 IRON SOLUTION (Fe-SOLUTION) for PES-N MEDIA

Fe ₂ SO ₄	100 mg
Na ₂ EDTA	600 mg
Water to	1000 mL

A.2.5 Fe-SOLUTION FOR PES MEDIA

Fe(NH ₄) ₂ (SO ₄).6H ₂ O	100 mg
Na ₂ EDTA	600 mg
Water to	1000 mL

A.2.6 PII METAL SOLUTION

Na ₂ EDTA	100 mg
H ₃ BO ₃	114 mg
FeCl ₃ .6H ₂ O	4.9 mg
MnSO ₄	16.4 mg
ZnSO ₄ .7H ₂ O	2.2 mg
CoSO ₄ .7H ₂ O	0.48 mg
Water to	100 mL

A.2.7 1M NaNO₃

NaNO ₃	4.25 g
Water to	50 mL
Autoclave	

A.2.8 1M NH₄Cl

NH ₄ Cl	2.67 g
Water to	50 mL

A.2.9 0.48% GERMANIUM OXIDE

Germanium oxide	0.48 g
Water to	100 mL

Add 1 mL to 2L of ASW

A.3 SOLUTIONS FOR RNA ISOLATION

A3.1 3M SODIUM ACETATE

Sodium acetate	2.46 g
Water to	10 mL

A.4 SOLUTIONS FOR ENZYMATIC ASSAYS

All stock solutions and water were either filter sterilised or autoclaved before being used to make the necessary extraction buffers, assay mixes and stop solutions required for enzymatic assays.

A4.1 STOCK SOLUTIONS

A4.1.1 0.2 M DTT

DTT (Sigma)	0.15 g
Sterile water to	5 mL

A4.1.2 2 M MgCl₂

MgCl ₂	40g
Water to	100mL
Autoclave	

A4.1.3 4 M HYDROXYLAMINE

Hydroxylamine	2.78 g
Water to	10 mL
Filter sterilise	

A4.1.4 0.85 M MONOSODIUM GLUTAMATE

Monosodium glutamate	0.96 g
Water to	10 mL
Filter sterilise	

A4.1.5 0.4 M ATP

0.2 g ATP added to 1ml autoclaved, distilled water.

A4.1.6 10% FeCl₃

FeCl ₃	1 g
Water to	10 mL

A4.1.7 24% Trichloroacetic acid (TCA)

TCA	24 g
dH ₂ O	100 mL

A4.1.8 6 M HCl

Add 5.89 mL of HCl (10.16 M) to 4.11 mL of water

A4.1.9 2 mM NADH
NADH
Water to
Filter sterilise

1.5 mg
1 mL

A4.2 NR ASSAY MIX

1M KNO₃ 0.2 mL
0.5 M EDTA 2 µl
1 M Tris (pH 8) 0.5 mL
Sterile water to 10 mL

A4.3 GS FORWARD ASSAY MIX

1 M Tris 0.15 mL
4 M Hydroxylamine 0.071 mL
2 M MgCl₂ 0.168 mL
0.85 M Monosodium glutamate 1.2 mL
Sterile water to 3 mL
Adjust to pH 7.5

A4.4 GS TRANSFERASE ASSAY MIX (1 reaction)

2 M Imidazole 120 µl
0.2 M Glutamine 75 µl
0.02 M MnCl₂ 7.5 µl
0.02 M ADP 10 µl
1 M Potassium arsenate 10 µl
4 M Hydroxylamine 7.5 µl

A4.5 1% SULPHANILAMIDE

Sulphanilamide 1 g
3 M HCl 100 mL
Filter sterilise

A4.6 0.02% *n* - (1 - NAPHTHYL) ETHYLENEDIAMINE DIHYDROCHLORIDE (NEDI)

NEDI 0.02 g
Water to 100 mL
Filter sterilise

A4.7 GS ASSAY STOP MIX

10 % FeCl₃.6H₂O 5 mL
24% TCA 1.25 mL
6 M HCl 0.625 mL
Water to 15 mL

A.5 SOLUTIONS FOR PROTEIN ISOLATIONS AND WESTERN BLOTS

A5.1 PROTEIN EXTRACTION BUFFER

1M Tris pH 7.5 (0.5M)	5 mL
0.5M EDTA (10mM)	0.1 mL
1% Triton X-100	0.2 mL
2% Mercaptoethanol	0.2 mL
Sterile water	4.5 mL

A5.2 0.1 M AMMONIUM ACETATE

Dissolve 3.85 g ammonium acetate in 500 mL methanol

A5.3. UREA LYSIS BUFFER (ULB)

Urea	24.0 g
Dissolve in 25 mL water by continuous stirring and then add:	
CHAPS	0.25 g
Thiourea	7.6 g

A5.4 0.1 M HCl

Perform a 1:100 dilution using 10.18 M HCl

A.5.5 5X SAMPLE APPLICATION BUFFER (SAB)

Tris-Cl (pH 6.8)	250 mM
DTT	500 mM
SDS	10%
Glycerol	10 mL
Water	5 mL

Add a small amount of Bromophenol blue, just enough to generate a deep blue colour.

A5.6 PONCEAU S staining solution

Ponceau S (Sigma)	0.1 g
Acetic Acid	5 mL
Sterile water	100 mL

Do not autoclave. Light sensitive. Reusable.

To visualise the protein on a nitrocellulose membrane:

Following protein transfer onto nitrocellulose membranes, incubate the membranes in 250 mL of Ponceau S stain for 15 min. Thereafter, rinse membranes in distilled water for to aid visualisation of transferred proteins. Ponceau S stain is removed by rinsing the membranes in 1X TBS (Appendix 5.7) with agitation to remove the majority of the stain.

A5.7 10X TBS

Tris Base	50.5 g
NaCl	73.14 g
Water to	500 mL
Adjust to pH 7.4 with HCl	
Autoclave	

A5.8 TBS-T (0.1% Tween in 1X TBS)

Tween-20	0.1 mL
10X TBS	100 mL
Water to	1000 mL
Filter sterilise	

A5.9 BLOCKING BUFFER (5% skim milk)

Skim milk	5g
10X TBS	10 mL
Water to	100 mL
Filter sterilise	

A5.10 TOWBIN BUFFER

Tris base	3.03 g
Glycine	14.42 g
Methanol	200 mL
Water to	1000 mL

A. 6 SOLUTIONS FOR CHLOROPLAST ISOLATIONS

A6.1 CHLOROPLAST ISOLATION BUFFER

1M Tris (pH 7.5)	1.5 mL
0.5 M EDTA	1.5 mL
Sorbitol (Merck)	8.7 g
PEG 4000	3 g
Water to	30 mL
Autoclave	

A.7 SOLUTIONS FOR IMMUNOLOCALISATION STUDIES

A7.1 PHOSPHATE BUFFERED SALINE (PBS)

NaCl	80.0 g
KCl	2.0 g
Na ₂ HPO ₄ ·7H ₂ O	17.8 g
KH ₂ PO ₄	2.4 g
Water to	1000 mL
Adjust pH to 7.4 with NaOH	
Autoclave	

A7.2 BASE BUFFER (BB)

10x PBS (pH 7.4)	10 mL
NaCl	2.34 g
Water to	100 mL
Filter sterilise.	

A7.3 2.5% GLUTARALDEHYDE IN BB

25% glutaraldehyde (Sigma)	1 mL
10x BB	1 mL
Water to	10 mL
Do not autoclave. Filter sterilised and freshly prepared.	

A7.4 IMMUNOLOCALISATION FIXATIVE

10% paraformaldehyde	4 mL
25% glutaraldehyde	0.04 mL
10x BB	1 mL
Sterile water to	10 mL

A7.5 2% URANYL ACETATE

Uranyl acetate (Sigma)	5 g
100% Methanol	25 mL
Filter sterilise before use. Store at 4°C	

A7.6 REYNOLDS LEAD CITRATE

Lead citrate (Sigma)	1.33 g
Sodium citrate (Saarchem)	1.76 g
Ultrapure water	30 mL
1 M NaOH	8 mL
Ultrapure water to	50 mL

Dissolve lead citrate and sodium citrate in 30 mL water and shake for 1 min. Allow solution to stand for 30 min before adding NaOH. Make up to 50 mL.

A7.7 PBS /1%BSA (PBS BSA)

10x PBS (pH 7.4)	1 mL
BSA (Roche)	0.1 g
Water to	10 mL
Filter sterilised and freshly prepared.	

A7.8 PBS (1% GLUTERALDEHYDE)

25 % glutaraldehyde	100 µl
10x PBS (pH7.4)	250 µl
Sterile water to	2.5 mL
Do not autoclave and freshly prepared.	

APPENDIX B

Identification of suitable reference gene (RG) for qPCR analysis, optimisation of western hybridisation, evaluation of the efficacy of chloroplast isolation method and optimisation of immuno-localisation experiments

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B 1. DETERMINATION OF A SUITABLE REFERENCE GENE (RG)

Gene expression studies that employ techniques such as qPCR, require that the expression data be normalised in order to reduce the influence of variations in RNA extraction yield, reverse-transcription (RT) yield and efficiency of amplification (Bustin *et al.*, 2009). Differences in RNA quality can be normalised by assessing RNA quality using agarose gel electrophoresis or measuring the 280:260 ratio whereas the amount of RNA can be quantified spectrophotometrically. Thus, a known quantity of high quality RNA can be used in subsequent RT reactions. However, contaminants from RNA extraction such as phenol or relative differences in transcript degradation may affect RNA quantification and/or reverse transcription. Therefore, in addition to standardising the amount of RNA and ensuring that RNA of a high quality that is not degraded is used in subsequent RT reactions, researchers commonly include internal controls/reference genes (RGs) for data normalisation (Nolan *et al.*, 2006). A reference gene can be defined as a stably expressed gene whose abundance is correlated with the total amounts of mRNA present in a sample (Bustin *et al.*, 2009). In reality, it is unlikely that the expression of a gene will remain constant no matter the treatment/stress imposed on the organism. Thus, it is good practice to determine suitable RGs for different experimental conditions by establishing that its expression is invariant under the tested condition.

The task of finding suitable RGs for nitrogen nutrition experiments is difficult as the *G. gracilis* genome has not been sequenced. However, our research group has identified *G. gracilis* genes that participate in various metabolic processes, and primer sets that amplify these genes are available. The following *G. gracilis* genes: *Hsp 70*, 18S rRNA, sugar epimerase, hypothetical protein 245, hypothetical protein 801 and putative serine protease (*PSP*) were evaluated for suitability as a RG. qPCR was performed using cDNA synthesised from RNA isolated from nitrogen deplete *G. gracilis* as described (4.2.3). A single biological repeat was performed due to cost concerns. The qPCR profile was the same as described (4.2.3), with the exception that the annealing temperature of each primer set for each gene differed (Table 1).

Table 1. Candidate RG for use in qPCR evaluation of NR and GS₁ transcript abundance.

Gene	Origin	Ta (°C)	Reference
Hsp 70 ^a	Isolated from heat shocked <i>G. gracilis</i> RNA	60	McLachlan (unpublished data)
18S rRNA ^a	Ribosomal RNA	59	Ealand (PhD thesis, 2011)
Sugar Epimerase ^a	Found not to be regulated disease stressed <i>G. gracilis</i>	59	Ealand (PhD thesis, 2011)
Hypothetical protein 245 ^a	Found not to be regulated disease stressed <i>G. gracilis</i>	60	Ealand (PhD thesis, 2011)
Hypothetical protein 801 ^a	Found not to be regulated disease stressed <i>G. gracilis</i>	60	Ealand (PhD thesis, 2011)
PSP ^b	Putative serine protease found to be up-regulated in disease stressed <i>G. gracilis</i>	59	Ealand (PhD thesis, 2011)

^a Genes not suitable for use as a RG. ^b Genes suitable for use as a RG. Annealing Temperatures (Ta) are indicated.

Microarray analysis indicated that the expression of the sugar epimerase, hypothetical protein 245 and a hypothetical protein 801 was invariant when *G. gracilis* was subjected to disease stress (Ealand, 2011). Further tests evaluating their suitability as RGs indicated that the expression of the genes encoding the sugar epimerase and hypothetical protein 245 were invariant in control (cultured in nitrogen replete media without disease elicitors) and disease (cultured in nitrogen replete media with disease elicitors) conditions. This was not the case when *G. gracilis* was subjected to nitrogen starvation. Amplification plots indicated that amplification of candidate genes were detected after 35 cycles (Figure 1A, 1D & 1E) and/or genes were regulated with Ct values differing by two to five units (Figure 1A-D). Even the routinely used RG 18S rRNA was not suitable for use as a RG in the present study (Figure 1A). However, *PSP* proved to be a suitable RG for evaluation of *GgNR* and *GgGS₁* expression, as *PSP* expression

remained constant over the time course of the nitrogen deprivation experiments. The suitability of *PSP* as a RG for nitrogen nutrition studies was further evaluated by testing the expression of the gene under various nitrogen growth conditions that included initial culture in nitrogen replete media (PES ASW), followed by 18 days of culture in nitrogen lacking media (PES-N ASW), after which the alga was re-supplied with nitrogen. Thalli were sampled at the following times; A0, A3, A7, L2, L6, L10, L14, L18, R3 and R6 where 'A', 'L' and 'R' correspond to nitrogen replete, nitrogen lacking and re-supply of nitrogen, respectively, and the numbers represent time (days). RNA was isolated from each of the samples, converted to cDNA and qPCR was performed. Once again, a single biological repeat was completed as costs needed to be considered. *PSP* maintained stable expression over all nitrogen conditions and Ct values were generally within one unit of each other (Figure 2A & B).

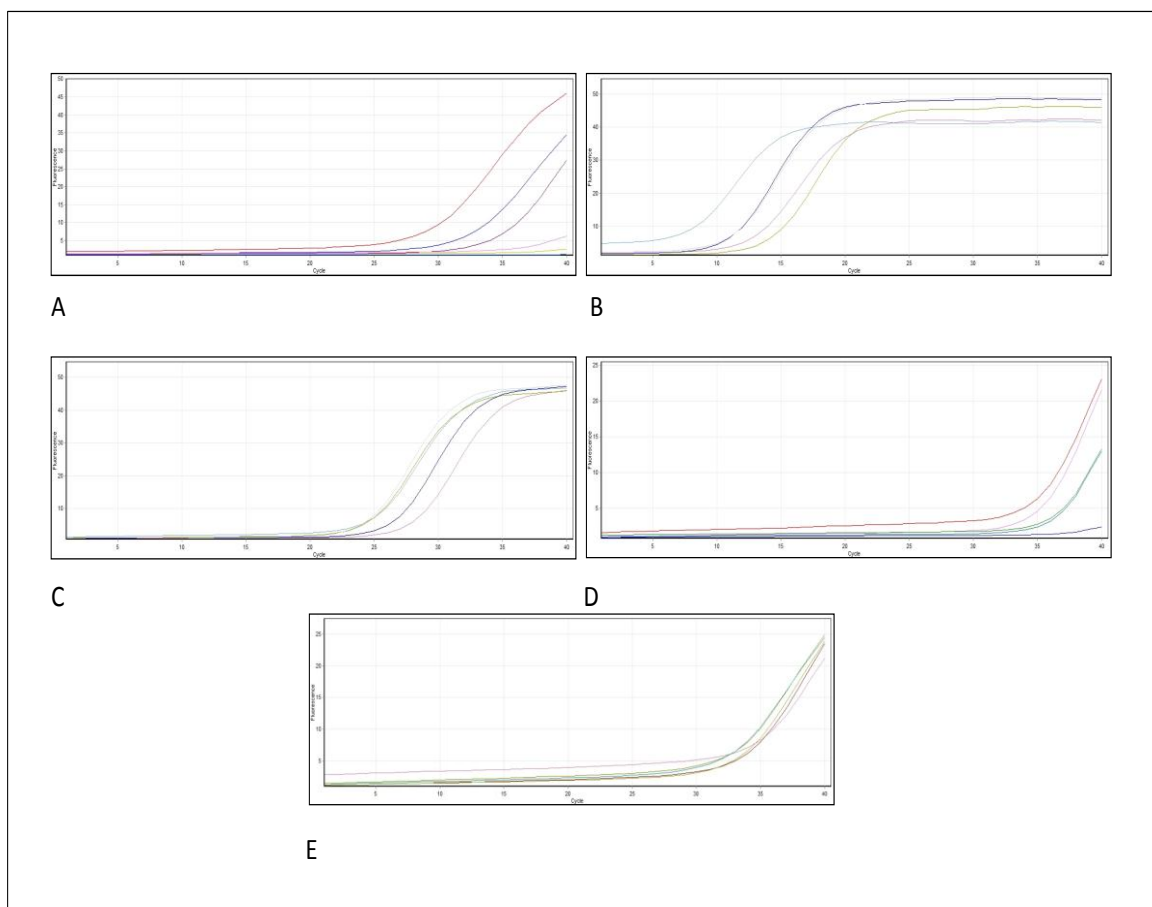


Figure 1. Amplification plots for (A) 18S rRNA, (B) hypothetical protein 245, (C) Hsp70, (D) hypothetical protein 801 and (E) sugar epimerase. Gene expression was evaluated after 2, 6, 10 and 14 days of culture in nitrogen lacking media. A control (nitrogen replete) sample was also tested.

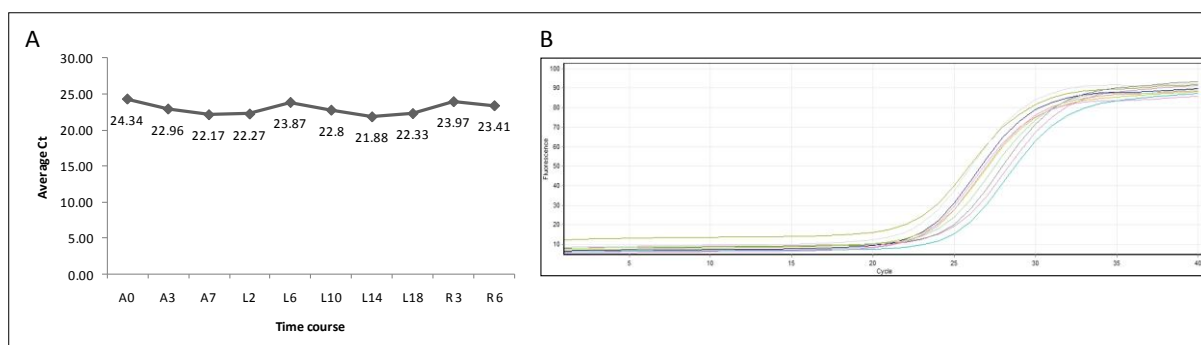


Figure 2. (A) graph representing the Ct values of RG *PSP* when cultured under differing nitrogen conditions. 'A' refers to culturing in nitrogen replete media, 'L' to culturing in nitrogen lacking and 'R' to the re-supply of nitrogen following nitrogen starvation. Numbers represent time in days. The A0 sample represents thalli before transfer to PES ASW as described (3.2.1). qPCR reactions were performed in triplicate and mean Ct values ($n = 1$) were automatically calculated in the Rotor-Gene 6000 software. (B) amplification plot of *PSP* gene expression.

Thus, *PSP* met the criteria of a RG as it was stably expressed over differing nitrogen conditions. Ideally when completing qPCR analysis, multiple RGs should be employed when evaluating gene expression (Bustin *et al.*, 2009). However, this is not always possible, particularly when working with non-model organisms as sequence information is limited. Thus, future studies should investigate which other *G. gracilis* genes are suitable RGs for evaluating gene expression in nitrogen nutrition studies. This can be achieved by examining microarray data of Lebi (2006) and Ealand (2011) to identify genes whose expression was unchanged in nitrogen deprivation and disease stress, respectively. The expression of these genes could then be examined under differing nitrogen treatments as described (Appendix B 1) and their suitability as RGs can be assessed using programmes such as geNORM (Vandesompele *et al.*, 2002). However, this was beyond the scope of the present study. Since *PSP* expression remained unchanged no matter the nitrogen treatments, its use as a RG to evaluate *GgNR* and *GgGS₁* was indeed valid. Once other RGs for nitrogen nutrition studies have been validated, expression data in the present study can be examined once again to confirm *GgNR* and *GgGS₁* expression data.

B 2 OPTIMISATION OF WESTERN HYBRIDISATION

B 2.1 Confirmation that AS08 210 NR and GLN1-2 cross react with *G. gracilis* NR and GS isoforms, respectively.

Successful western hybridisation requires that the antibody employed cross reacts with the target protein. The cross reactivity of commercially available antibodies (known to detect NR and GS in plants and green algae) with *G. gracilis* NR and GS isoforms was evaluated.

Total protein was extracted (4.2.5) from control (Time 0, nitrogen replete) and day 14 nitrogen starved *G. gracilis* samples. Thereafter, western hybridisation analysis was performed as described (4.2.6). Western hybridisation analysis confirmed that the approximately 110 kDa polypeptide visualised using AS08 210 NR antibodies was indeed *G. gracilis* NR (Figure 3) as indicated by the positive *Arabidopsis* NR control. Initially primary (1:1000) and secondary (1:4000) antibody dilutions corresponded to those described by the manufacture (Agrisera) for the detection of *Arabidopsis* NR. In subsequent western hybridisation analysis, a range of secondary antibody dilutions were tested. A secondary antibody dilution of 1:20 000 resulted in the visualisation of *G. gracilis* NR and a reduction in non-specific binding of lower molecular weight polypeptides (data not shown). Thus, it was established that the optimal primary and secondary antibody dilutions for *G. gracilis* NR were 1:1000 and 1:20 000, respectively.

Similarly, initial western hybridisation analysis was performed according to the conditions described for the detection of *Arabidopsis* GS isoforms, as outlined by the manufacturer (Agrisera). These conditions proved to be optimal for the visualisation of *G. gracilis* GS isoforms. The 43 kDa *G. gracilis* GS polypeptide visualised using the GLN1-2 antibody aligned with GS₂ of *Arabidopsis* (~ 44 kDa) (Figure 4). Therefore, it is likely that the 43 kDa *G. gracilis* protein represents GS₂ while the lower molecular weight polypeptide detected in protein samples from nitrogen starved thalli (Chapter 4), corresponded to GS₁.

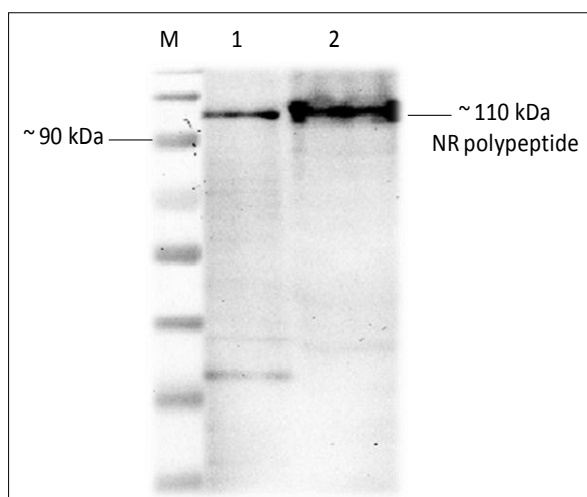


Figure 3. Testing cross reactivity of AS08 210 NR antibody to *G. gracilis* NR. Forty micrograms of protein isolated from *G. gracilis* nitrogen replete thalli and fifteen micrograms of *Arabidopsis* leaf protein were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with 1:1000 dilution of AS08 210 NR primary antibody and a 1:4000 dilution of the secondary antibody (alkaline phosphatase goat anti-rabbit IgG conjugate). Lane 1 represents the *G. gracilis* sample and lane 2 the *Arabidopsis* sample. PageRuler™ Prestained Protein Ladder (Fermentas), was used to size polypeptides.

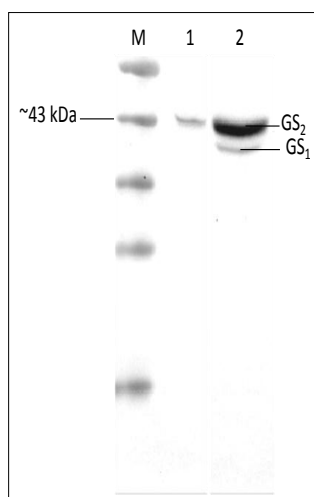


Figure 4. Testing cross reactivity of GLN1-2 antibody to *G. gracilis* GS isoforms. Thirty micrograms of protein isolated from *G. gracilis* nitrogen replete thalli and 0.5 µg of *Arabidopsis* leaf protein were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with 1:10 000 dilution of GLN1-2 primary antibody and a 1:20 000 dilution of the secondary antibody (alkaline phosphatase goat anti-rabbit IgG conjugate). Lane 1 represents the *G. gracilis* sample and lane 2 the *Arabidopsis* sample. PageRuler™ Prestained Protein Ladder (Fermentas) was used to size polypeptides.

B 2.2 Determination of the optimal amount of protein required to visualise *G. gracilis* NR and GS isoforms

Once it was confirmed that the respective commercial antibodies cross reacted with *G. gracilis* NR and GS proteins, the optimal amount of protein required for visualisation was tested. The aim of these experiments were performed to establish the amount of protein required to detect target proteins of nitrogen replete and deplete *G. gracilis*, so as to standardise the amount of protein used in subsequent western hybridisation analysis.

A range of total protein concentrations (20-50 µg) were tested. Total protein was isolated from nitrogen replete and day 14 nitrogen starved algal samples. NR was not detected in the total protein extracts of nitrogen starved algae but visualised in those of nitrogen replete algae (Figure 5). It may be likely that NR was either not present in day 14 nitrogen starved samples or the amount of NR present was too low to allow detection. When completing western hybridisation it is suggested that the amount of protein loaded onto mini-polyacrylamide gels should not exceed 5-20 µg/lane as non-specific cross reactions, higher background and diffuse protein bands may be detected (www.molecularstation.com/protein/western-blot/; Thermo Scientific Western Blotting Handbook, 2010). When 20 µg of total protein was loaded in protein gels, *G. gracilis* NR in nitrogen replete samples was faintly detected. Therefore, 20 µg of total protein may not be sufficient to detect NR in nitrogen deplete samples. Since NR was easily visualised when 30–50 µg of total protein was loaded onto mini-polyacrylamide gels, it was decided that 40 µg of total protein would be used in subsequent western hybridisation analysis experiments evaluating NR expression.

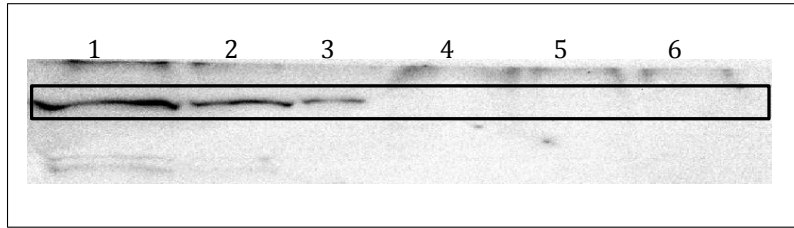


Figure 5. Western hybridisation analysis testing the amount of total protein required to detect *G. gracilis* NR. A range of total protein concentrations were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with 1:1000 dilution of AS08 210 NR primary antibody and a 1:20 000 dilution of the secondary antibody (alkaline phosphatase goat anti-rabbit IgG conjugate). Lanes 1-3: 50, 30 and 20 μ g protein isolated from Time 0 nitrogen replete *G. gracilis* and lanes 4-6: 50, 30 and 20 μ g protein isolated from day 14 nitrogen starved *G. gracilis*. *G. gracilis* NR is outlined by the black box.

Similar to NR, a range of protein concentrations (10-35 μ g) were tested in order to determine the amount of protein required to visualise *G. gracilis* GS isoforms. *G. gracilis* GS₂ was detected in nitrogen replete thalli whereas both isoforms were detected in nitrogen starved thalli (Figure 6). When 10 μ g of total protein isolated from nitrogen starved algae was used, *G. gracilis* isoforms were not detected (data not shown). These experiments indicated that 30 μ g of total protein would be sufficient to detect GS isoforms in nitrogen replete and nitrogen starved *G. gracilis*.

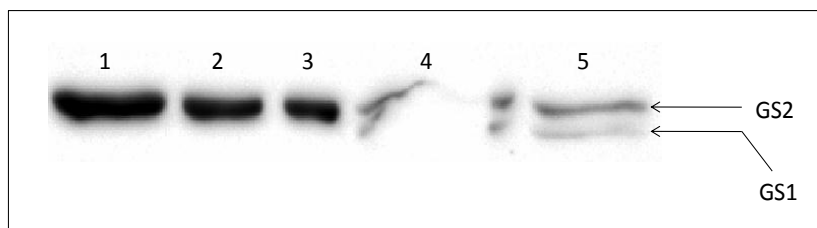


Figure 6. Western hybridisation analysis testing the amount of total protein required to detect *G. gracilis* GS. Differing amounts of total protein were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with 1:10 000 dilution of GLN1-2 primary antibody and a 1:20 000 dilution of the secondary antibody (alkaline phosphatase goat anti-rabbit IgG conjugate). Lanes 1-3: 35, 20 and 10 μ g protein isolated from Time 0 nitrogen replete *G. gracilis* and lanes 4 & 5: 35 and 20 μ g protein isolated from day 14 nitrogen starved *G. gracilis*. *G. gracilis* GS₁ and GS₂ isoforms are indicated by the black arrows.

B 3 ASSESSMENT OF THE EFFICACY OF THE CHLOROPLAST ISOLATION METHOD

Hapgonian *et al* (2002) found that the inclusion of polyethylene glycol (PEG) 6000 resulted in the isolation of intact chloroplasts. Omission of this compound from the chloroplast extraction buffer resulted in the lysing of chloroplasts. Thus, the efficacy of the isolation method at isolating intact *G. gracilis* rhodoplasts was assessed. Following rhodoplast isolation (5.2.2.1), re-suspended rhodoplasts were loaded onto a two-step sucrose gradient and ultracentrifuged at 112 000 x *g* for 1 h. Thereafter, the intactness of separated rhodoplasts was assessed microscopically (Figure 7). Microscope observations confirmed that the method described by Hapgonian *et al.* (2002) successfully isolated intact *G. gracilis* rhodoplasts as a uniform distribution of chlorophyll autofluorescence was detected. Cell lysis would have cause the release of pigments.

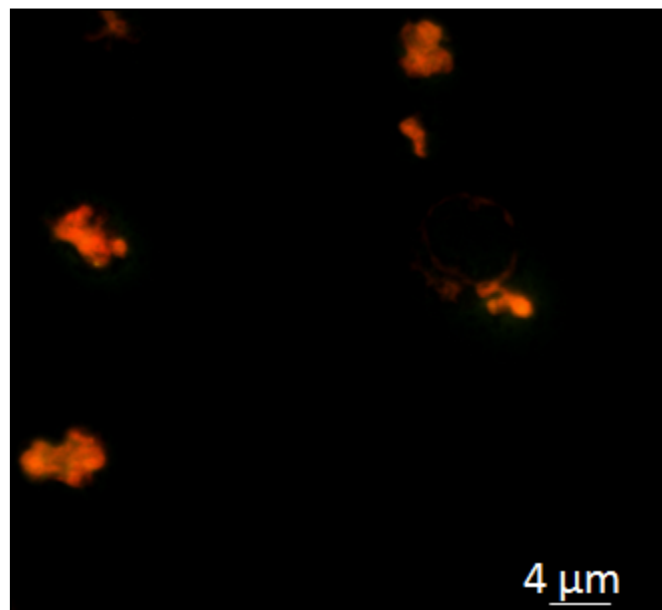


Figure 7. Microscopic evaluation of *G. gracilis* chloroplasts. Chloroplast preparations were purified with a 2-step sucrose gradient and 'intactness' evaluated with a Nikon Diaphot -TMD inverted microscope (fitted with a Nikon-epifluorescence attachment) equipped with a 510 nm emission filter.

B 4 OPTIMISATION EXPERIMENTS PERFORMED FOR IMMUNO-GOLD LOCALISATION STUDIES

B 4 1. The effect of fixative on antigenic properties of *G. gracilis* tissue samples

Problems associated with fixation methods in immunocytochemical studies are that high concentrations or prolonged fixation in glutaraldehyde may result in the loss of antigenic properties of the sample. This in turn can cause a reduction in gold labelling or no detection of the gold labelled target protein. Antigenic properties of tissue samples can be retained by reducing fixing time in glutaraldehyde or omitting osmium tetroxide (Bergman *et al*, 1985 and Kamachi *et al*, 1987). It is important to use a method of fixation that preserves the antigenicity and the cellular structure of the sample.

The GLN1-2 antibody was used to test the effect of different concentrations of glutaraldehyde (Ga) and paraformaldehyde (Pf) on the antigenic properties of *G. gracilis* tissue samples. Western hybridisation analysis was performed as described (4.2.5) with the exception that following incubation in blocking buffer, blots were incubated in the appropriate fixative combination for 30 min or overnight, before the addition of the primary antibody. The following concentrations and combinations of fixative were tested: 2% PF & 1.5% Ga, 3% PF & 1% Ga, 4% PF & 0.5% Ga (Figure 8). Compared to the control (no fixative), concentrations of glutaraldehyde greater than 0.5% resulted in a marked decrease in the chemiluminescent signal of GS isoforms. Although incubation in 4% PF & 0.5% Ga reduced GS₂ signal, GS₂ was still easily detected in nitrogen deplete samples, GS₁ signal was faint. The 30 min incubation indicated that extended time in fixative was responsible for the reduction in signal. However, these results were encouraging as GS isoforms could still be detected in nitrogen starved samples (that have low levels of GS₁ and GS₂) even after overnight incubation in fixative. In addition, the difference in separation/migration of the GS₁ and GS₂ isoforms of protein samples in lanes 4 & 5 and lane 1 & 3 is a consequence of analysing the samples on separate denaturing polyacrylamide gels.

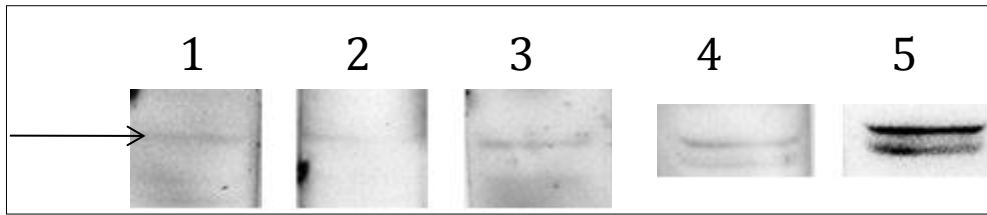


Figure 8. Western hybridisation analysis using polyclonal antibodies (GLN1-2, Agrisera) to detect GS isoforms in *G. gracilis* after incubation in differing concentrations of glutaraldehyde (Ga) and paraformaldehyde (PF). Lane 1, 2PF:1.5 Ga; Lane 2, 3PF:1Ga; Lane 3, 4PF:0.5Ga, Lane 4, 4PF: 0.5Ga and Lane 5, control. All incubations in fixative were completed overnight except for Lane 4, where the membrane was incubated in fixative for 30 minutes. The control was not incubated in fixative. Proteins were analysed by completing 12 % SDS – PAGE and subsequently transferred onto nitrocellulose membranes. Thirty micrograms of total protein isolated from day 14 nitrogen starved *G. gracilis* was loaded on gels. The arrow indicates the ~ 43 kDa GS₂ isoform.

It was decided that thalli would be fixed in 4% PF & 0.1% Ga. Weak fixation conditions, similar to these, have been used with great success to preserve the antigenic and structural properties of tissue in many immuno-gold localisation studies to date (Brangeon *et al*, 1989; Sakai *et al*, 2005). In addition, despite the marked reduction in signal following overnight incubation, it was decided that overnight fixation of samples would be performed. The algal thallus is quite thick, and an overnight fixation was chosen to ensure that complete fixation would occur.

B 4.2. Testing Non-immune serum (NIS)

To ensure that the NIS (Agrisera) did not react with *G. gracilis* proteins, western hybridisation was performed using NIS as the primary antibody (Figure 9). Duplicate protein samples isolated from nitrogen replete and nitrogen starved *G. gracilis* were loaded and separated on 12% acrylamide gels. Following SDS-PAGE, gels were transferred to a nitrocellulose membrane that was then cut in half. Each half contained protein from nitrogen replete and nitrogen starved alga. Thereafter, each half was probed with either a 1:10 000 dilution of NIS or GLN1-2 and a 1:20 000 dilution of the secondary antibody (alkaline phosphatase goat anti-rabbit-IgG conjugate). The two halves were visualised simultaneously.

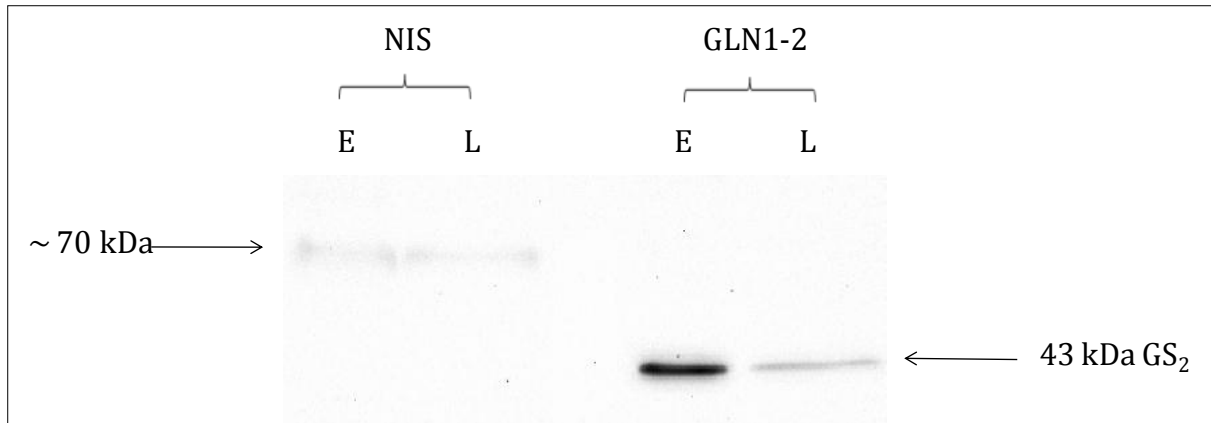


Figure 9. Test of cross reactivity of non-immune serum (NIS). Thirty micrograms of protein was separated by 12 % SDS – PAGE and transferred to nitrocellulose membranes. Protein extracted from day 0 nitrogen replete (E) and day 10 nitrogen starved (L) *G. gracilis*. Membranes were probed with either a 1:10 000 dilution of NIS or the primary antibody GLN1-2. A secondary antibody (alkaline phosphatase goat anti-rabbit-IgG conjugate) dilution of 1:20 000 was used. A 70 and 43 kDa polypeptide was visualised when membranes were probed with NIS and GLN1-2, respectively.

The NIS reacted faintly with a 70 kDa protein in protein extracts of nitrogen replete and nitrogen starved *G. gracilis*. Ideally, the NIS should not react or have affinity for proteins in the test sample. Thus, to overcome this problem, it was decided that the NIS would be pre-absorbed to total protein extracts of nitrogen starved and nitrogen replete *G. gracilis*. However, despite the cross reactivity of NIS with *G. gracilis* total protein extracts, the NIS did not seem to have specificity for GS isoform

APPENDIX C

RNA, qPCR and western hybridisation controls

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C.1 RNA and qPCR CONTROLS

C 1.1 RNA Quality

RNA was not subject to ribonuclease degradation and was suitable for use in subsequent reverse transcription reactions. The 28S and 18S ribosomal RNA bands were easily detected and intact in all RNA preparations for all nitrogen treatments (Figure 1A-C).

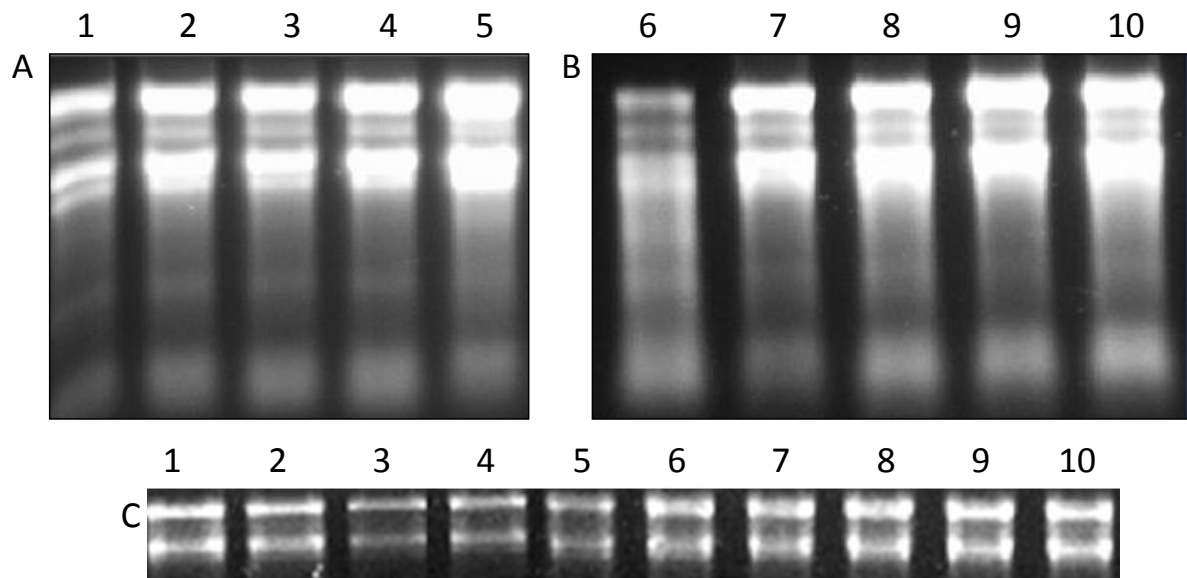


Figure 1. Assessment of RNA quality and integrity by electrophoresis of 1 µg total RNA through 1.2% formaldehyde gel. Total RNA was isolated from (A) nitrogen starved, (B) nitrogen replete and (C) nitrate, ammonium and nitrate+ammonium treated *G. gracilis* using the peqGOLD plant RNA isolation kit. Representative RNA gels are shown. (A & B): Lane 1 & 6: Control (Time 0); lanes 2-5: 2, 6, 10 and 14 days following culture in nitrogen deplete (PES-N) ASW and lanes 7-10: 2, 6, 10 and 14 days following culture in nitrogen replete (PES) media. (C): Lane 1: Control (Time 0); lanes 2-4: 0.5, 2 and 96 h following nitrate treatment; lanes 5-7: 0.5, 2 and 96 h following ammonium treatment and lanes 8-10: 0.5, 2 and 96 h following nitrate+ammonium treatment.

C 1.2 Genomic DNA (gDNA) contamination

gDNA contamination of RNA samples was evaluated as described (4.2.3). *GgGS₁* was only amplified in the positive control (gDNA) sample (Figure 2). Every RNA sample tested was free of gDNA contamination as indicated by the absence of the *GgGS₁* transcript.

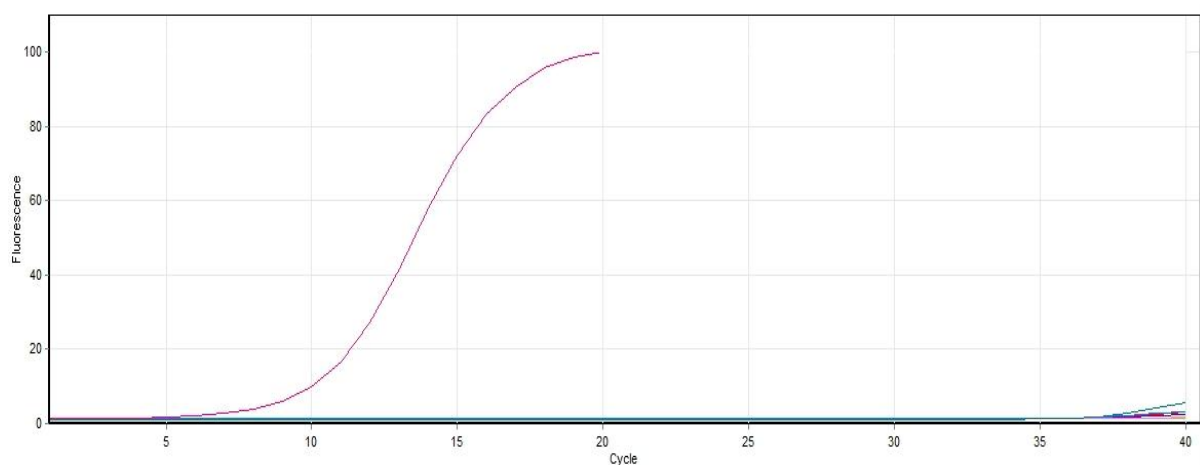


Figure 2. Representative qPCR run profile of *GgGS₁* amplified from gDNA and RNA . No amplification of *GgGS₁* was detected in RNA samples after 40 cycles. *GgGS₁* was detected after approximately 10 cycles in the positive control (gDNA) sample (purple curve).

C 1.3 Ct Values

Ct values of each biological repeat (BR) in all nitrogen treatments were invariant (Table 1 and 2) and were generally within one unit of each other. In cases where Ct values differed by two units, differences may be attributed to degradation of mRNA transcripts, different reverse transcription (RT) efficiencies and slight differences in the amount of RNA used in RT reactions.

Table 1. Ct values of the RG *PSP* in each biological repeat (BR) of nitrogen deplete and replete *G.gracilis*

Time (d)	N-DEplete			N-REplete		
	BR1	BR2	BR3	BR1	BR2	BR3
0	21.36	21.36	22.98	22.31	26.3	25.71
2	21.24	21.09	22.26	22.29	25.29	27.74
6	19.99	21.48	22.02	23.7	24.61	26.83
10	20.76	20.08	21.78	24.89	24.54	26.93
14	23.73	22.1	24.57	23.04	25.02	27.56

To overcome this, duplicate RT reactions for each time point could have been performed and pooled. This was not feasible in the present study due to cost concerns.

Table 2. Ct values of the RG *PSP* in each biological repeat (BR) of nitrogen starved *G. gracilis* re-supplied with nitrate (NO_3^-), ammonium (NH_4^+) and nitrate+ammonium ($\text{NO}_3^- + \text{NH}_4^+$)

Time (h)	NO_3^- TREATMENT			NH_4^+ TREATMENT			$\text{NO}_3^- + \text{NH}_4^+$ TREATMENT		
	BR1	BR2	BR3	BR1	BR2	BR3	BR1	BR2	BR3
0	16.15	20.99	18.36	15.6	20.99	20.99	18.36	18.36	18.36
0.5	16.62	21.13	19.09	14.51	21.44	20.91	19.09	18.26	17.73
2	16.76	21.21	17.93	15.38	20.96	21.23	17.93	17.48	18.52
96	16.69	21.99	18.17	15.9	21.74	21.6	18.17	18.34	18.27

C 1.4 Melt and standard curves

Representative melt and standard curves of *GgNR* (Figure 3), *GgGS₁* (Figure 4) and *PSP* (Figure 5) are presented. The melt curves and efficiencies were constant and reproducible for all biological repeats of nitrogen replete, deplete and nitrogen sources

experiments. Melt curve peaks of samples corresponded with positive controls (plasmid harbouring the target gene) thereby indicating that the target gene was amplified. A single peak also indicated that non-specific amplification and primer-dimer formation did not occur in qPCR reactions. This was further validated by high qPCR efficiencies that were greater than 90% for the amplification of *GgNR* and *GgGS₁*.

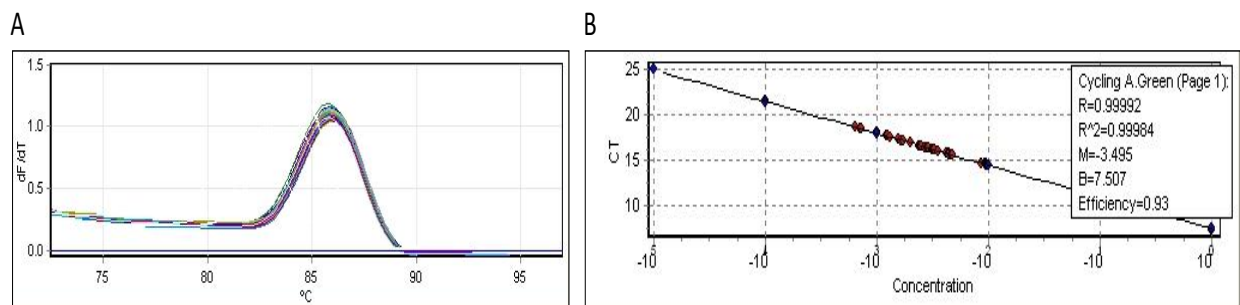


Figure 3. Representative qPCR profile of the gene of interest *GgNR* for biological repeats 1 and 2 of nitrogen starved thalli. (A) The melt curve and (B) the standard curve of the gene.

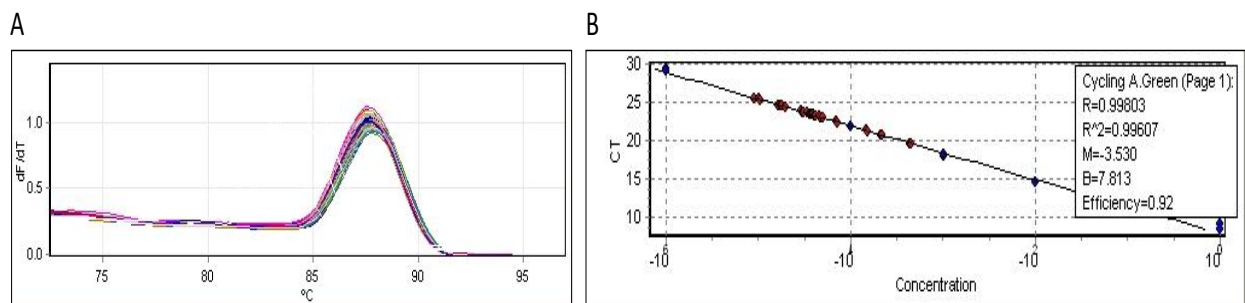


Figure 4. Representative qPCR profile of the gene of interest *GgGS₁* for biological repeats 1 and 2 of nitrogen starved thalli. (A) The melt curve and (B) the standard curve of the gene.

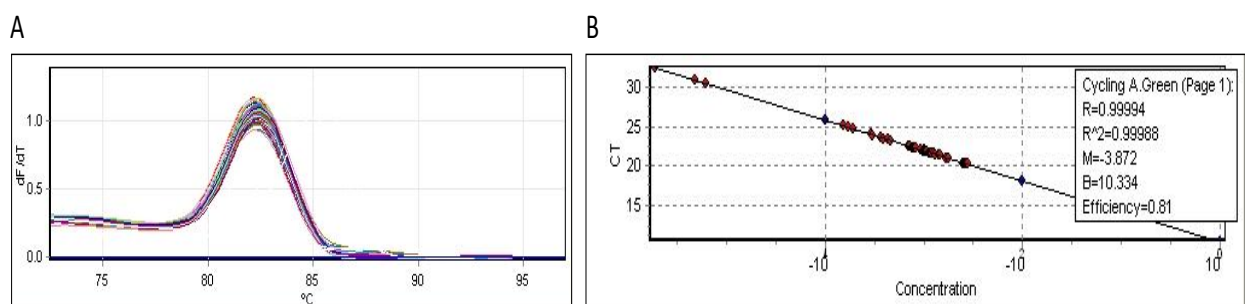


Figure 5. Representative qPCR profile of the reference gene *PSP* for biological repeats 1 and 2 of nitrogen starved thalli. (A) The melt curve and (B) the standard curve of the gene.

C 2 WESTERN HYBRIDISATION CONTROLS

C 2.1 Confirmation of equal loading

Coomassie and Ponceau S staining was performed to determine whether proteins were equally loaded and transferred to nitrocellulose membranes. Both stains confirmed that proteins were equally loaded and transferred for all biological repeats of nitrogen replete, deplete and nitrogen sources experiments (Figure 6-11). The AS08 210 NR primary antibody detected primarily NR polypeptides, however, prolonged exposure (greater than 1000 s) resulted in increased background (Figure 6C and 7 C). In general, when *G. gracilis* total protein extracts contained minimal amounts of NR, the detection time needed to be extended, resulting in the detection of background. However, the background was far reduced and signal was poor compared to the positive NR signals. Lower molecular weight proteins have also been detected when the AS08 210 NR antibody was used in the detection of NR in the monocot *Hordeum vulgare* (Agrisera, product information) and may represent degradation products.

Western hybridisation indicated that only GS polypeptides were detected in *G. gracilis* total protein extracts (Figure 9C, 10C, 11C&D).

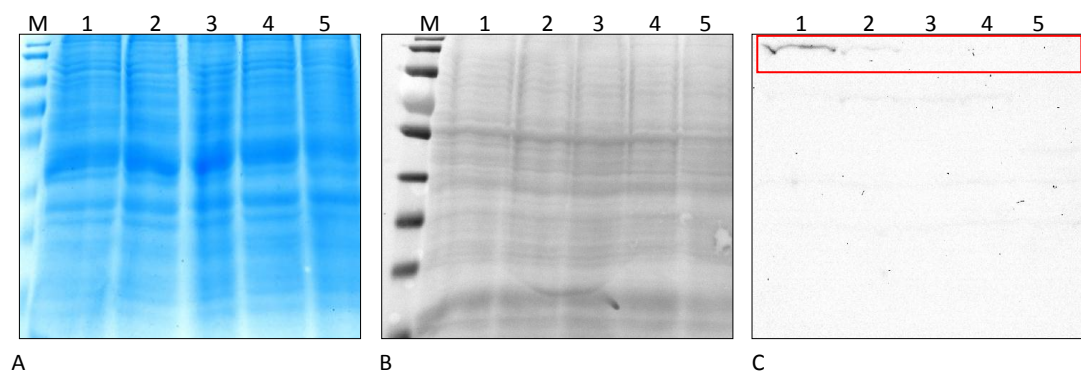


Figure 6. Representative western hybridisation analysis of NR protein expression in *G. gracilis* cultured in nitrogen deplete (PES-N) media over fourteen days. (A) Forty micrograms of protein was separated by 10% SDS-PAGE, (B) transferred to nitrocellulose membranes and stained with Ponceau S. (C) The membranes were probed with a 1:1000 dilution of AS08 210 NR primary antibody and a 1:20 000 dilution of the secondary antibody (alkaline phosphatase goat anti-rabbit IgG conjugate). NR proteins were detected using the Immun-Star™ WesternC™ Chemiluminescent Kit (Bio-Rad) (A). NR proteins are outlined by the red box. Lane 1: control (C) sample and lanes 2-5: 2, 6, 10 and 14 days of culture in nitrogen deplete media. PageRuler™ Prestained Protein Ladder (Fermentas) was used to size polypeptides.

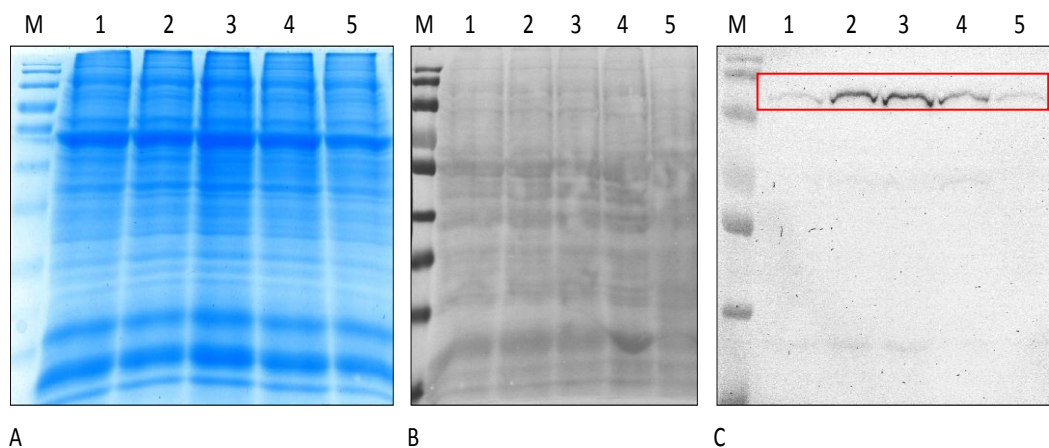


Figure 7. Representative western hybridisation analysis of NR protein expression in *G. gracilis* cultured in nitrogen replete (PES) media for fourteen days. (A) Forty micrograms of protein was separated by 10% SDS-PAGE, (B) transferred to nitrocellulose membranes and stained with Ponceau S. (C) The membrane were probed with a 1:1000 dilution of AS08 210 NR primary antibody and a 1:20 000 dilution of the secondary antibody (alkaline phosphatase goat anti-rabbit IgG conjugate). NR proteins were detected using the Immun-Star™ WesternC™ Chemiluminescent Kit (Bio-Rad). NR protein are outlined by the red box. Lane 1: control (C) sample and lanes 2-5: 2, 6, 10 and 14 days of culture in nitrogen replete media, respectively. PageRuler™ Prestained Protein Ladder (Fermentas) was used to size polypeptides.

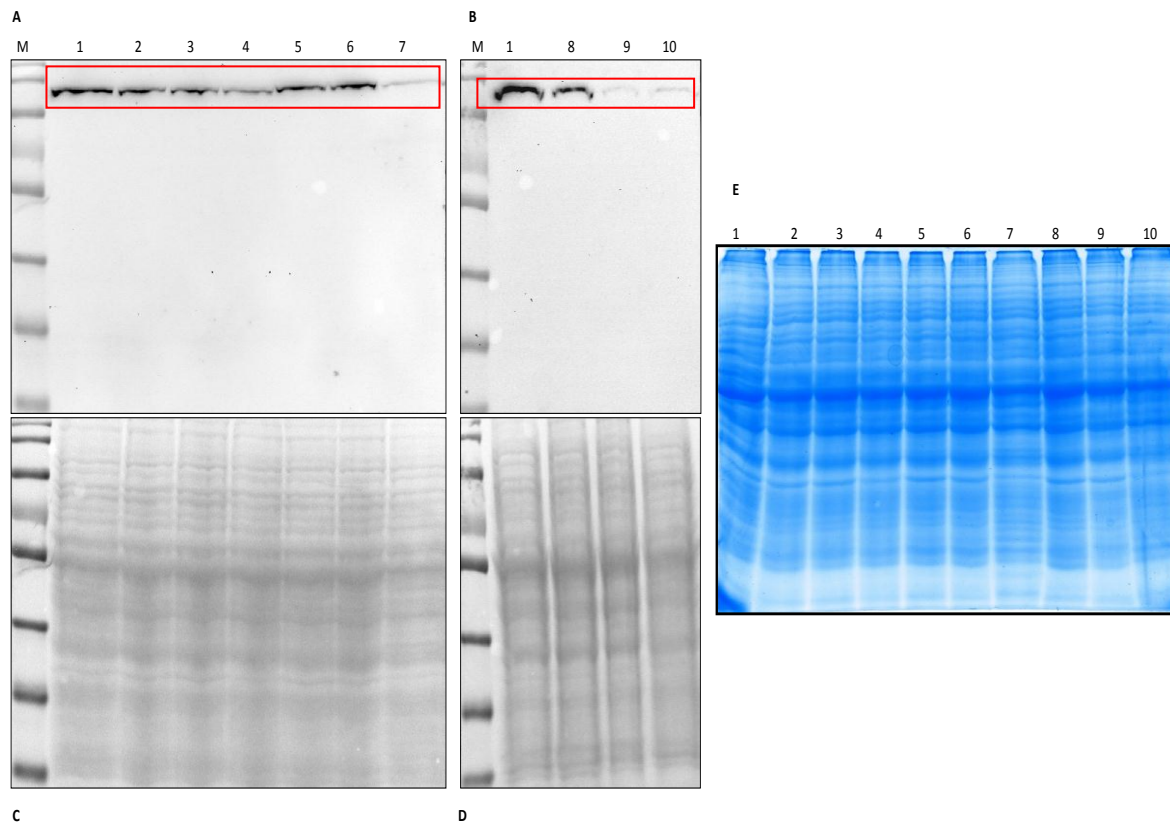


Figure 8. Representative western hybridisation analysis of NR protein expression following re-supply of (A) nitrate, ammonium and (B) nitrate+ammonium to nitrogen starved *G. gracilis*. Forty micrograms of protein was separated by 10% SDS-PAGE, transferred to (A & B) nitrocellulose membranes and stained with Ponceau S (C & D). The membranes were probed with a 1:1000 dilution of AS08 210 NR primary antibody and a 1:20 000 dilution of the secondary antibody (alkaline phosphatase goat anti-rabbit IgG conjugate). NR proteins were detected using the Immun-Star™ WesternC™ Chemiluminescent Kit (Bio-Rad). NR proteins are outlined by the red box. PageRuler™ Prestained Protein Ladder (Fermentas) was used to size polypeptides. (E) Polyacrylamide gels were stained with coomassie to assess whether proteins were equally loaded. Lane 1: control (C) and lanes 2-4: 0.5, 2 and 96 h following nitrate treatment; lanes 5-7: 0.5, 2, 96 h following ammonium treatment and lanes 8-10: 0.5, 2 and 96 h following nitrate+ammonium treatment.

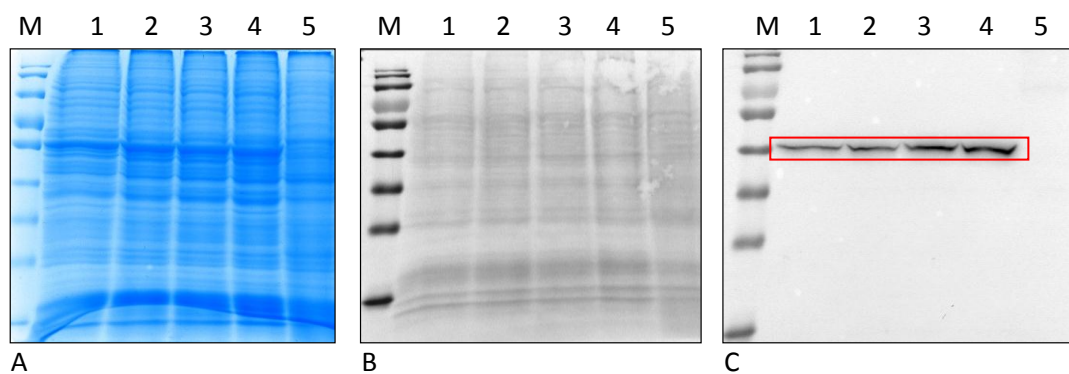


Figure 9. Representative western hybridisation analysis of GS protein expression in *G. gracilis* cultured in nitrogen free (PES-N) media for fourteen days. **(A)** Thirty micrograms of protein was separated by 12% SDS-PAGE **(B)** and transferred to nitrocellulose membranes. **(C)** The membranes were probed with a 1:10 000 dilution of GLN1-2 primary antibody and a 1:20 000 dilution of the secondary antibody (alkaline phosphatase goat anti-rabbit IgG conjugate). GS₁ and GS₂ proteins were detected using the Immun-Star™ WesternC™ Chemiluminescent Kit (Bio-Rad) GS₂ proteins are outlined by the red box. Lane 1: control (C) and lanes 2-5: 2, 6, 10 and 14 days of culture in nitrogen deplete media. PageRuler™ Prestained Protein Ladder (Fermentas) was used to size polypeptides.

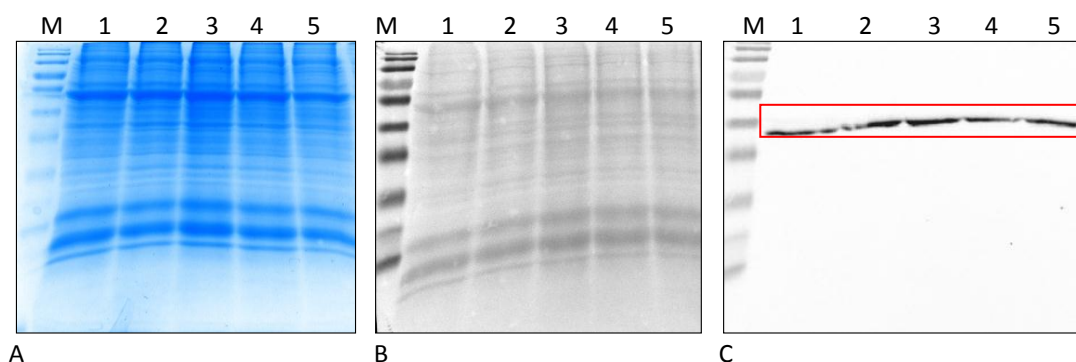


Figure 10. Representative western hybridisation analysis of GS₁ and GS₂ protein expression in *G. gracilis* cultured in nitrogen replete (PES) media over fourteen days. **(A)** Thirty micrograms of protein was separated by 12% SDS-PAGE **(B)** and transferred to nitrocellulose membranes. **(C)** The membranes were probed with a 1:10 000 dilution of GLN1-2 primary antibody and a 1:20 000 dilution of the secondary antibody (alkaline phosphatase goat anti-rabbit IgG conjugate). GS proteins were detected using the Immun-Star™ WesternC™ Chemiluminescent Kit (Bio-Rad) **(A)**. GS₂ proteins are outlined by the red box. Lane 1: control (C) sample and lanes 2-5: 2, 6, 10 and 14 days of culture in nitrogen replete media. PageRuler™ Prestained Protein Ladder (Fermentas) was used to size polypeptides.

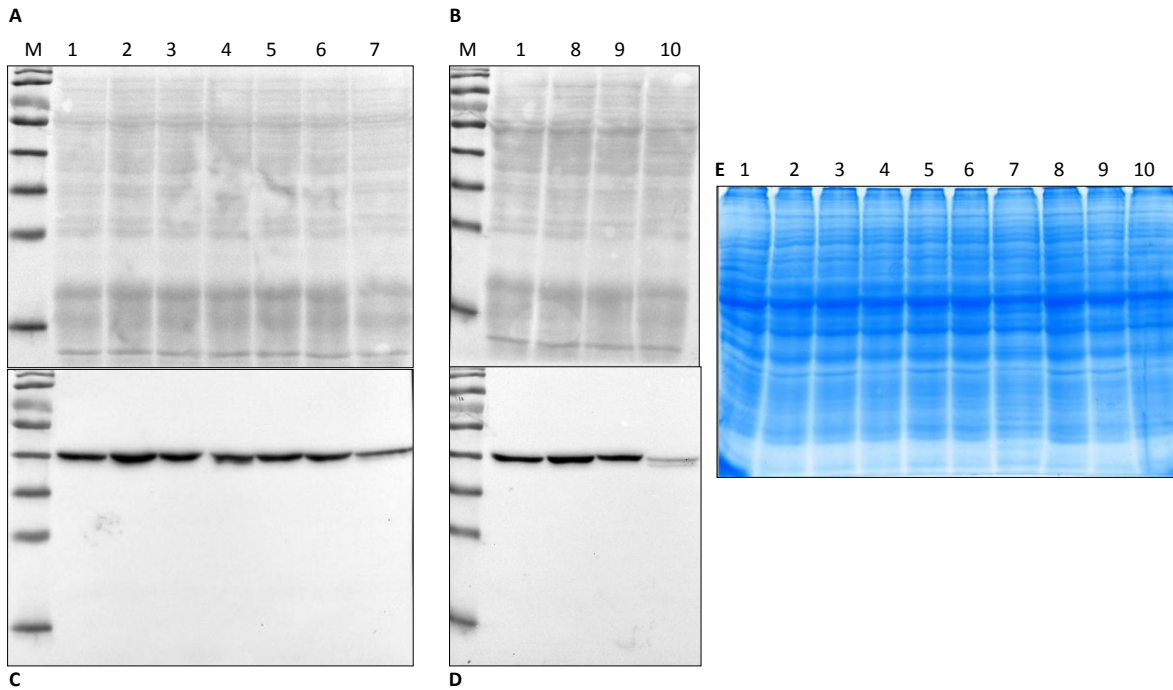


Figure 11. Representative western hybridisation analysis of GS₁ and GS₂ protein expression following re-supply of (A) nitrate, ammonium and (B) nitrate+ammonium to nitrogen starved *G. gracilis*. Thirty micrograms of protein was separated by 12% SDS-PAGE, transferred to (A & B) nitrocellulose membranes and stained with Ponceau S (C & D). The membrane were probed with a 1:10 000 dilution of GLN1-2 primary antibody and a 1:20 000 dilution of the secondary antibody (alkaline phosphatase goat anti-rabbit IgG conjugate). GS₁ and GS₂ proteins were detected using the Immun-Star™ WesternC™ Chemiluminescent Kit (Bio-Rad). PageRuler™ Prestained Protein Ladder (Fermentas) was used to size polypeptides. (E) Polyacrylamide gels were stained with coomassie to assess whether proteins were equally loaded. Lane 1: control (C) and lanes 2-4: 0.5, 2 and 96 h following nitrate treatment; lanes 5-7: 0.5, 2, 96 h following ammonium treatment and lanes 8-10: 0.5, 2 and 96 h following nitrate+ammonium treatment.