ABSTRACT

JAIN, SIDDHARTH KISHORE. Selection of Nitrogen Source Affects Physiology and Metabolism in *Dunaliella viridis*. (Under the direction Dr. Heike Sederoff, Dr. Michael Flickinger, and Dr. Amy Grunden).

Photosynthetic, saltwater-tolerant microalgae are the most sustainable feedstock to produce carbon-neutral biofuels. Nitrogen is an essential macronutrient that is generally provided via synthetic fertilizers, usually made from the Haber-Bosch process, which unsustainably utilizes more energy to produce the nitrogen than the potential energy generated by the biofuels. Alternatively, organic nitrogen sources can be used to recycle assimilated nitrogen to the algae without the addition of fertilizer nitrogen. After extraction of the oil and starch for biofuel production, the spent algal biomass contains all the assimilated nitrogen, phosphate, and sulfates from the fertilizer in proteins and polynucleotides. These can be hydrolyzed to release free amino acids and nucleotides for new algal growth and provide a potentially self-sustaining model for reutilization of nitrogen. This thesis aims to study the effects of utilization of free amino acids as the source of nitrogen in comparison to traditional inorganic nitrogen sources on cellular physiology of the microalgae *Dunaliella viridis*. First, the passage of assimilated nitrogen through key amino biosynthetic pathways is briefly described with special attention paid to the intersection of nitrogen metabolism with carbon metabolism in photosynthetic microalgae.

In the experimental section, a study describing the use of glutamine, glutamic acid, histidine, cysteine, and tryptophan as the selected organic nitrogen sources and potassium nitrate and ammonium chloride as the inorganic nitrogen sources is described. The biochemical composition of *Dunaliella viridis* including the lipid, carbohydrate, protein, and free and proteinaceous amino acid content was analyzed at two stages – at the beginning and end of log phase. Nitrogen and carbon metabolism changes were compared between the conditions tested by differential transcript quantification of seven key metabolic genes. *Dunaliella viridis* grown in nitrate were found to be consistently high in cell density, display low signs of cellular stress, and not accumulate lipids. Samples grown in ammonium were found to have a narrow concentration gradient where growth was possible, with the optimal concentration found at about 0.5 mM, above which ammonia caused cellular toxicity and cell lysis. 0.5 mM ammonium was sufficient for high cell growth and promote lipid accumulation as soon as it was depleted. Tryptophan, glutamine and cysteine were found to be the best amino acids for high cell densities and protein formation. Glutamine was found to be unstable in media, decomposing to ammonium which might potentially lead to cellular toxicity. Algae grown on glutamate and histidine mimicked physiological aspects of nitrogen starvation by accumulating lipids and carbohydrates and promoting protein catabolism recovery measures such as ammonium recycling via NADH-GOGAT.

Selection of Nitrogen Source Affects Physiology and Metabolism in *Dunaliella viridis*.

by

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A thesis submitted to the Graduate Faculty of

North Carolina State University

in partial fulfillment of the

requirements for the degree of

Masters of Science

Biomanufacturing

Raleigh, North Carolina

2015

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DEDICATION

I dedicate this thesis to my friends, family, and mentors - and to the coffee growers of the world, without whom this work would never have been completed.

I also dedicate it to the giants in this field, on whose shoulders I have attempted to stand to add a few micrometers to the depth of knowledge on this subject.

BIOGRAPHY

I grew up in New Delhi, India in a family of engineers going back three generations. I am deeply committed to pursuing technological solutions to current environmental problems. I believe microbiology and biotechnology have solutions to many of the current world problems such as food, fuel, and renewable sources of essential chemicals. To gain the necessary skills to create solutions for these problems, I finished a Bachelor's in Engineering in Biotechnology from VIT University, India and a Master's of Science in Biomanufacturing from NC State University.

ACKNOWLEDGMENTS

I’d like to acknowledge two sets of people, besides my advisers, who have helped and enabled me to commit myself to this task:

The first set, are my family and friends who have tolerated my occasional prolonged social absences, motivated me to keep at the task and finish it, and encouraged me to give it my best possible shot whenever I might waver towards ‘good enough’.

The second set is divided into the faculty and staff at BTEC, and my lab mates in the Plant and Algal Labs under Dr. Sederoff.

In BTEC, I would like to acknowledge the help I received from Dr Nat Hentz and Brian Mosley who taught and troubleshot amino acid analysis with me, as well as all my professors who helped me hone my written word.

From the lab, in particular, I would like to acknowledge Soundarya and Colin, who initially taught me the basics of cultivating the algae and performing some measurements and Jake, who taught me almost everything else and answered at least ten questions a day towards the end. I’d also like to thank Sathya and Maria for teaching RNA and qPCR related techniques and for providing everything I needed, whenever I needed it.

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CHAPTER 1

Nitrogen Assimilation in Photosynthetic Marine Microalgae – A Review

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Chapter 1

ABSTRACT

The many fates of nitrogen utilized by a marine alga are highly dependent on the amount and type of nitrogen source present, the kind of assimilation pathways the organism has, the habitat the organism lives in, availability of other nutrients, and the specific requirements of the cell at that stage of its life cycle. Many algal species use multiple assimilatory pathways for different nitrogen sources, and even multiple pathways for the same source; thus maintaining flexibility in their energy utilization strategy, as all uptake systems require some form of energy to function. A comparison of different assimilatory pathways for nitrogen, keeping their ecological significance in mind, is provided in this review. The passage of the assimilated nitrogen through key amino biosynthetic pathways is briefly covered with special attention paid to the intersection of nitrogen metabolism with carbon metabolism in photosynthetic microalgae.

1.1. INTRODUCTION

Nitrogen (N) is one of the mineral macronutrients that algae require in large quantities and its metabolism is an energy intensive process requiring almost 50% of the carbon (C) content of a cell. Several N sources have been characterized for different algal species, including inorganic sources like nitrate and ammonium, and organic sources like urea, amino acids, and peptides.[1–3] Most species contain multiple assimilatory pathways for a range of sources, often with several isoforms of transporter proteins associated with the uptake of the same source. For example, *Chlamydomonas reinhardtii* (hereafter *Chlamydomonas*) contains eight putative ammonium transporters and thirteen putative nitrate/nitrite transporters.[4] Assimilatory pathways of different sources often regulate each other, producing a complex control system in environments with multiple nitrogen sources. In *Chlamydomonas* and *Dunaliella salina* it has been shown that ammonium is preferentially taken up over nitrate, and the presence of ammonium also actively inhibits the transcription of nitrate transport genes. [1,5] Besides ammonium and nitrate, other organic sources of nitrogen have also been reported to be utilized by microalgae such as urea[5,6], amino acids[2,5,7], and hydrolyzed peptides[7–9] – although uptake of peptides as a nitrogen source is often limited by the size of the molecule and not widely reported for microalgae in their natural environment. [10]

This review captures the current understanding about the assimilation of nitrogen from various sources into the metabolite pool of marine microalgae and the differences between the treatments of various N sources among related microalgal species, specifically, in their uptake, utilization, and catabolism of nitrogenic metabolites. The pathways involved in the uptake of nitrogen in the form of nitrate, ammonium, urea, and amino acids in marine micro- and macroalgae are described. Further, the biosynthetic and degradation pathways of key amino acids are consolidated including any pertinent known differences among algal species. The role of nitrogen in carbon metabolism and photorespiration will also be addressed briefly.

1.2. NITROGEN IN THE MARINE ENVIRONMENT

Nitrogen exists in a diverse range of biologically active and inert states in the oceans; the specific proportions of which help define varied marine environments and their diversity of microorganisms. The composition of bioavailable nitrogen states in a particular environment does not remain constant, as it fluctuates based on the residence times of the different compounds in a marine layer and the metabolism of the microbial community in that environment. This necessitates a robust and malleable nitrogen assimilation capability in marine microorganisms. As these microorganisms are both producers and consumers of marine nitrogen, complex temporal and spatial patterns of nitrogen compound distributions exist at any given point in the ocean. [10]

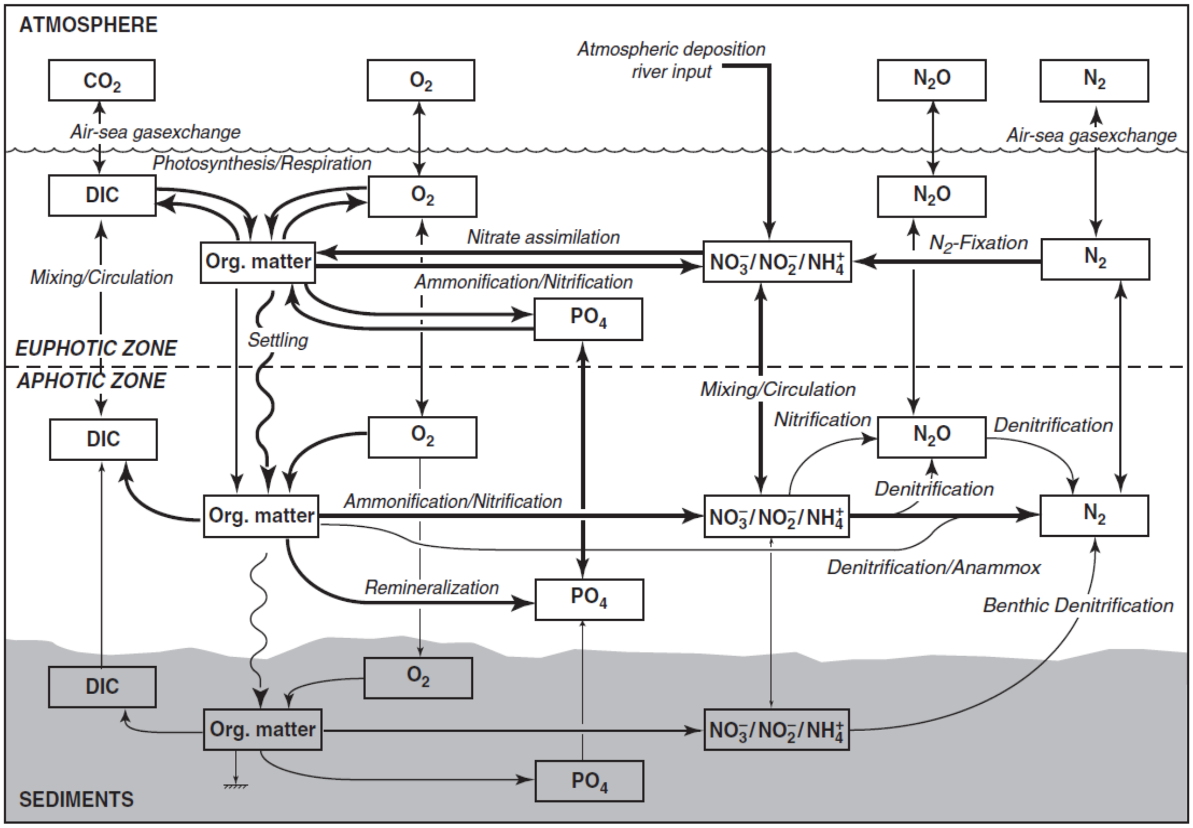

Figure 1.1. Representation of the marine nitrogen cycle and its coupling with the cycles of carbon, phosphorous, and oxygen. Taken from Nitrogen in the Marine Environment (2008).[10]

The nitrogen cycle shown in Figure 1.1, describes the biogeochemical cycle that is understood to demonstrate the flow of nitrogen through various organic and inorganic compounds in the ocean. Nitrogen enters the ocean by diffusion of dinitrogen and nitrous oxide gases into the water, where it is assimilated by nitrogen fixing cyanobacteria, which can make up to more than 90% of photosynthetic biomass in the nutrient-poor deep oceans. These cyanobacteria provide the bulk of the bioavailable forms of nitrogen such as nitrate, nitrite, and ammonium for other marine microorganisms such as bacteria, algae, or dinoflagellates. The nitrogen and carbon cycle interact as cyanobacteria also fix carbon dioxide diffused into the ocean making it available for other heterotrophic microbes in the ocean. Cyanobacteria and photosynthetic macro- and microalgae provide the bulk of photoassimilated carbon in the oceans, except for coastal regions and sediments where higher plants and benthic algae provide a higher percentage of fixed carbon. Cyanobacteria and phototrophic algae mostly reside in the euphotic zone of the ocean where sunlight is still available to fuel photosynthesis. In deeper parts of the ocean, the microbes are generally chemolithotrophs or chemoheterotrophs subsisting on settling organic matter or inorganic N and C circulating down the layers. Some of these deep ocean heterotrophs are also denitrifiers; converting organic nitrogen into dinitrogen gas or nitrate/nitrite, thus completing the cycle. Thus, it can be concluded that only a few types of nitrogen compounds support the microbial ecology of the oceans.

The two main nitrogen sources – ammonium and nitrate, must satisfy the bulk of the nitrogen requirements for the marine microbiota. This is self-evident by the fact that despite ammonium being present in very limited quantities in the oceans, its turnover rate is very high and is able to sustain a high biological demand from both prokaryotes as well as marine eukaryotes. For example, in Atlantic coastal waters, 78% of the total ammonium was assimilated by heterotrophic bacteria. Thus, despite ammonium being the preferred nitrogen source for marine eukaryotes as well, alternative nitrogen assimilatory pathways, such as nitrate, are utilized too. Assimilation of nitrate is more energetically demanding as it requires the synthesis of two extra enzymes, energy expenditure in the form of ATP and NADPH, and the utilization of an active transport system. The most energy intensive form of nitrogen import of all, however, is still nitrogen fixation from dissolved dinitrogen as mentioned above.

These limitations are increasingly highlighting the inevitability of dissolved organic nitrogen compounds (DON) as a source of nitrogen for phytoplankton and bacteria. Urea has been shown to be the most important organic source of carbon for oceans, accounting for 15-18% of total nitrogen uptake and up to 50% of the total in some coastal areas.[10] Bacteria have been shown to be the primary urea producers[10] in the marine environment and phytoplankton the main consumers[11], which points toward a sequence of nitrogen assimilation in marine environments involving nitrogen fixation, uptake of nitrate/ammonium by bacteria/eukaryotes, secretion of urea, and uptake of urea by phytoplankton. Dissolved free amino acids (DFAA) are also a relatively abundant form of nitrogen available in the marine environment and multiple studies have shown their uptake by marine microalgae. [2,5,12,13]

Marine microalgae by necessity of the environment they inhabit, must be at least moderately halotolerant. Saltwater lakes and hot springs also provide similar, if not more extreme, environments and the microbes inhabiting them have additional modifications that enable them to thrive in such environments. Only bacteria and archaea were originally thought to be able to survive in such environments, but halophlic fungi such as some *Aspergillus* and *Penicillium*[14] have been isolated in hypersaline environments as well as green algae such as *Dunaliella salina* and *Picochlorum* SE3.[15] Evidence of gene transfer from bacterial origins has been established in these eukaryotes, leading to speculation that they have assimilated superior genes in their environment to enable them to prosper in these harsh conditions. In the context of nitrogen metabolism, high salinity often results in a decrease in utilization of organic nitrogen sources, although the *Picochlorium* genome did encode urea assimilation genes.[16] No urea transporter genes have been found in *Dunaliella viridis* to date (based on unpublished transcriptomic sequences by Murphree et al.). Understanding the nutritional conditions prevalent in the environments of these microalgae will help provide the rationale behind the assimilatory pathways that have evolved in these organisms for nitrogen utilization.

1.3. NITROGEN UPTAKE IN EUKARYOTIC ALGAE

Nitrogen is often the limiting nutrient for the growth of photosynthetic microorganisms. Consequently, the first enzymatic step after nitrogen uptake becomes the rate-limiting step in the assimilation of nitrogen into the cell. [17–19] All major forms of nitrogen used by microalgae are transported into the cell using active transporter proteins in the membrane. Most of these protein families are highly conserved across species and are even common with land-dwelling plants.[5] These active membrane transporters import the nitrogen source into the cell and flux through associated enzyme steps, ultimately to form ammonium in the chloroplast, which enters into the GS-GOGAT pathway and integrates with further downstream nitrogen metabolic pathways.[3,17] Key differences arise among species on how they regulate these pathways, flux capacities of their transporters, and kinetics of their enzymes.

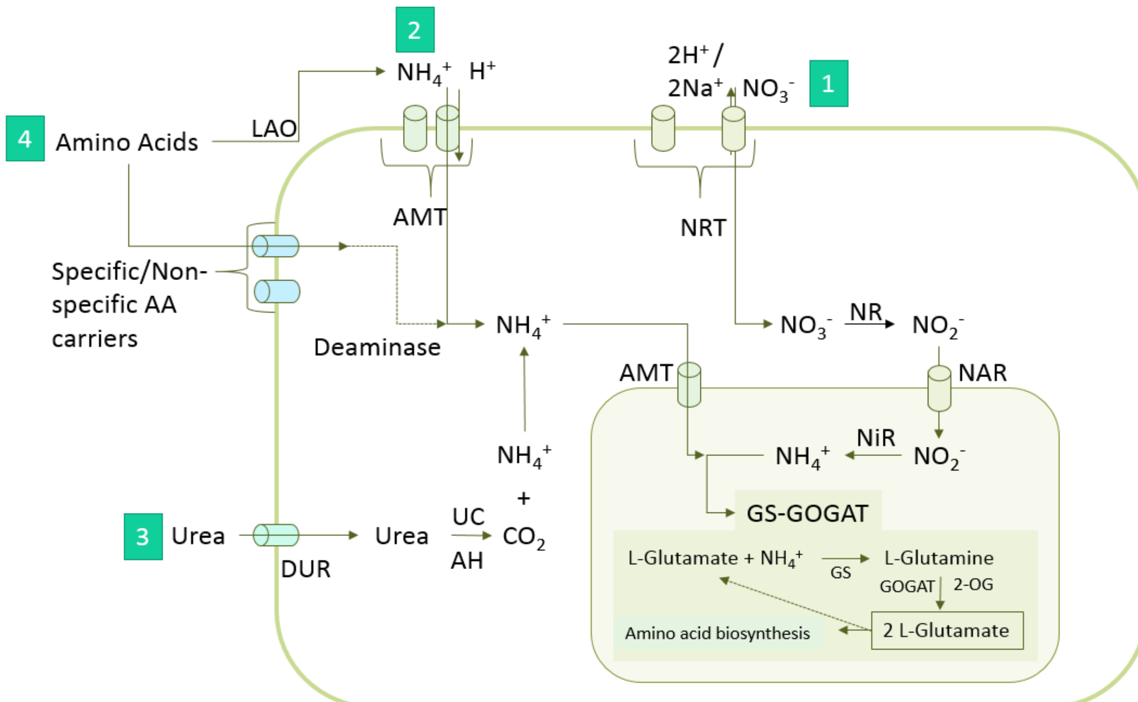

Figure 1.2. Generalized nitrogen assimilatory pathways in marine algae. Adapted from multiple sources.[4,5,17,20] Abbreviations: NRT - Nitrate transporters; NR – Nitrate reductase; NAR – Nitrite transporters; NiR – Nitrite Reductase; GS-GOGAT – Glutamine synthetase/ Glutamine oxoglutarate amidotransferase; AMT – Ammonium transports; DUR – Urea transporters; UC – urea carboxylase; AH – allophanate hydrolase; LAO – L-amino acid oxidase; AA carriers – amino acid transporters.

The main nitrogen sources covered in this review are nitrate, ammonium, urea, and select amino acids. The generalized assimilation pathways for these sources in marine algae are given above in figure 1.2.

1.3.1. Nitrate Assimilation

The assimilation of nitrate is carried out by a combination of three families of proteins: nitrate/nitrite transporters (NRT1, NRT2 and NAR*)*, nitrate reductase (NIA or NIT), and nitrite reductase (NII). In general, nitrate/nitrite is imported into the cell via a transporter from the Nrt or Nar families, converted into nitrite by nitrate reductase, transported into the chloroplast via another transporter and converted into ammonium via nitrite reductase. Ammonium is assimilated into amino acids via the GS-GOGAT pathway. [17]

1.3.1.1. Nitrate Transporters

In higher plants and algae, there are two main families of nitrate transporters identified at a genetic level – *NRT1* and *NRT2.* In *Arabidopsis thaliana*(hereafter *Arabidopsis)*, 53 *NRT1* genes and seven *NRT2* genes have been identified.[21] In *Chlamydomonas*, one *NRT1* gene, five *NRT2,* and seven *NAR* genes have been identified.[5] The *NAR* family of genes in *Chlamydomonas* which encode a nitrate/nitrite transporter (*NAR1)* and six nitrite transporters (*NAR2),* have not been shown to be present in the *Arabidopsis* genome. These nitrate transporter (NRT) gene families can be further categorized based on their functional localization – across the plasma membrane (Pm), or embedded in the chloroplast inner envelope membrane (Chl); or based on their affinity for nitrate and regulation of their expression– constitutive low-affinity (cLANT), inducible low-affinity (iLANT), constitutive high-affinity (cHANT), and inducible high-affinity(iHANT).[22] There is no clear link between the genetic classification of these genes as *NRT1* and *NRT2* and their location (Pm or Chl) or functional type. Most photosynthetic eukaryotes contain at least one Pm NT gene and one Chl NRT gene and some combination of low- and high-affinity transport systems.[23] In plants, the most well characterized nitrate transport system is in *Arabidopsis*, with 16 of the 53 NRT1 proteins having been characterized and their functions elucidated. [24] It is known that NRT1 transporters are not only specific for nitrate, and can also transport polypeptides – hence their alternative name - proton-dependent oligopeptide transporters. In *Arabidopsis,* it was found that NRT1.1 in particular can have a wide variety of responses to nitrate based on different protein structure level nitrate transduction mechanisms.[25] For example, the phosphorylated form of NRT1.1 has been shown to transport auxin into the cell in low nitrate conditions.[26] NRT2 proteins on the other hand, have been shown to be specific for nitrate in *Chlamydomonas* and require the NAR family of proteins to perform their function.[24]

All NT systems described in the literature currently are considered to be active energy-dependent transport systems. The most common coupling has been found to be with proton pumps or H^+ ATPases with an efflux of two protons for import of one nitrate molecule. [4,20,27] In marine environments, the nitrate import may instead be coupled with sodium ion efflux ($2Na^+:NO_3^-$). This system has only been shown to be functionally active in the aquatic plant *Z. marina,* but is possibly the main system used by marine microalgae as well.[20,28] Published findings on marine nitrate transporters are few. A PCR-based gene characterization study compared *nrt2* transporter gene presence in marine microalgae *Dunaliella salina, Dunaliella tertiolecta,* and *Emiliania huxleyi,* to marine diatoms *Thalassiosira weissflogii, Skeletonema costatum, Ditylum brightwellii,* and *Chaetoceros muelleri.*[29,30] The nature of their efflux system was not explicated, but their constitutive or nitrate-inducible expression was shown. Most of the species listed above were shown via direct PCR to have at least two different *nrt2* genes each. The *nrt2* genes found in the two *Dunaliella* species were found to be phylogenetically clustered together and distant from other green algae. The complete transcriptional repression of NRT2 genes in the presence of ammonia was also shown in most species, as expected from previous results. However, in diatoms, only a slight repression, without the loss of function was found, hinting at an ammonium-resistant nitrate assimilation mechanism. The ecological significance of this might be the apparent prevalence of diatoms across the world's oceans.

The most well-characterized nitrate transporter is NRT1.1 isolated from *Arabidopsis*, renamed AtNPF6.3 according to the new nomenclature standard for nitrate transporters for plants proposed by Leran (2014).[31] NRT1.1 (AtNPF6.3) was found to be a unique type of transporter - a dual-affinity transporter capable of high-affinity or low-affinity import of nitrate based on a difference in phosphorylation site on the protein. This phosphorylation control was found to be inducible by low or high levels of nitrate in the environment. Besides upregulation by nitrate, some genes in this family and NRT2.1 have been also shown to be repressed by reduced nitrogen compounds, suggesting a self-regulating model for functional control.[17]

The NRT2 family of transporters are just as widely distributed as the NRT1 family. Their structure is referred to as a carrier-type structure with twelve transmembrane domains divided into two sets with a central loop connecting the sets of domains.[4,17] They have been studied in *Chlamydomonas* as they have a unique mechanism of action involving the use of an auxiliary protein from another family – NAR. This family of genes also codes for nitrite transporters, detailed in section 3.1.3.

1.3.1.2. Nitrate Reductase

Nitrate Reductase (NR) is the first enzyme in the assimilation of nitrate, and reduces nitrate to nitrite by using pyridine nucleotides as electron donors. The structure of NR is well conserved throughout eukaryotes and photosynthetic eukaryotes in particular, and involves a homodimer-based structure with each monomer being a 105-kD polypeptide that contains three prosthetic groups - FAD, cytochrome b, and molybdenum-molybdopterin (Mo-MPT or Moco), attached to three functional domains separated by two short hinge regions.[5,32] In eukaryotes, NR is regulated by nitrate, light, and carbon metabolism, at the transcriptional, translational, and post-translational level.[33] In *Chlorella vulgaris, Volvox carteri,* and *Chlamydomonas* it is also induced during nitrogen starvation and repressed by ammonium and metabolites of ammonia.[32,33] However, in *Dunaliella tertiolecta,* this nitrogen-depletion activation was not observed.[33] Furthermore, in almost all of these organisms, there is only one *NIA/NIT1* gene encoding NR. In vascular plants, such as *Arabidopsis*, there are at least two structural loci identified as coding for NR. For the control of NR in vascular plants, there is an additional regulatory mechanism involving phosphorylation of NR at Ser-534 in the first hinge region of the enzyme and the binding of a 14-3-3 protein.[5] This post-translational modification regulates NR activity and protein stability/turnover in response to light and metabolic changes.[34] The *Chlamydomonas* version of NR does not utilize this regulatory regime, as it lacks the conserved sequence for the required binding in the first hinge region. *D. tertiolecta* lacks this conserved region as well, as do other photosynthetic microalgae.[33]

NR in *Chlamydomonas* on the other hand, appears to be regulated by two controls – the redox state of plastoquinone(PQ) in the chloroplast, and the availability of nitrate. If the PQ pool is oxidized, NR activity is downregulated and vice versa.[35] Further, it has been shown to be inactivated in the absence of nitrate.[5,35] The significance of the redox control of NR is that it provides evidence for a link between nitrogen assimilation and photosynthesis as carbon fixation was also found to be lowered, with the oxidation of PQ when exposed to light. [35] This is justified by the necessity of carbon fixation to keep pace with nitrogen assimilation, as carbon fixation provides the carbon backbones for amino acids. This is considered necessary to keep potentially toxic intermediates after this step - nitrite and ammonium, from building up. Similar light- and nitrate-dependent control between NR activity and photosynthesis is also reported in red macroalgae *Gracilaria tenuistipitata* and *Kappaphycus alvarezii,* the brown alga *Laminaria digita,* and the green algae *Ulva rigida* and *Selenastrum minutum*.[36–39]

As mentioned previously, the seasonal and geographical prevalence of nitrate in marine environments has a role to play as well. Seasonal variations in NR activity have been reported in red, brown, and blue-green intertidal macroalgae in north Atlantic coastal waters in the US and UK.[32,38–41] This has been attributed to fluctuations in nitrate content, with a buildup of nitrate in the winter in intertidal algal tissues when nitrate is plentiful, and utilization of this nitrate in summer when environmental nitrate is low due to blooms of phytoplankton. However, while nitrate levels are high in the winters, irradiance is low, leading to lower photosynthetic rates, and a disjoint in nitrogen assimilation and carbon fixation. Thus, most of the nitrate taken up by the algae remains unassimilated and stored in vacuoles.[40] This is in contrast with what is seen in microalgae that display a tight link between N- and C-metabolism. Geographical variations also account for higher prevalence of NR genes in some environments over others, such as the case of the cyanobacterium *Prochlorococcus* displaying a higher occurrence of metagenomic NR transcripts in Caribbean and Indian Ocean coastal waters compared to the Sargasso Sea and Northern Pacific Ocean.[42]

1.3.1.3. Nitrite Transporters

After the conversion of nitrate into nitrite, it is transported into the chloroplast via another family of transporters localized in the inner chloroplast membrane. The *NAR* family of genes code for nitrite transporters. In *Chlamydomonas, NAR1.1* codes for a chloroplast nitrite transporter. A single copy of *NAR1* has been found in *Ostreococcus* and other algae as well with evidence from putative translocation regions in the gene transcripts towards a plastidic localization and activity as a nitrite transporter.[20]

1.3.1.4. Nitrite Reductase

Nitrite reductase (NiR), the last enzyme in nitrate assimilation, catalyzes the reduction of nitrite into ammonium using reduced ferredoxin as an electron donor.[5] NiR is coded by the *NII* family of genes. *Chlamydomonas* is believed to have only one gene of this type, *NII1.* NiR appears to be well conserved in plants and eukaryotic algae in both structure and regulation of its gene.[20] However, it has been reported in a red alga, *Cyanidioschyzon merolae,* that the activity of NiR is instead performed by a sulfite reductase (SiR), and the alga lacks NiR altogether.[43] Two types of NiR are described in literature – Fd-dependent and NADH-dependent.

1.3.1.5. Signaling and Regulation

Apart from being a nutrient, nitrate also serves as a signaling molecule for plants and algae, regulating the expression of over a 1000 genes, in some cases, such as in *Arabidopsis.*[44,45] Besides regulating genes related to its own uptake and assimilation, nitrate has also be found to regulate genes involved in ammonium uptake, downstream amino acid synthesis (such as asparagine synthetase), and genes related to the pentose phosphate pathway. In brief, a meta-analysis of microarray studies performed on plants and algae to elucidate nitrate effects on gene transcription revealed at least ~300 genes that were consistently affected by nitrate, and sometimes as much as 10% of the entire transcriptome.[46]

The main proteins currently known to be involved in extracellular nitrate sensing in plants and algae are the NRT1.1 (AtNPF6.3 or CHL1) dual-affinity nitrate transporter in *Arabidopsis* and the newly characterized NZF1 protein, a zinc finger transcriptional factor in *Chlamydomonas.*[47,48] In *Arabidopsis,* NRT2.1 is also involved in nitrate sensing.[49] The NRT1.1 nitrate signaling mechanism was elucidated in an important study by Ho et al[50], asserting that the phosphorylation of a threonine residue at position 101 is dependent on the presence of nitrate in the soil, and the main kinase involved is CIPK23, which phosphorylates NRT1.1 in response to low nitrate levels. The signaling activity of NRT1.1 was found to be independent of its transport activity. For nitrate signaling, nitrate is required to bind to one of two binding sites on NRT1.1 depending on concentration levels. At low nitrate concentration, nitrate will bind to the high affinity binding site, expose the T101 residue, induce CIPK23 mediated-phosphorylation, and thus induce high-affinity transport of nitrate into the cell. At high nitrate concentration, nitrate binds to the low-affinity binding site, blocks the T101 residue, and prevents CIPK23 from phosphorylating T101. Nitrate also regulates plant root development via NRT1.1. At low nitrate levels, NRT1.1 transports auxin into the cell, away from lateral roots, thus suppressing growth. At high nitrogen, auxin transport is inhibited, allowing auxin to stimulate lateral root development.[49]

In *Chlamydomonas,* a new study indicates that NZF1, a transcription factor, performs a signaling function by polyadenylating *NIT2* in six different ways.[47] Nitrate presence induces two varieties of long polyadenlyated transcripts while absence of nitrate produces four short transcripts. The authors hypothesize that the long form of the transcript is more amenable to export from the nucleus for translation than the short form, regardless of the fact that the short form contains the complete coding sequence as well. *Nit2* is the main gene controlling nitrate assimilation in *Chlamydomonas,* coding for a transcriptional factor that upregulates expression of the main nitrate assimilatory proteins.[44] Additionally, NIT2 has also been demonstrated to play a negative regulatory role for ammonium assimilatory proteins.[51] *Chlamydomonas –nit2* mutants are unable to grow on nitrate as a sole nitrogen source as they are unable to synthesize all of the necessary proteins.[44]

1.3.2. Ammonium Assimilation

Ammonium assimilation requires far less energy than nitrate reduction and assimilation – 15 ATP for nitrate reduction vs 5 ATP for ammonium assimilation, thus is assumed to be the energetically favorable nitrogen form for plants and cyanobacteria.[52] Ammonium assimilation begins with import of ammonium into the cytoplasm via plasma membrane localized transporters with further transporters present on chloroplast and mitochondrial membranes.[5] Ammonium assimilatory enzymes are present in the chloroplast, hence the main flux for ammonium ions is understood to be directed there to be assimilated by the GS-GOGAT pathway. Diffusion of ammonia gas into the cell also occurs at high pH, which is expected to be significant for marine microalgae living in salty waters. The ammonium from outside the cell is assimilated into the cell's ammonium pool which also contains ammonium from protein turnover, photorespiration, and other catabolic reactions that is subsequently incorporated into a carbon backbone by the GS-GOGAT pathway. (Figure 1.2.)

Glutamine synthetase (GS) catalyzes the reaction between glutamate and ammonium and produces glutamine. Glutamine:- oxoglutarate amidotransferase (GOGAT) catalyzes the transfer of the amide group from glutamine to 2-oxoglutarate to produce two glutamate molecules. A third enzyme, glutamate dehydrogenase, catalyzes the reversible amination of 2-oxoglutarate to form glutamate.[1,4,5,53] As a reversible reaction, this enzyme's main role in some organisms (including *Chlamydomonas*) is in the deamination direction to provide 2-oxoglutarate and ammonium.[5] The 2-oxoglutarate is then utilized in the GOGAT-mediated reaction, which is a more efficient catalysis than the one mediated by GDH.[12]

1.3.2.1. Ammonium Transporters

Ammonium transporters are encoded by the ubiquitous *AMT* family of genes. Transporters in this family act as symports importing NH_4^+ with H^+ in a 1:1 ratio by importing ammonia and 2 H^+ which is equilibrated inside the cell to ammonium and H^+.[20] Structurally, ammonium transporters are trimeric and contain 11 transmembrane domains.[54] In organisms that prefer nitrate over ammonium, ammonium transporter activity is repressed in the presence of nitrate.

As mentioned previously, *Chlamydomonas* contains eight putative *AMT* genes distributed widely across the genome which have been characterized.[51] This is the highest number of ammonium transporters characterized for a single species yet. This multitude of transporters could be required for responding to changing environmental nutrient conditions in the microalga's habitat, providing functional redundancy, and functioning in inter-compartment transport. In, *Dunaliella viridis,* two putative AMT genes have been found that have ~60% amino acid identity to *Chlamydomonas* genes *CrAMT1.3* and *CrAMT1.4*, and are discussed further in Chapter 2 of this thesis. Previously, Song et al (2011), had isolated two putative transporter genes from *D. viridis* cDNA libraries – *DvAMT1.1* and *DvAMT1.2.*[55] These were found to have high similarity with *CrAMT1.3* and *CrAMT1.4,* in agreement with findings detailed chapter 2. *CrAMT1.3* was found to be localized to the plasma membrane and *CrAMT1.* to the chloroplast membrane.[51] No such localization has been proven for the two *D. viridis* homologs yet, but should be expected because of the functional necessity to have these transporters in those two locations for ammonium assimilation. A smaller number of putative ammonium transporters as compared to *Chlamydomonas* in the halotolerant marine microalga *Dunaliella* might indicate a lower preference for ammonium as a nitrogen source, or a reduced reliance on active transport of ammonium into the cell. The latter is possible, as *Dunaliella* thrives at a high pH where free diffusion of NH_3 is more likely. Further, toxicity due to excess intracellular ammonium has been noted in plants and cyanobacteria[56–58], hence a rudimentary ammonium import system may serve *Dunaliella* well enough to avoid excess intracellular ammonium.

The green algae *Micromonas* has been shown to contain *AMT2* genes of bacterial origin in addition to *AMT1* genes.[11] A pelagophytic brown alga *Aureoumbra lagunensis,* known to cause harmful algal blooms, was found to contain two *AMT* genes of which only one increased in transcript count with a decrease in available N in the form of ammonium.[59] Such increase in transcription of *AMT* genes in response to nitrogen limitation has been well documented in *Chlamydomonas, Dunaliella,* and *Miromonas* as well. [51,55,60] The *Chlamydomonas* gene *AMT1.1* and the *Dunaliella* gene *DvAMT1.1* were found to have increased transcript levels in response to nitrogen starvation.[51] An examination of expressed sequence tag contigs in the red alga *Porphyra* revealed 17 distinct contigs putatively coding for ammonium transporters divided between two species – *P. purpurea* (10 contigs) and *P. umbilicalis* (seven contigs) suggesting a high level of importance of this pathway for the red seaweed's survival.[61] However, these numbers may be over- or underestimated due to alternative splicing, fragmented

ESTs, and assembly errors in the study. In the photosynthetic-fermentative mixotroph *Chlorella protothecoides,* one putative ammonium transporter was discovered using cDNA hybridization studies.[62] This transporter gene, *CpAMT1* was found to be dissimilar to *CrAMT1.1* and *DvAMT1.1* in that it was not repressed by the presence of nitrate. This is instead similar to the regulatory mechanism of *CrAMT1.4.* Other *Chlorella* species have also been found to have novel ammonium assimilation pathways.[63] An endosymbiotic alga for the ciliate *Paramecium bursaria* has been found to be completely absent in GS expression and have low expression of GDH genes, presumably performing the activity of GOGAT albeit at a lower efficiency.[64]

1.3.2.2. GS-GOGAT-GDH Pathway

The GS-GOGAT pathway has remained fairly conserved in almost all forms of life. Most ammonium utilizing organisms have multiple copies of the genes encoding GS, GOGAT, and GDH. Marine algae that prefer ammonium as a nitrogen source over all else have evolved an efficient GS-GOGAT system. *Chlamydomonas* contains four GS genes, two GOGAT genes and three GDH genes. The expression levels for these enzymes are species-dependent, nitrogen source-dependent, and have also been shown to be dependent on salinity.[65]

In *Chlamydomonas,* GS occurs in two isoforms – GS I (encoded by *GLN1* and *GLN4*) and GS II (encoded by *GLN2* and *GLN3*). Both isoforms are homooctomers. GS I is localized in the cytosol and GS II is localized in the chloroplast. In *Chlamydomonas,* GS I is more abundant in nitrate-grown cells and GS II is more abundant in ammonium-grown cells.[5] However, in *D. salina*, the activity of GS was not found to be affected by the N source.[66] A third family, GS III, has been found to occur in some bacteria and diatoms, such as the marine diatom *Skeletonema costatum.*[67] In a recent study on prasinophytes, in *Micromonas pusilla,* one gene encoding GS II and two for GS III were found, along with one GS III gene in *Ostreococcus* and no GS II genes.[60] While the localizations of GS I and GS II are already known, GS III is predicted to have a localization of either in a chloroplast or in the mitochondria. This study was the first report of GS III in green algae. Phylogenetically, the GS III found in these prasinophytes was found to be dissimilar to the ones found in cyanobacteria and closer to GS III found in other eukaryotes and vascular plants. The authors propose a viral mediated horizontal gene transfer to have occurred in the past that enabled some descendent populations to acquire this gene, which is still found to be absent in all other green algae. However, like in other green algae, Murphree et al (manuscript in preparation), indicate the presence of RNA transcripts corresponding only to GS I and GS II in *Dunaliella viridis*. Likewise, in *Emiliana huxleyi,* only genes with sequence similarity to isoforms I and II were identified.[68] Unlike other algal GS proteins which are octomers, the *E. huxleyi* GS proteins were found to be hexamers, with GS II having more mass than GS I.[69]

In photosynthetic algae, all forms of GS are regulated by light and nitrogen source. Light promotes GS activity and nitrogen assimilation in general, in most species while the effect of nitrate and ammonium as the nutrient source affects GS functioning differently in different species. However, N deprivation stimulates GS activity in most algae tested including *E. huxleyi*[68]*, Synechococcus*[70]*,* and *Phaedactylum*[71].

In *Chlamydomonas,* GOGAT is present as two isoforms, one NADH-dependent and the other ferredoxin-dependent (Fd) encoded by *GSN1* and *GSF1* respectively. Although data is inconclusive, Fd-GOGAT is considered to be the primary pathway for ammonium assimilation along with GS1. NADH-GOGAT has also been shown to be involved with primary assimilation as its activity increases with increase in extracellular ammonium, but it is primarily utilized for ammonium recycling from intracellular sources such as protein degradation and photorespiration. Another hypothesis is that the choice of isoform depends upon the choice of reductant available, which depends upon the growth strategy being employed by the cell at the time - photosynthetic or mixotrophic. For example, *Chlamydomonas* can utilize acetate in the dark as a carbon source and act as a mixotroph, while *D. viridis* can take up amino acids and have a mixotrophic growth. *D. viridis* has putative transcripts for both kinds of GOGAT isoforms. Three putative isoforms of GDH are present in the *Chlamydomonas* genome – GDH1, GDH2, and GDH3. Unlike the mainly chloroplastic or cytosolic localization of the two enzymes above, GDH is primarily present in the mitochondria.

1.3.2.3. Ammonium Toxicity by Futile Cycling

Toxicity arising from high intracellular free ammonium is already recognized as a huge concern for terrestrial agriculture arising from excessive fertilizer usage and proximity to livestock rearing.[56] Although it has been reported to a lesser extent in marine algae[57], there is evidence to show that it can be of concern to photosynthetic microalgae. *Chlamydomonas,* for example does not accumulate nitrate, nitrite, or ammonium intracellularly to avoid any toxic effects and instead chooses to use active exporting mechanisms to remove these metabolites.[72] Various hypotheses have been posited for the reason behind the toxicity of high ammonium levels for plants and algae ranging from concerns about acidification of the soil and extracellular media to pH imbalances inside the cell.[56] However, the most accepted hypothesis currently to explain this phenomenon is the so-called futile cycling of ammonium, or rapid import and export of ammonium by the cells in conditions of high ammonium availability. Due to its transport as ammonia, its activity as a weak base disrupts the proton gradients generated across the inner mitochondrial membrane by respiration or by the light reaction across the thylakoid membrane. Transport also depends on the activity of the proton-ATPase in the plasma membrane which maintains a proton gradient essential for electrogenic transport. Evidence has been provided in plant roots, in which a study found an increase in oxygen consumption of 41% between low-ammonium and high-ammonium environments.[56] This increase in energy expenditure was attributed to active efflux of ammonium through the plasma membrane. A similar effect was witnessed earlier in barley as well.[73]

^{13}N tracer and membrane potential experiments have been used to ascertain the mechanism of influx/efflux of ammonium to gain understanding about this process in plants. The first question to be answered was the nature of the molecule that is cycled through the membrane – whether it is ammonia or ammonium. In barley, at high extracellular ammonium (10 mM), it was found that the influx of ammonia/ammonium ions did not lead to a reduction in membrane polarization, indicating that the molecule being transported is ammonia. Further, efflux was stimulated in these plant roots by high external alkaline pH. A decrease in potassium availability also contributed to high ammonia efflux. An increase in ammonia influx also rapidly increased the ratio of efflux to influx, and the amount of ammonia efflux.[74]

The evidence provided for the futile cycling of ammonium at high pH/alkaline conditions leads to concerns about such mechanisms being active in marine microalgae. In Chapter 2 of this thesis, evidence for ammonium toxicity is provided for *Dunaliella viridis.* At concentrations above 2 mM ammonium in a brackish media, *D. viridis* shows a rapid decline in biomass and restricted growth, whereas at 5 mM ammonium, the algal cells appear to have completely lysed with a dramatic reduction in cell count. However, marine cyanobacteria such as *Synechococcus* have not been shown to be affected by this mechanism adversely.[75]

As ammonia has been discerned to be responsible for this process, it follows that besides active transporter systems, passive diffusion of ammonia gas through the lipid bilayer or via aquaporins are possible avenues for influx/efflux of ammonium.[74] Aquaporins are proteins embedded in the lipid bilayer allowing for bidirectional passive diffusion of small molecules. Evidence for diffusion of ammonia through these aquaporins has been shown previously.[75] The proportion of ammonia gas to ammonium ions (the summation is referred to as 'total ammonia') depends upon the pH of the environment. At pH 8, the median pH for seawater, the proportion of ammonia in the total ammonia content is only 10%. However, higher pH exponentially increases the fraction of ammonia in the medium. At pH 9, the fraction of ammonia becomes 43% of the total ammonia content. Temperature and salinity also affect the fraction of ammonia in the medium. In brief, a unit increase in pH leads to a ten-fold increase in ammonia, a unit increase in temperature (℃) leads to a two-fold increase, while an increase in salinity only minimally decreases the percentage of ammonia.

In general, a higher dissolved ammonium content is obtained in freshwater compared to seawater – a median of 3 µM of ammonium is usually found in surface seawater while freshwater sources have reported ammonium values ranging from 300-680 µM. A strategy to mitigate the harmful effects of ammonium ions may also be to store the assimilated ammonium as free amino acids.

1.3.3. Urea Assimilation

As mentioned previously, urea accounts for over 50% of the total dissolved nitrogen uptake in some marine environments. Thus, it is an ecologically important nutrient source for a wide range of marine organisms. Bacteria assimilate urea via specific urea transporters and then hydrolyze it via a urease enzyme to produce carbon dioxide and ammonium. Urea assimilation in green algae and fungi does not follow the same pathways. Urea assimilation in green algae and yeast involves the use of a two-enzyme complex that performs the function of urease viz. urea carboxylase and allophanate hydrolase - together referred to as ATP:urea amidolyase – first described in *Chlamydomonas*.[76] Expectedly, this enzyme complex's activity is inhibited in the presence of ammonium. Urea is imported into the cytoplasm via an active transport system encoded by the *DUR* family of genes which include at least two different transport systems for high- and low-affinity transport of urea into the cell. Transport of urea at low concentration has been shown for *Chlamydomonas* following derepression of transporter activity after ammonium depletion up to a K_m of 5.1 µM.[76] High affinity transport of urea is widely found in organisms that utilize it, as the environmental concentration of urea in the oceans is usually low, at <1 µM, lower than inorganic sources like nitrate or ammonium.[77] Marine species that can utilize urea as a sole organic source can often outcompete with other surrounding microbes. It has been reported that urea was found to support the growth of harmful algal blooms around the world. The increased human use of urea as a fertilizer could have an effect on coastal natural urea levels, thus altering the microbiome of these locations to favor the species that can utilize urea more efficiently.[78]

The most abundant urea transporter transcript in marine microalgae is DUR3. It is a Na^{2+} symporter, like the other marine transporter systems discussed previously. Together, these transporters are referred to as the 'Sodium:solute symporter family' of transport proteins.[79] Phylogenetic analysis of *DUR3* ESTs in marine algae revealed two distinct lineages – a red algal tree with species such as *Phaeodactylum tricornutum* and *Emiliania huxleyi* and the green algal tree with *Chlamydomonas, Arabidopsis, and Ostreococcus.* Interestingly, *Ostreococcus* and *Arabidopsis* clustered closer together, while *Chlamydomonas* was more distantly related. Most organisms listed above contained multiple copies of the *DUR3* gene. Besides DUR3, other proteins that enable uptake of urea include: low affinity urea carriers, aquaporins, and passive urea/amide channels. The reason for the close clustering of *Ostreococcus* and *Arabidopsis* may be due to the presence of the single nickel-containing urease coding gene in its genome as opposed to the two enzyme system detailed above. A single urease enzyme is also present in *Micromonas.*[15]

Marine algae generally have a lower rate of urea uptake in the presence of nitrate or ammonium due to a change in the functioning of the transporters.[76] Light and diel fluctuations in urea uptake have also been studied in marine algae, with maximal uptake recorded during the day. This is probably due to the energy requirements of the active transport that requires reducing equivalents from photosynthesis. Urea is often generated within the cell as well, as a product of arginine or purine degradation.[79] The urea cycle is often used to store excess assimilated ammonium in a non-toxic form and generate other useful intermediates such as ornithine and arginine.

1.3.4. Amino Acid Assimilation

After urea, the most important class of organic nitrogen sources for marine algae is amino acids. Dissolved free amino acids are present in the ocean in a wide variety and sufficient quantities to serve as sufficient replacements for the more desired nitrogen sources. Regulation of amino acid metabolism is highly complex as the assimilation of one type of amino acid often represses the assimilation of another, and a web of such repression-derepression results. Further, carbon source related restrictions on amino acid utilization have also been reported. Thus, the choice and method of amino acid uptake in marine microalgae is diverse.

Extracellular deamination of amino acids resulting in uptake of ammonium ions has been reported by a few studies as well as utilization of specific or non-specific amino acid transporters.[5] Extracellular deamination is catalyzed by the L-amino oxidase (LAO1) enzyme found in the periplasm of unicellular algae.

1.3.4.1. Amino Acid Transporters

Evidence for amino acid transporters has been reported for marine algae. In *Chlamydomonas,* the only amino acid found to be transported by an active carrier is arginine.[80] However, there is evidence of direct uptake of cysteine in *Chlamydomonas* as well.[81] In *Chlorella vulgaris,* at least seven different transport systems have been described:[63] a general non-specific transport system for most neutral and some acidic amino acids (alanine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, valine), a system for short chain neutral amino acids (alanine, glycine, serine, proline), another for basic amino acids (arginine, histidine, lysine), a special system for acidic amino acids(aspartate, glutamate, asparagine) and specific systems for methionine, glutamine, and threonine. In the green alga *Volvox,* a high-affinity and highly specific carrier for arginine has been identified and was shown to fluctuate in activity in a regular cycle during the asexual life stage of the alga.[82] A low-affinity, low-specificity carrier system for leucine has been described for the brown alga *Giffordia mitchellae.*[83]

In *Chlamydomonas,* a number of genes encode for two families of amino acid transporters – Amino Acid/Auxin Permeases, and Amino Acid Polyamines. These transporters putatively code for both plasma membrane translocation and intracellular transport and more work needs to be done to characterize the individuals in this family.

1.4. AMINO ACID BIOSYNTHESIS

Marine algae that utilize primary inorganic nitrogen sources such as nitrate and ammonium by necessity have the ability to synthesize the full range of amino acids that they require for protein production. Free amino acids also serve functions in metabolic cycling, and other cellular signaling processes. Limited information is available for amino acid biosynthesis for marine algae. The information that is available is found via comparisons with higher plants, on data which has in turn been inferred from fungal and bacterial sources.[5] The majority of amino acid biosynthesis in plants and photosynthetic algae occurs in the plastid as energy produced from photosynthesis is readily available, along with the newly assimilated nitrogen as ammonium. A brief overview of possible amino acid biosynthesis pathways is given below with details provided on specific knowledge related to microalgae.

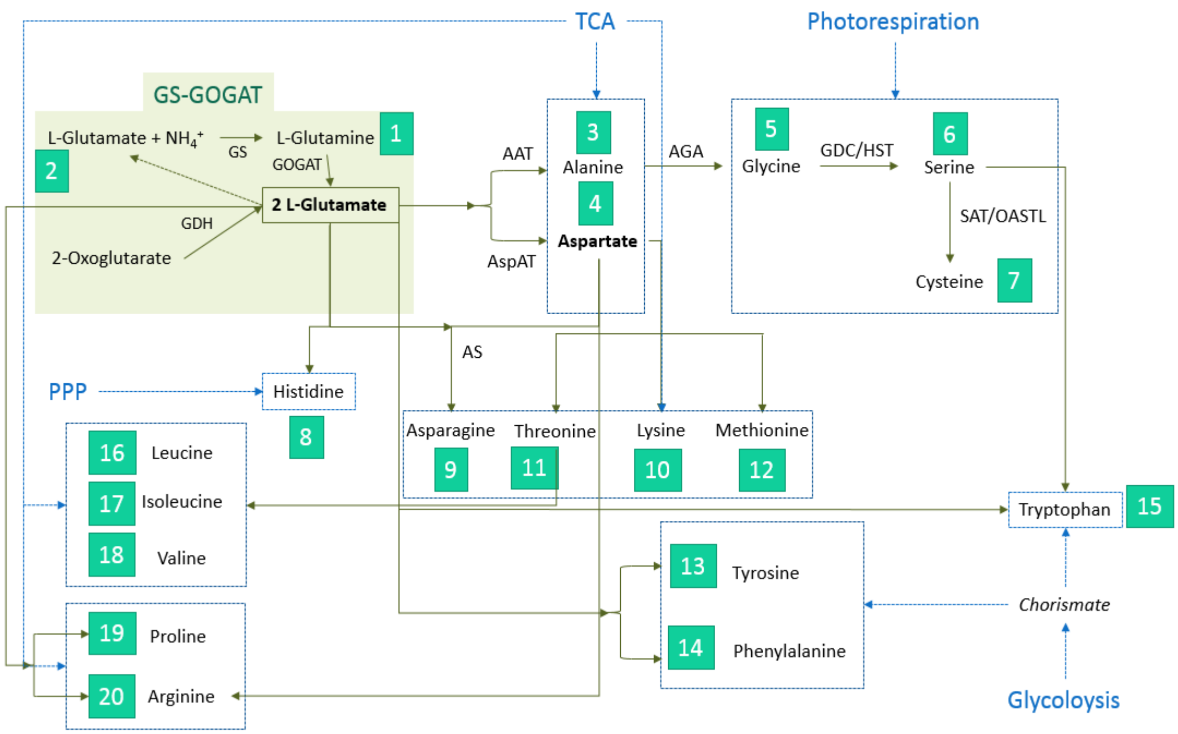

Figure 1.3. Generalized amino acid biosynthesis pathways. Green arrows indicate flow of nitrogen (in the form of ammonium), blue arrows indicate source of carbon skeletons from carbon metabolic pathways. Abbreviations: GS – glutamine synthetase; GOGAT - Glutamine oxoglutarate amidotransferase; GDH - glutamate dehydrogenase; AAT – alanine aminotransferase; AspAT – aspartate aminoatransferase, AGA - alanine-glyoxylate aminotransferase; GDC/HST - glycine decarboxylase complex/ serine hydroxymethyltransferase; SAT/OASTL - serine acetyltransferase/ O-acetylserinethiolase; AS – asparagine synthetase

1.4.1. Products of GS-GOGAT and Carbon Metabolism (1-8)

The first amino acids produced are glutamine and glutamate during ammonium assimilation by the GS/GOGAT pathway. As mentioned previously, the carbon skeleton for glutamine/glutamate synthesis is provided by 2-oxoglutarate, a product of the TCA cycle. Further amino acid biosynthesis draws ammonium from this pool of glutamate and with the help of transaminases (or aminotransferases) which catalyze the transfer of ammonium from glutamate to other carbon chains. Alanine and aspartate are synthesized by transamination of pyruvate and oxaloacetate, respectively by the action of specific transaminases; alanine aminotransferase (also known as glutamic-pyruvic transaminase or AAT) and aspartate transaminase (also known as aspartate aminotransferase, glutamic-oxaloacetic transaminase, or AspAT) respectively.

Serine and glycine can be produced from carbon donor molecules originating from photorespiration. Glycine is produced by the transamination of glyoxylate, a photorespiratory intermediate, and fatty acid degradation product, by alanine-glyoxylate aminotransferase (AGA) with alanine as the N donor. Serine is synthesized in a two-enzyme process requiring glycine decarboxylase and serine hydroxymethyl-transferase – utilizing two molecules of glycine as substrate and producing one molecule of serine, carbon dioxide and ammonia.

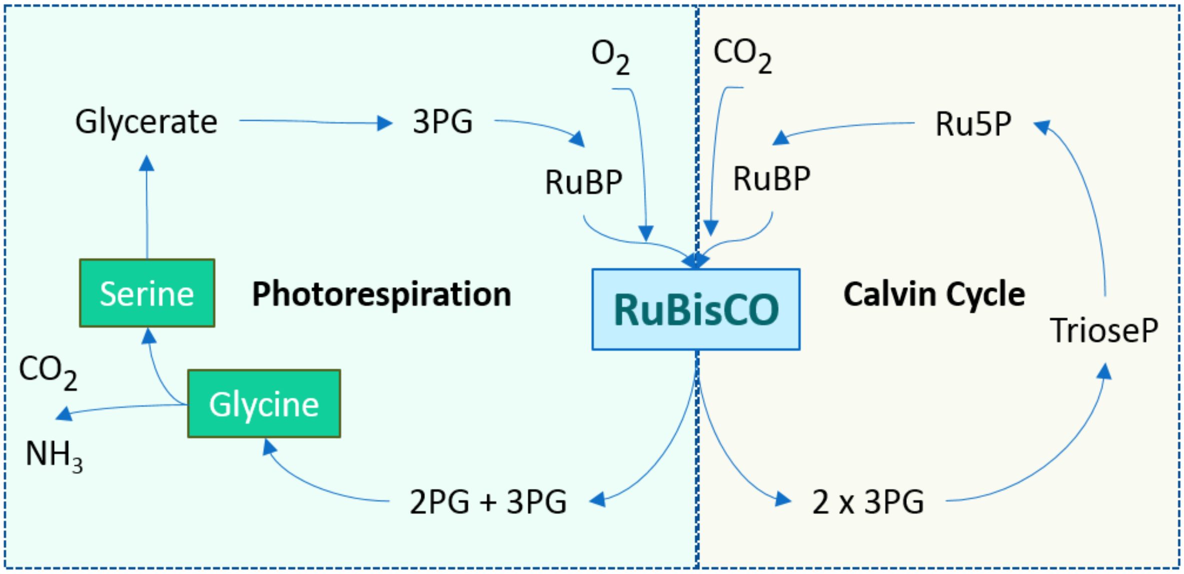

Figure 1.4. Dual action of RuBisCO in carbon assimilation via Calvin cycle and oxygen metabolism in photorespiration. Both pathways take place in the chloroplast stroma of a cell. The production of glycine and serine during photorespiration is highlighted. Abbreviations: 2PG – 2-phosphoglycolate; 3PG – 3-phoshoglycolate; 3PG - 3-phoshoglycerate; RuBP – Ribulose bisphophate; Ru5P – Ribulose-5-phosphate; TrioseP – Triose phosphate. The 2-PG generated in the first step of photorespiration by RuBisCO is dephosphorylated into glyoxylate in the mitochondria before being converted into glycine as described in the text.

Cysteine is derived from serine in two steps in which an acetyl group is transferred from acetyl-CoA to serine – a step catalyzed by serine acetyltransferase (SAT). This O-acetylserine is condensed with sulfide to generate cysteine and acetate, and this step is catalyzed by O-acetylserinethiolase.

Histidine biosynthesis involves a series of 10 conversion steps catalyzed by nine different enzymes when starting from ribose -5- phosphate. Table 4.1-1 below lists the substrate, enzyme, and product for each step as is currently understood for most plants including *Chlamydomonas.* This pathway is conserved very well in plants. An identical pathway is described for the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*. There are some differences in prokaryotes and yeast, such as reaction 3 (Table 1.1); in prokaryotes the reaction is carried out by two separate proteins and in yeast, this enzyme complex contains an additional histidine dehydrogenase enzyme. In nature, the enzyme catalyzing reaction 8 (Table 1.1), histidine phosphate phosphatase, is encoded by two distinct gene families – *Escherichia coli hisB* and yeast *his2.* The unicellular marine algae *Ostreococcus* contains the bacterial variety. Neither family was represented in the genomes of *Arabidopsis* or *Chlamydomonas,* hence it was proposed that a third type of enzyme performs this function. Recently, an enzyme called myoinositol monophosphatase-like2 (IMPL2) has been shown to perform HPP-like functions in *Arabidopsis,* thus plugging the gap in knowledge about this pathway for *Arabidopsis.* A similar sequence has been found in *Chlamydomonas* as well, but no functional confirmatory studies have been performed yet. Interestingly, overexpression of HIS3 in *Chlamydomonas* resulted in higher environmental nickel tolerance and overexpression of this and other genes of the histidine biosynthetic pathway in 'hyperaccumulator' plants produced similar results, the most pronounced results achieved by overexpression of HIS1. A possible cause for this phenomenon may be the chelating effect of free and polyhistidine on nickel which is well reported and has useful applications in protein purification using Ni columns and PolyHis-tagged proteins.

Table 1.1. Enzymes involved in histidine biosynthesis in Chlamydomonas. Abbreviations: R-5P – Ribulose -5- phosphate; RPPK - ribose-phosphate pyrophosphokinase; PR-ATP Transferase – N' -5'-phosphoribosyl-ATP transferase; PP-ATPPH/ PP-AMPCH - phosphoribosyl-ATP phosphohydrolase/ phosphoribosyl-AMP cyclohydrolase; PRF - phosphoribosyl formamino-5-aminoimidazole-4-carboxamide ribonucleotide; PRuF - phosphoribulosyl formamino-5-aminoimidazole-4-carboxamide ribonucleotide; ImGP - imidazoleglycerol phosphate; AICAR - 5-aminoimidazole-4-carboxamide ribonucleotide; IAPA - imidazoleacteol phosphate aminotransferase; HPP – histidine phosphate phosphatase; histidine dehydrogenase.

Reaction	Substrate	Enzyme	Product	Cr Genes
1	R-5P	RPPK	Phosphoribosylpolyphosphate (PRPP)	*RPPK1* and *RPPK2*
2	PRPP	PR-ATP transferase	Phosphoribosyl-ATP	*HIS1*
3	Phosphoribosyl-ATP	PP-ATPPH/ PP-AMPCH	PRF	*HIS4*
4	PRF	PRF Isomerase	PRuF	*HIS6*
5	PRuF	IGP synthase	ImGP + AICAR	*HIS7 (bifunctional protein)*
6	ImGP	IGP dehydratase	imidazoleacteol phosphate (IAP)	*HIS3*
7	IAP	IAPA	Histidinol-P	*HIS5*
8	Histidinol-P	HPP	Histidinol	*No such gene found, unknown type of enzyme*
9,10	Histidinol	HDH	Histidine	*HDH1*

1.4.2. Aspartate-derived Amino Acids (9-12)

The synthesis of aspartate leads to the synthesis of four more amino acids – asparagine, threonine, lysine, and methionine. Asparagine is generated by the amidation of aspartate catalyzed by asparagine synthetase, with the source of the ammonium group speculated to be glutamine or ammonia. The synthesis of threonine, lysine, and methionine have a common first few steps beginning with the committing enzymatic reaction catalyzed by asparagine kinase that phosphorylates asparagine, followed by reduction to aspartate-4-semialdehyde.

Asparagine synthetase is present in eukaryotic algae in two forms, A and B which use ammonia or glutamate as ammonium source.[84] *Chlamydomonas* and higher plants appear to have only one form (encoded by the *ASN2* gene), with the ability to do both reactions but ammonia assimilation does not appear to be significant, hence are presumed to preferentially utilize glutamate as the N donor.[85] Marine unicellular algae *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* on the other hand, appear to have putative coding sequences for both forms.[84]

Threonine, lysine, and methionine synthesis follow a common pathway for two steps till aspartate-4-semialdehyde. At this point, the pathway diverges for lysine towards a five step conversion from A4S till lysine, while the pathways for threonine and methionine continue to be common 4 two more steps till O-phosphohomoserine. Then, threonine synthase catalyzes its conversion into threonine.

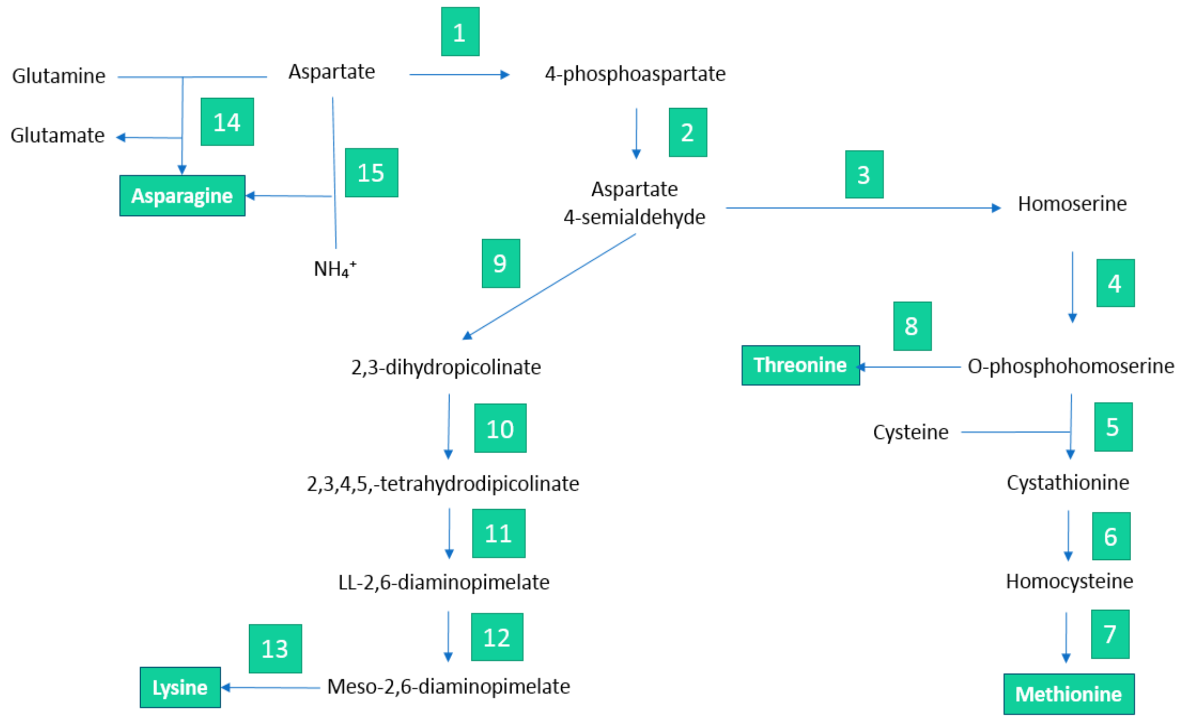

Figure 1.5. Pathways for Lysine, Threonine, and methionine synthesis. Picture from Bromke, 2013.[84] Enzymes listed by number are as follows: (1) aspartate kinase, (2) aspartate semialdehyde dehydrogenase, (3) homoserine dehydrogenase, (4) homoserine kinase, (5) cystathionine-γ-synthase,(6) cystathionine-β-lyase, (7) methionine synthase, (8) threonine synthase, (9) dihydrodipicolinate synthase, (10) dihydrodipicolinate reductase, (11) LL-diaminopimelate aminotransferase, (12) diaminopimelate epimerase, (13) diaminopimelate epimerase, (14) asparagine synthetase, (15) ammonia: aspartate ligase

All of the genes listed in figure 1.5 have been located in the genomes of *Chlamydomonas, Ostreococcus, T. pseudonona,* and *P. tricornatum.*[84] Besides the pathway listed above, there are five other slightly varying pathways for lysine biosynthesis present in other organisms, however the pathway shown in figure 1.5, known as the diaminopimelate (DAP) pathway has been proven to be biologically active in and dominant in plants and the marine eukaryotes listed above.

1.4.3. Aromatic Amino Acids (13-15)

In both vascular plants and microalgae such as *Chlamydomonas,* aromatic amino acids are synthesized by the shikimate pathway starting from Chorismate and resulting in the three aromatic amino acids – phenylalanine, tyrosine, and tryptophan. The carbon skeletons for these amino acids are provided by chorismate, which is produced by a seven-step shikimate pathway beginning with erythrose-4-phosphate and phosphoenolpyruvate, both products of glycolysis.[5]

Starting with chorismate, the pathway for tryptophan diverges from phenylalanine and tyrosine. A six step conversion to tryptophan follows from chorismate via anthranilate and indole. The pathways for tyrosine and phenylalanine are not very well characterized in algae, but the web shown in Figure 1.6 indicates the likely pathway as derived from plant studies.

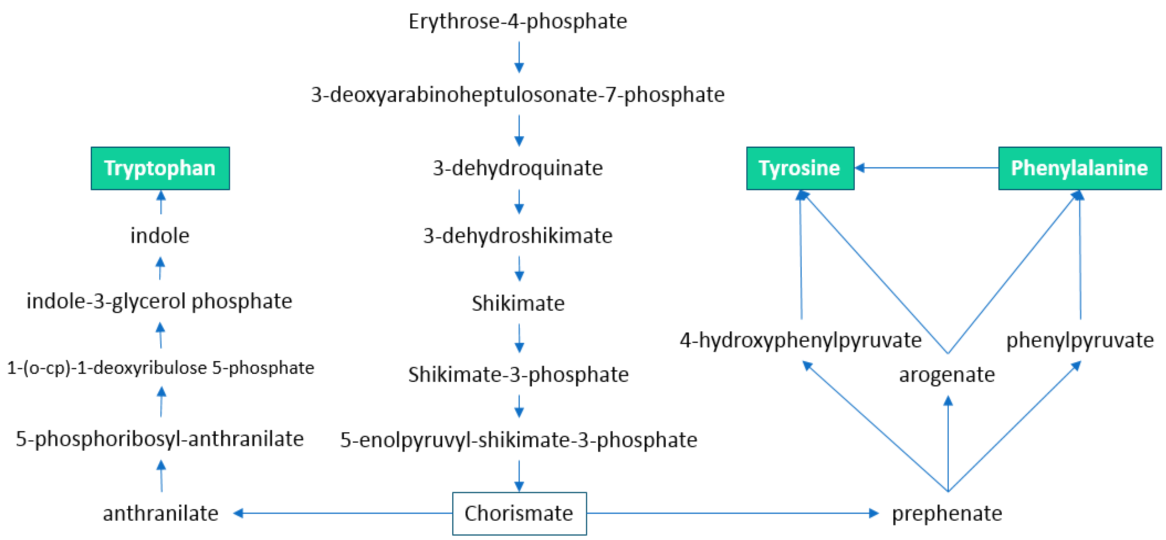

Figure 1.6. Common aromatic amino acid biosynthetic pathway in plants, algae, and bacteria.[5]

1.4.4 Branched-Chain Amino Acids (16-18)

Like the pathways above, these amino acids – leucine, isoleucine, and valine, are most probably synthesized in microalgae in the same manner as in prokaryotes. Four enzymes which have dual specificity start with 2-ketobutyrate and pyruvate, and follow their subsequent products to form isoleucine and valine. A branch from the last precursor before valine, leads to leucine biosynthesis via four additional reactions. The first common enzyme catalyzing the conversions of 2-ketobutyrate to 2-acetohydroxybutyrate and of pyruvate to 2-acetolactate is called acetohydroxy acid synthase (AHAS) and is targeted by three classes of herbicides.[5] The main regulation for the biosynthesis of these amino acids is provided by feedback control, focused towards AHAS.

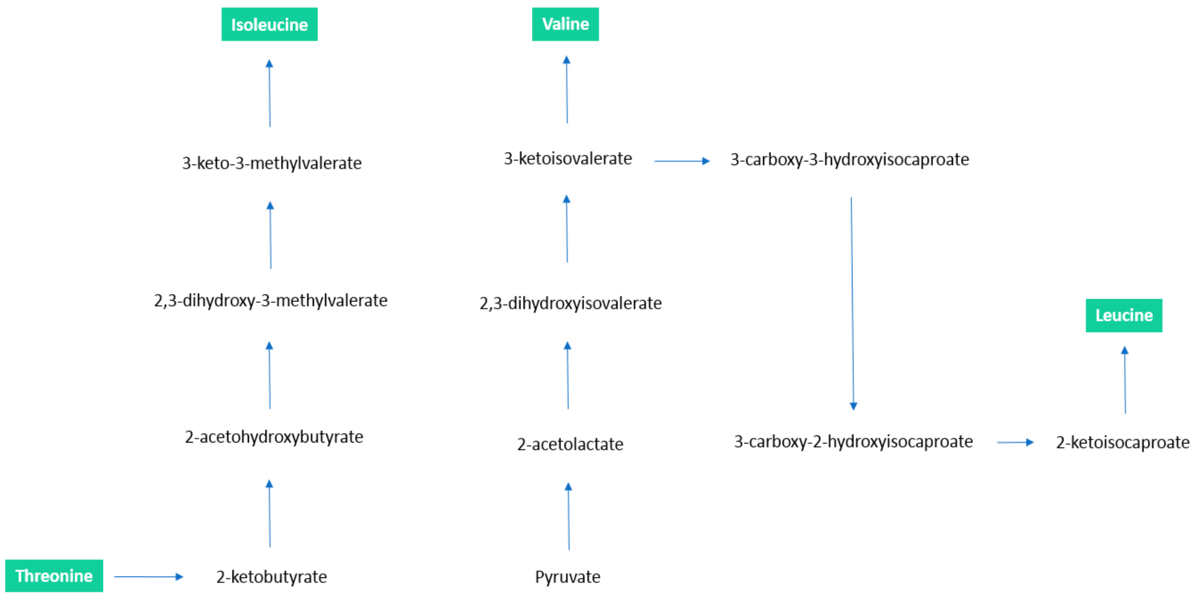

Figure 1.7. Pathways for isoleucine, valine, and leucine.*[5]*

1.4.5. Arginine and Proline (19-20)

The arginine and proline biosynthetic pathways are complex processes and has been well characterized in *Chlamydomonas.* Briefly, they are linked with the urea cycle through ornithine, and originate from glutamate. Arginine obtains its three ammonium groups from glutamate via ornithine, which receives another from carbamoylphosphate and finally one from aspartate. Proline can be synthesized using glutamate directly, or via the ornithine metabolite pool.[84]

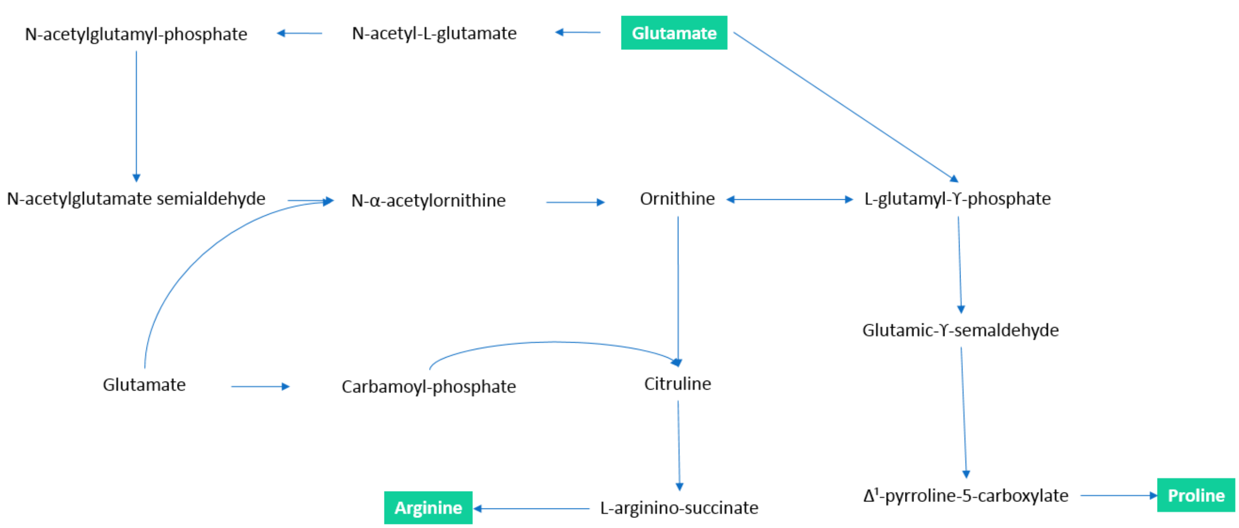

Figure 1.8. Pathways for arginine and proline biosynthesis.*[5]*

1.5. ROLE OF NITROGEN IN CARBON METABOLISM

Up to 55% of the cell's carbon is linked with nitrogen assimilation and metabolism.[86] The precise mechanisms by which the uptake of one affects the other has been a subject of research and there is much to be discovered about the regulatory mechanisms governing the balancing of C/N ratios in algae. Although the study of unicellular algae is important to understanding these mechanisms across a range of plants, there has been a greater effort to understand the interactive regulation between C- and N-metabolism in C3 plants which may also help guide future research for the same in algae.[87] Huppe & Turpin (1994)[86] provide a comprehensive review on the interaction between C/N metabolism in algal cells while Bloom (2015)[87] provides an updated version of the same for C3 plants. Finally, Palmucci et al (2011) provide an ecological and evolutionary perspective on this topic for marine microalgae.[88]

1.5.1. Nitrogen and RuBisCO

The first intersection of C-metabolism with N assimilation is during the GS-GOGAT pathway when carbon skeletons from ketoacids are utilized to synthesize the amino acids glutamine and glutamate. Carbon skeletons, energy, and electron donors (NADH, Fd), which the enzymes of the GS-GOGAT pathway require to function, are essential for amino acid biosynthesis. The most important enzyme in the Calvin Ben cycle is Ribulose-1,5-bisphosphate carboxylase/oxygenase or RuBisCO, which catalyzes the first step of carbon fixation – the carboxylation of ribulose-1,5-bisphosphate (RuBP) by carbon dioxide to produce two molecules of 3-phosphoglycerate.[89] Because of its slow activity, a larger amount of it is required to provide enough carbon to the cell. Thus it is the most abundant enzyme in plants representing over 55% of the total protein content at times,[90] and by extension, it is often cited as the most abundant enzyme in the world.[91] However, new evidence has come to light that RuBisCO does not actually account for a high percentage of total protein content in marine phytoplankton. In a recent study[89], it was found that RuBisCO accounted for less than 6% of the total protein content in a representative sample of microalgae tested including *Thalassiosira weissflogii*, *Thalassiosira oceanica*, *Chaetoceros mulleri*, *Skeletonema costatum*, *Phaeodactylum tricornutum*, *Emiliana huxleyi*, *Isochrysis galbana* and *Chlamydomonas.* Besides accounting for less than 6% of total protein content in all the species tested, RuBisCO also accounted for less than 2.5% of the total protein in the case of nitrogen limitation. According to the authors' calculations of apparent enzyme activity, this represents the minimum amount of enzyme required for supporting these organisms and thus might not be the N-sink that RuBisCO usually is in land plants.

1.5.2. Effects of Nitrogen Limitation

In today's oceans, photosynthetic organisms are usually N-limited.[92] Thus the allocation of assimilated carbon depends upon the availability and type of nitrogen source. In environments with enough nitrogen, the carbon is pooled from recently photosynthesized metabolites with carbon assimilation acting as the limiting factor, while in N-limited environments, carbon fixation and assimilation are usually downregulated to maintain compositional homeostasis. This feedback however, is not the same across species and depends upon the temporal availability of the nutrients and how long the starvation condition lasts. For example, depletion of a primary nitrogen source might lead to a temporary slowdown in C-fixation until alternative pathways are assembled for utilization of other nitrogen sources. In cases of true deprivation or when alternative pathways do not exist in the organism, maintaining the C:N that was previously the norm might not be the route of choice either. In such cases, a higher C:N ratio might result wherein the metabolic pathways flux towards C-compounds that require less or no nitrogen such as starch or lipids – as a form of emergency storage of energy. These two options - between carbohydrate and lipids as storage, are not equivalent in energy requirements. For 1 mol of C, conversion to starch requires 3.17 mol of ATP and 2 mol of NADPH whereas conversion to storage lipids (tripalmityl glycerol) requires 3.88 mol ATP and 2.84 mol NADPH. The choice then, ultimately depends upon the energetic capacity of the cell; limited energy might favor carbohydrates as storage while when energy is not limiting the cell might choose to benefit from the higher energy density lipids as storage. The ecological and evolutionary impact of this decision was studied by Palmucci et al using Fourier transform infrared spectroscopy of eleven microalgal species in a marine environment.[88]

They observed that there were three distinct strategies employed by the microalgae in response to lower nitrogen (in the form of nitrate) availability and starvation, namely:

1. No effect from nitrogen conditions: The C:N ratio was maintained constant and nitrogen limitation only resulted in reduction in carbon fixation to maintain homeostasis. *Thalassiosira pseudonana* and *Dunaliella parva* fell in this category. No major shifts in metabolite pool ratios were observed even under complete deprivation only a reduction in absolute terms in the cells.

2. Lipid storage, reduction in carbohydrates: In the face of nitrogen limitation, *Phaeodactylum tricornutum* and *Thalassiosira weissflogii* chose to completely realign their metabolite pool by reducing their protein and carbohydrate pools and increasing their lipid content. However, in nitrogen starvation conditions, *T. weissflogii* was previously reported as having accumulated carbohydrates instead whereas *P. tricornutum* was previously reported for maintaining its preference for storing energy in high-energy lipid molecules.

3. Lipid storage, carbohydrates unchanged: Most species responded to nitrogen limitation by directing flux towards lipid production without reducing the carbohydrate content. These species were also found to be more tolerant of reducing nitrogen levels and maintained a steady C:N ratio till a lower nitrogen level than the other species, a threshold level beyond which resulted in lipid accumulation. Some halotolerant species such as *Dunaliella* require an osmolyte, such as glycerol, to maintain cellular integrity at high pH, which could explain the observation of steady carbohydrate content.

The effects of nitrogen starvation on subsequent nitrogen uptake were studied in *Selenastrum* and *Chlorella*.[93] In N-starved *S. minutum,* ammonium addition led to a decrease in Ribulose biphosphate (RuBP) concentration and a drop in photosynthetic carbon fixation. In a previous study, it was found that these effects were not linked to uncoupling of the photosynthetic electron flow, but due to ammonium assimilation itself.[94] The precise mechanism is unknown as yet. In *Chlorella,* there was a drop in the RuBP concentration but there was no drop in RuBisCO activity as the RuBP concentration remained above the binding site density of RuBisCO, maintaining binding site saturation for carbon fixation. In *Selenastrum,* ammonium assimilation also lead to an increase in utilization of oxygen in the mitochondrial electron transport chain activity. This led to a net reduction in oxygen evolution during the light phase. A similar response was observed in *Chlorella* as well.

The effects of nitrogen limitation and starvation in microalgae has been well studied due to their potential use as producers for economically viable chemicals and fuels.[95] The production of many of these chemicals such as carotenoids[96] and triacyl-glycerides[97] are induced under N-stress conditions hence it is important for commercial manufacturers to ascertain the optimal type and duration of nitrogen deprivation to produce the maximum amount of their desired products. Understanding the underlying metabolic regulations involved in these production pathways allows better control of the microalgae used in industrial production.[97,98]

Recently, a study[99] on the partitioning of nitrogen among different physiological pools during nitrogen deprivation was performed on three microalgae – *Chlorella sorokiniana, Ettlia oleoabundans,* and *Nannochloropsis salina.* It was found that the trends of some of nitrogen fractions varied among the species while others were common albeit to varying degrees. All species were found to increase the fraction of nitrogen in proteins to varying degrees, decrease the fraction of N as RNA, and maintain the chlorophyll fraction; while the fraction of N as free amino acids was found to be variable among the algae.

In another comparative study between red, green, and yellow-green algae, namely, *Porphyridium cruentum*, *Scenedesmus incrassatulus* and *Trachydiscus minutus,* the effects of nitrate deprivation and recovery were studied on key enzymes at the intersection of nitrogen and carbon metabolism.[100] The changes in metabolic activities due to nitrogen limitation were found to vary between these species as well. The main constant found though, was the activity of GS which remained high and stable through most conditions in all species. The activity of NAD-GDH was found to decrease in *P. cruentum* and *S. incrassatulus,* while it increased in *T. minutus.* The activity of NADP-GDH was found to remain the same in *P. cruentum,* and increase in *S. incrassatulus* and *T. minutus.* These results provided clues towards the different strategies adopted by these algae in response to nitrogen limitation. The upregulation of activities of GS and NADP-GDH in *S. incrassatulus* might help reassimilate ammonium released from degradation of proteins or amino acid catabolism in these algae whereas the high and constant activity of GS and NADP-GDH in *P. cruentum* might indicate a strategy to wait for nitrogen levels to come back to normal and achieve a rapid recovery, while subsisting on nitrogen storage compound degradation products. The strategy employed by each algae probably has its basis on the ecology of its natural environment. *S. incrassatulus* is a freshwater alga and as mentioned previously, freshwater sources generally have sufficient nitrogen in the form of ammonium and low nitrate, hence an upregulation in GS and GDH may be a response to a switch to ammonium assimilation. *P. cruentum,* is a ubiquitous alga, more often found in brackish water, hence may focus more on capturing and storing nitrogen in storage compounds called phycobiliproteins during nitrogen repletion – to be used in case of scarcity.[101]

1.7. CONCLUSION

Nitrogen enters the algal cell through a mix of active and passive transporters, aquaporins, and gaseous diffusion. Inorganic N is reduced to ammonium and catalyzed through the GS-GOGAT cycle to form glutamate. Glutamate serves as a precursor for all of the other amino acids through various pathways that intersect with carbon metabolism through the need for the carbon backbones in the amino acids. Organic N sources like amino acids may be oxidized to produce free ammonium that is channeled through the GS-GOGAT cycle or are incorporated directly into their pathways for further catabolism.

The combination of surface proteins that enable uptake of one N source versus the other is a function of the alga's habitat, micro-environment, season and other factors. To develop a more holistic understanding of the diverse mechanisms of nitrogen assimilation in marine algae, an approach has been made in this review to keep the big picture about the ecology of marine algal habitats in mind while discussing molecular-level interactions, enzyme activity, and genomic responses of these algae to various nitrogen sources present in the natural marine environment. The sum total of these metabolic responses to the nitrogen present in marine environments is then presented as the physiological makeup of different algal species in various habitats utilizing different nitrogen sources. Naturally, these responses are not solely due to the effect of nitrogen source, but due to the interactive effects of all nutrients, co-habiting species, and other environmental conditions. The compounding effects of temperature, pH and salinity were not discussed in detail in this review, but along with nitrogen represent the other main effectors of metabolic activities in marine environments.

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Chapter 2

Selection of Nitrogen Source Affects Physiology and Metabolism in *Dunaliella Viridis*

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ABSTRACT

Photosynthetic, saltwater-tolerant microalgae are a sustainable feedstock to produce carbon-neutral biofuels. Nitrogen is an essential macronutrient that is generally provided via fertilizers, usually made using the synthetic Haber-Bosch process, which unsustainably utilizes more energy to produce the nitrogen than the potential energy generated by the biofuels. Alternatively, organic nitrogen sources can be used to recycle nitrogen to the algae without the addition of synthetic fertilizer nitrogen. Hydrolyzed spent algal biomass after removal of starch and oils, containing free amino acids, could provide a potentially self-sustaining model for reutilization of nitrogen. This thesis aims to study the effects of free amino acids as the source of nitrogen on cellular physiology of the microalgae *Dunaliella viridis* in comparison with traditional inorganic nitrogen sources. Glutamine, glutamic acid, histidine, cysteine, and tryptophan were selected as the organic nitrogen sources in this study and potassium nitrate and ammonium chloride selected as the inorganic nitrogen sources.

The main factors studied under the different nitrogen conditions were: the carbon storage in main macromolecules (carbohydrates, lipids, and proteins), cellular growth rate, photosynthesis, and photorespiration. The biochemical composition and cell density of *Dunaliella viridis* was quantified at two stages – at the beginning and end of their growth log phase, including the lipid, carbohydrate, protein, and free and proteinaceous amino acid content. Metabolic changes were inferred via differential transcription of seven key metabolic genes using quantitative PCR. It was found that nitrogen starvation and amino acids that mimicked starvation-like conditions promoted lipogenesis. Inorganic nitrogen sources and tryptophan promoted protein production. Lipid accumulation and cell growth were inversely related, and conditions which promoted growth also promoted photorespiration. Ammonium was found to be the most optimal nitrogen source for the purpose of lipid production. A lower starting concentration of ammonium compared to other sources was sufficient to achieve high growth during the log phase, while also inducing lipid accumulation towards the end of the log phase.

2.1. INTRODUCTION

2.1.1. Organic Nitrogen Sources for Biofuel Production

Marine microalgae have the ability to utilize many different organic and inorganic nitrogen sources depending on availability in their natural habitat.[1] This, combined with their photosynthetic ability and low mineral nutrient requirements position them as important organisms to produce valuable animal feeds, chemicals, and fuels in a sustainable way with a positive carbon footprint.[2] For large scale production of marine microalgae, carbon can be obtained from the atmosphere or waste flue gases from industrial plants.[3] Thus, nitrogen becomes the most expensive and environmentally impactful nutrient necessary for growth.[4] For large scale cultivation, inorganic nitrogen sources from synthetic fertilizers become the most viable option.[5,6] These fertilizers are finite and resource intensive, and their use competes with usage in crops for feed and food.[3] Furthermore, their use to grow microalgae for biofuels becomes a net energy-negative endeavor, as a larger amount of energy is used to synthesize the fertilizers using the Haber-Bosch process than is produced in the form of biofuel.[7] A study estimates that algal biofuels produced 10 billion gallons per year in the US would account for almost 44% of the ammonium fertilizer market, thereby affecting the availability of synthetic fertilizers for food crops.[8] Thus, alternative nitrogen sources must be considered for use in large scale cultivation of microalgae.

Organic nitrogen sources such as urea[9] and amino acids[10] can be potential nitrogen sources for sustainable production of marine microalgae.[11] An intriguing possibility is the inclusion of a biomass recycling step in a large scale cultivation of microalgae for biofuels or biochemicals.[7,12] This would separate the carbon-rich product of interest and leave the nitrogen-rich biomass. The nitrogen rich fraction, in the form of proteins and polynucleotides, could be recycled and used as a source for nitrogen and other macronutrients such as potassium, phosphorous, and sulfur.[13] The proteins and polynucleotides, could be further hydrolyzed thermally[14], chemically[15], or enzymatically[16] to yield simpler molecules like amino acids and nucleotides that could be assimilated by microalgae. The use of amino acids as a nitrogen source has been reported in marine microalgae.[10,11,17,18] A schematic of an algal biorefinery with nutrient recycling is given below in Figure 2.1.

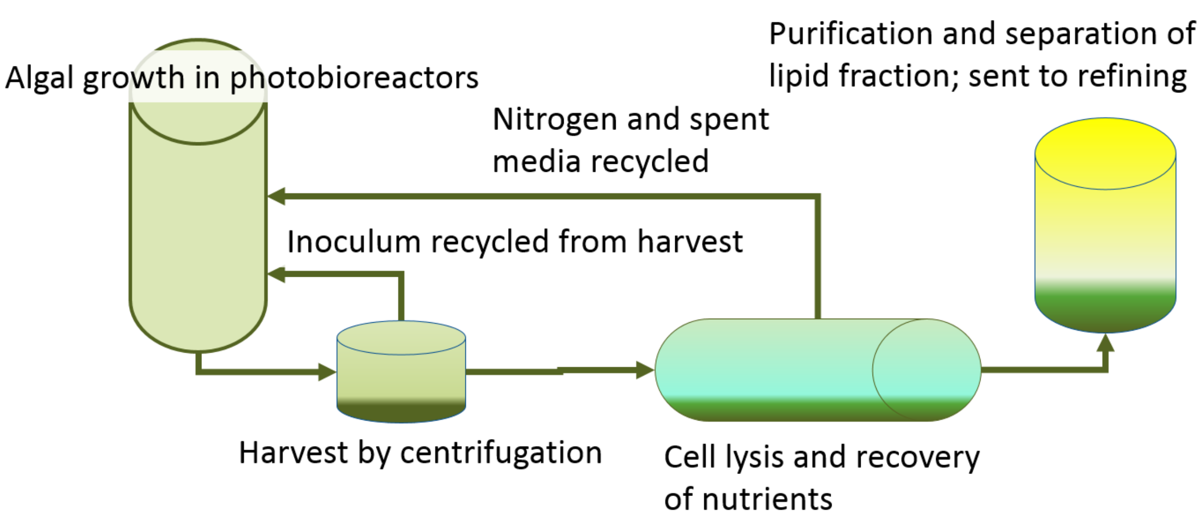

Figure 2.1. Schematic of an algal biorefinery with nutrient recycling.

The use of inorganic nitrogen sources such as nitrate and ammonium have been extensively studied in the context of biofuel or biochemical production in marine microalgae.[19–21] The use of organic sources, less so. Thus, steps towards understanding the effects of utilization of organic nitrogen in marine microalgae must be taken. Nitrogen metabolism affects carbon metabolism as well.[22] Nitrogen limitation results in carbon metabolism channeling carbon through pathways generating storage compounds such as starch or lipids instead of metabolically active intermediates. Nitrogen repletion results in an induction of the photosynthetic machinery and assimilation of more carbon to balance the excess assimilated nitrogen.[23] Eventually, a study of the effects of organic nitrogen uptake on the production of economically important bioproducts must be undertaken. First, however, a general understanding of the differences in physiology and metabolic responses of marine microalgae grown with organic or inorganic nitrogen sources must be gained. *Dunaliella viridis*[24] (hereafter, *Dunaliella)* is a halotolerant, marine, photosynthetic microalga chosen for this study of physiological and metabolic responses to different nitrogen sources in marine microalgae.

2.1.2. Nitrogen Metabolism in *Dunaliella viridis*

Dunaliella has commercially compelling properties such as the ability to tolerate high salinity conditions (up to 10 M NaCl)[25], to produce a high percentage of lipids in its biomass[26], and grow on organic nitrogen sources such as the amino acids glutamine, cysteine, histidine, and tryptophan (this study and Murphree et al, *in preparation*). Salinity tolerance allows *Dunaliella* to grow in seawater, thus not compete with human or agricultural use of freshwater. High lipid content provides an efficient means to produce high value bioproducts such as triglycerides, starch, terpenes and nutritional oils. Further, the ability to grow on amino acids suggests that *Dunaliella* can be a viable candidate for a ‘closed loop’, nitrogen-recycling, based approach to large scale cultivation. Besides these attributes, *Dunaliella* also lacks a cell wall[27], which, coupled with the preference for saltwater, allows easy cellular disruption by osmotic lysis, thus providing energy-efficient downstream processing to recover bioproducts.

Chapter 1 of this thesis outlines the main metabolic pathways involved in nitrogen assimilation by photosynthetic microalgae. From these pathways, seven nitrogen assimilation and metabolism genes were chosen for transcript analysis by quantitative PCR in this study as indicators of genomic responses to changes in N-source metabolism. These genes code for two ammonium transporters (DvAMT1;1, DvAMT1;2), NADH-dependent glutamine-oxoglutarate amidotransferase (NADH-GOGAT), alanine aminotransferase (AAT), aspartate aminotransferase (AspAT), NADP-dependent malate dehydrogenase (MDH), and a limiting CO_2 inducible protein (LCID). The first five transcripts were chosen as they represent key points in the nitrogen assimilatory process up to and including amino acid biosynthesis. Malate dehydrogenase and limiting CO_2 inducible protein were chosen to represent carbon metabolism in relation to nitrogen assimilation.

Ammonium transporters are membrane-spanning proteins that actively transport ammonium.[28] They are localized to the plasma membrane to transport ammonium into the cell, and in the chloroplast envelope to transport cytosolic ammonium into the chloroplast, to be assimilated into amino acid biosynthetic pathways. Mainly two ammonium transporter families have been found in algae – *AMT1* and *AMT2*.[29] In *Chlamydomonas reinhardtii,* eight putative ammonium transporter transcripts were found from the *AMT1* family[29], while the green alga *Micromonas* was found to have two *AMT2* genes.[30] Two ammonium transporters belonging to the AMT1 family have been previously characterized in *Dunaliella* – DvAMT1;1 and DvAMT1;2.[31] Both of these were also located in our transcriptome database, and their amino acid sequences were found to match *C. reinhardtii* ammonium transporters CrAMT1.3 and CrAMT1.4 up to ~60% identity each. However, these transcripts were found to be dissimilar to each other, sharing only ~40% of their sequences. Functional localization of these transporters has not been elucidated in *Dunaliella* yet, but it is likely that one is localized to the plasma membrane and one to the chloroplast envelope because of the functional necessity to have at least one ammonium transporter in each of these locations and only these ammonium transporter transcripts have been found in *Dunaliella*. Song and collaborators utilized WoLF PSORT to predict the localization of DvAMT1;1 and DvAMT1;2, and found that they might both be localized to the plasma membrane[31], though this has not been confirmed experimentally. Regardless, variation in ammonium transporter activity between different nitrogen supplies would help shed light on the diversity of nitrogen assimilation tactics in *Dunaliella.* Variation in the expression levels when grown with amino acids would be especially interesting to determine the mechanism of nitrogen uptake in *Dunaliella* when fed amino acids. Amino acids can be taken up either through membrane-bound active amino acid transporters or by external oxidation via a periplasmic enzyme called L-amino acid oxidase (LAO1), followed by import of ammonium.[18] Based on a search of our transcriptome database (Murphree et al. unpublished data), *Dunaliella viridis* lacks a putative LAO1 transcript. A study on the amino acid transporters in *Dunaliella* is currently underway. (Dums et al.) Nitrate transporters and the other proteins involved in its assimilation (nitrate reductase and nitrite reductase) were not studied here, to allow for more focus on organic nitrogen assimilation and amino acid metabolism.

The GS-GOGAT pathway is the primary cycle converting assimilated nitrate and ammonium to glutamate and making nitrogen bioavailable to the cell.[32] Glutamine synthetase (GS) catalyzes the energy-dependent synthesis of glutamine from ammonium and glutamate.[33] Glutamine oxoglutarate amidotransferase (GOGAT) then catalyzes the transfer of an amine group from glutamine to alpha-ketoglutarate to form two molecules of glutamate.[34] A search of our transcriptome database using *C. reinhardtii* GOGAT protein sequences has revealed two types of GOGAT present in *Dunaliella* – a ferredoxin-dependent form and an NADH-dependent form. Both perform the same activity, are probably localized to the chloroplast like in the case of *C. reinhardtii,* and complement each other depending upon the source of reducing compound available.[35] NADH-GOGAT has been previously found to increase activity in ammonium-grown *C. reinhardtii* cells and be involved in re-assimilation of ammonium from protein degradation.[35] Further, based on the published *Dunaliella* transcriptome by Srirangan et al.[36] and as yet unpublished work by Murphree et al., NADH-GOGAT had differential expression under nitrogen starvation conditions, hence this was the isoform chosen for analysis in this study.

Glutamate synthesis by the GS-GOGAT pathway leads to amino acid biosynthesis via the transfer of ammonium from glutamate to various carbon backbones provided by intermediates of the TCA cycle[37], glycolysis, pentose-phosphate pathway, and photorespiration, among others.[38] Alanine is one of the most easily synthesized amino acids directly from glutamate in a single step conversion. Alanine aminotransferase catalyzes the reversible transfer of ammonium from glutamate to pyruvate to produce alanine and alpha-ketoglutarate.[39] This is a key reaction as it produces alanine which is shown in this study to be one of the most abundant amino acids in *Dunaliella.* It is also a precursor for the amino acid intermediates in photorespiration - serine and glycine.[40] Variation in AAT correlated to variation in alanine abundance can provide clues towards photorespiratory functioning under different nitrogen conditions as well as push towards amino acid biosynthesis.[41] AspAT catalyzes the amination of oxaloacetate using the ammonium from glutamate to yield aspartate and alpha-ketoglutarate. Aspartate is an important amino acid as it leads to the biosynthesis of six other amino acids[39], thus AspAT catalyzes an important reaction that provides an indication towards the nitrogen metabolism status of a cell.

The Limiting-CO_2 inducible (LCI) protein, as its name implies, is induced under conditions of low intracellular carbon, and belongs to a family of low carbon-inducible proteins with at least five members – LCI A through E,[42] first isolated in *Chalmydomonas reinhardtii.* Based on a search in the transcriptome ESTs in *Dunaliella* a putative limiting CO_2 inducible protein with sequence similarity to both LCID and LCIE has been found (Alignment scores available in the Appendix). The main function of these LCI proteins appears to be the activation of the carbon concentrating mechanism in microalgae, which enables the uptake and storage of inorganic carbon from the environment.[43] Differential transcription of LCID/E transcripts would suggest activation of the carbon concentrating mechanism under the different growth conditions tested herein. NADP-Malate dehydrogenase catalyzes the conversion of oxaloacetate to malate in the chloroplast.[44] It is the most important enzyme in the malate shuttle, which is a cycle that provides reducing equivalents to the mitochondria using aspartate as a carrying molecule.[45] It has also been found to be influenced by light levels in plants and serve as a redox control to save plant cells from oxidative damage by light-induced reactive oxygen species in *Arabidopsis* and tomato plants.[46,47] In *Chlamydomonas reinhardtii,* it is found clustered close to nitrate assimilation gene loci.[44] This is theorized to be due to the high requirements of reducing equivalents during nitrate assimilation.[44] Thus, NADP-MDH is also linked to nitrogen assimilation and its transcript levels in *Dunaliella* under different nitrogen conditions can provide further information about the link between carbon and nitrogen metabolism.

2.1.3. Aim & Hypothesis

The aim of the experiments described herein were to characterize the effects of organic nitrogen sources compared to inorganic nitrogen sources on the physiology and metabolism of *D. viridis.* The hypothesis is that amino acids as N sources lead to mixotrophic or heterotrophic growth, as opposed to the autotrophic growth with inorganic N sources, leading to differences in cellular responses.

The overarching goal has been to identify nitrogen sources that promote lipid biosynthesis to aid in biofuel production. An evaluation of the various facets of the metabolic response has been made utilizing a combination of assay measurements and observations, detailed in the next section. The main aspects of the metabolic response that were compiled were: the carbon allocation to major components, growth rate, photosynthesis, and photorespiration. The assays and measurements used to observe these responses and the hypothesized responses for each are given below in Table 2.1.3-1.

Table 2.1. Consolidation of key factors examined.

Factor	Tested By	Hypothesized Response
C allocation to major components	Protein/Lipid/Carbohydrate Assays	Some nitrogen sources promote lipid accumulation at the cost of protein/carbohydrate content
	GOGAT activity	Protein degradation is a response to unfavorable nitrogen conditions
C Assimilation/ Photosynthesis	Chlorophyll	Higher chlorophyll in sources with more C-assimilation from photosynthesis
	LCID activity	Low CO2 concentration regulator, unknown function, no hypothesis
	Total macromolecular content	Total content of biomass reflects total C assimilated from photosynthesis and nitrogen sources with C
Growth rate	Cell Density/ Dry Weight	Lower growth rate is correlated with higher lipid production
Photorespiration	Glycine and Alanine levels	High free glycine and alanine indicate activation of photorespiratory pathway
	AAT transcription	High AAT transcript abundance shows higher free alanine generated for photorespiration.
Nitrogen Limitation	Biochemical analysis without N	N limitation inhibits growth and promotes lipid accumulation.

1.4. Experimental Design

In this study, the biochemical composition of *Dunaliella* was tested under five different nitrogen conditions in one study and eight different nitrogen conditions in another. Five conditions (growth on nitrate, ammonium, glutamine, all 20 amino acids, and no nitrogen) were assayed at the end of log phase to determine variation in lipids, carbohydrates, proteins, amino acids, and chlorophyll. In the study with eight test conditions (nitrate, ammonium, glutamine, glutamate, histidine, cysteine, tryptophan, and no nitrogen), all of the above assays were performed towards the beginning of log phase. Two experiments were performed to obtain physiological responses at different stages of growth for *Dunaliella.* One study compared the biochemical composition of *Dunaliella* grown in five different nitrogen conditions at 144 hours after inoculation. The other compared the biochemical composition and transcriptional variation after 48 hours under eight different nitrogen conditions. The 144 hour time point was used to study the physiological variation at the end of log phase, and the 48 hour time point was chosen because gene transcript abundance is adapted to different conditions fairly early, and that time point represented a balance between obtaining sufficient cell mass for physiological analysis while still being early enough to visualize fold variation in transcript abundance of key metabolism genes. Overviews of the experimental designs are given below with the compounds assayed and the specific methods chosen for each.

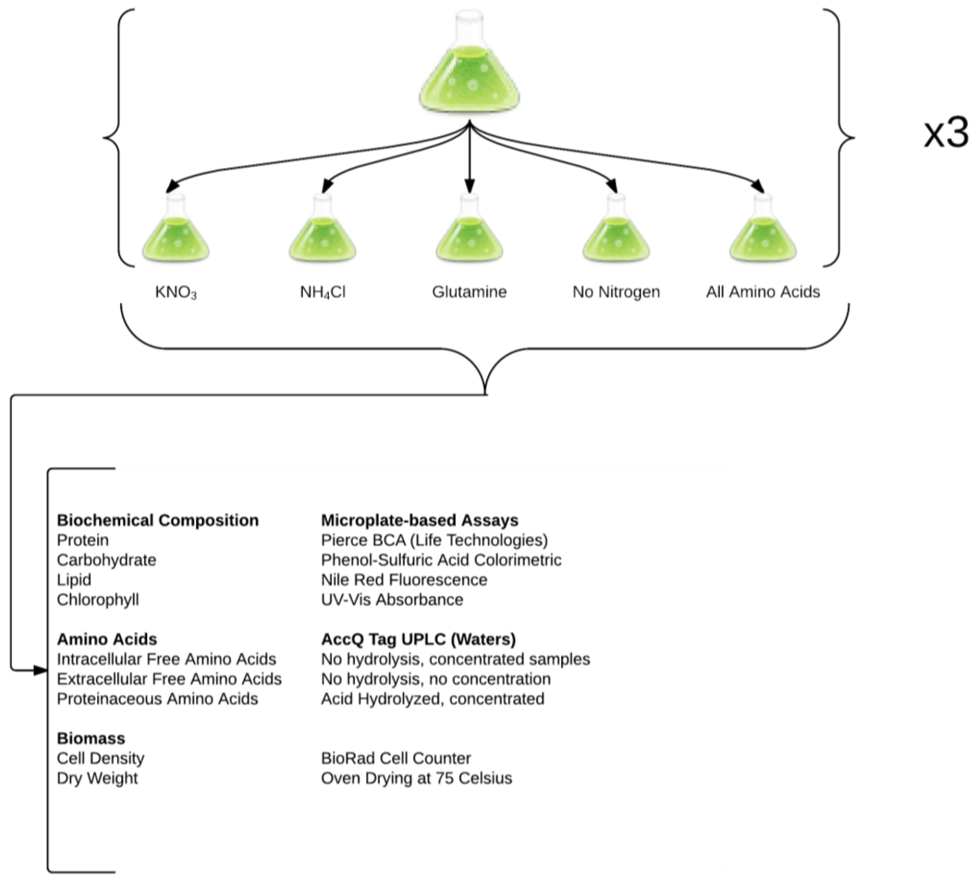

Figure 2.2. Experimental setup for the experiment lasting 144 hours. All conditions were tested in triplicate. All assays contained at least three technical replicates for each sample tested.

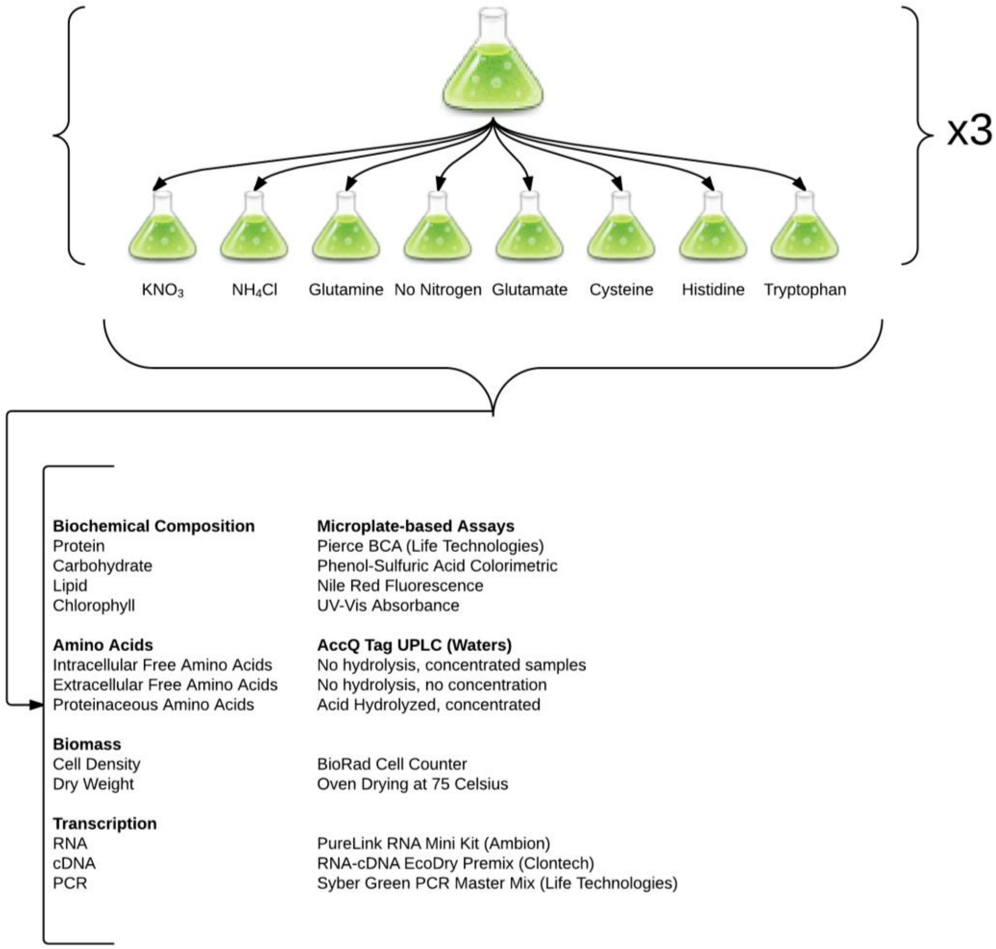

Figure 2.3. Experimental setup for the cultures harvested at 48 hours. Cells were grown in triplicate in all conditions. All assays were performed with at least three technical replicates as well.

2.2. METHODS

2.2.1. Cultivation and Harvest

Dunaliella viridis cultures used in these experiments descended from a single colony from cultures obtained from the Center for Applied Aquatic Ecology at North Carolina State University as described in Srirangan et al[36]. All cultures were grown in the same media supplemented with different nitrogen sources and grown at 21℃ under continuous soft white light at 12.81 klux without shaking or external CO_2 addition. Three biological replicates were performed for each condition. Each replicate was inoculated using the same parent culture with a starting concentration of 1 million cells at 0 h. The negative control with no nitrogen in the 144 hour experiment was grown differently; an initial culture was grown till 144 h with 5 mM potassium nitrate after which the medium was replaced with medium containing no nitrogen and grown for a further 144h.

A parent culture of 500 mL was grown for 120 h prior to beginning each experiment. All test cultures were then inoculated from this parent culture to obtain the starting concentration of 1 million cells/mL, as shown in figures 2.2 and 2.3. The test cultures were grown at a volume of 60 mL in static flasks.

After incubation for 144 h and 48 h in the two experiments, 50 mL was harvested and divided into a 10 mL aliquot for cell density, pH, protein, carbohydrate, lipid, and chlorophyll assays and a 40 mL aliquot for dry mass and amino acid analysis. From the 10 mL aliquot, two 1 mL aliquots for protein and carbohydrate analysis were centrifuged (13,000 rpm, 10 min) and stored as pellets at -80℃until analysis. Cell density, pH, lipid and chlorophyll assays were performed immediately after harvest by the protocols described below. For amino acid analysis and dry mass measurements, the 40 mL aliquot of the harvested culture was concentrated to 5 mL by centrifugation (4000 rpm, 15 min) and removal of supernatant. A 1 mL aliquot was used for amino acid analysis. The remaining 4 mL aliquot was centrifuged (13,000 rpm, 10 min) to be used for dry mass estimation in pre-weighed Eppendorf tubes by drying in an oven at 70℃for 24 h. The 1 mL aliquot for amino acid analysis was centrifuged at 13,000 rpm for 10 min). The supernatant was removed and stored separately for extracellular amino acid analysis and the pellet was resuspended in 400 µL distilled water to lyse the cells by osmotic shock. The lysed cells were stored for proteinaceous and intracellular amino acid analysis. All of these samples were then stored at -20℃until processed for amino acid analysis.

2.2. Protein

Protein was first extracted from cell cultures using a modified extraction protocol described by Barbarino et al.(2015)[48] and then assayed using a Bicinchoninic acid (BCA) assay (Thermo Scientific, Waltham, MA) according the manufacturer's instructions. Cell culture pellets for protein analysis were stored at -80℃ in 1 mL Eppendorf tubes until analysis was performed. The cells were lysed by adding 400 µL of distilled water to the frozen pellet and incubating at room temperature for 10 min with occasional vortexing. To separate soluble proteins and cell debris, the solution was centrifuged for 10 min at 13,000 rpm at 4℃. The soluble proteins in the supernatant (the water fraction) were transferred to a fresh tube. The protein in the pellet, which is comprised of the insoluble protein in the cell debris, was extracted from the pellet using 500 µL of 0.1N NaOH. 1 mL of 0.1 N NaOH was added to the pellet and incubated for 1 h at room temperature with intermittent vortexing followed by centrifugation (13,000 rpm, 5 min, 4℃) to obtain the NaOH fraction. The aforementioned water fraction and the NaOH fraction were combined to determine total protein content using the microplate procedure of the BCA assay kit according to the manufacturer's instructions. Bovine Serum Albumin (BSA) in a phosphate buffered saline solution at pH 7.4 (8 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl), provided with the kit, was used as the protein standard for standard curve generation in a concentration range of 0-200 µg/mL. Briefly, protein sample volumes of 150 µL were mixed with 150 µL of reagent provided in the kit and incubated for 2h at 37℃. The microplates were then read on a plate reader (Synergy HT multi-mode) at 562 nm. The standard curves obtained in both assays were consistent (R^2 > 0.99 for both plates) and precision of the assay was adequate with coefficient of variation between standard measurements in the range of 0-2% for intra-plate variation and 5-20% in inter-plate variation.

2.3. Carbohydrates

Total carbohydrates were quantified using a microplate-based phenol-sulfuric acid colorimetric method.[49] In this assay, sulfuric acid is used to break down complex sugars into furfural-like compounds which subsequently react with phenol to produce a yellow-gold color that detected using a UV-Vis spectrophotometer.[50] Glucose was used as a standard in the range of 0 – 200 µg/mL. Samples previously stored as pellets were washed with distilled water to remove the growth medium, as it reacts with the components in this assay. 1mL distilled water was added to the cell pellet to lyse the cells and the aqueous solution of the lysed cells were used in this assay. Briefly, 50 µL of 5% (w/v) phenol and sample were mixed in 96 well clear microplates, and then saturated with 250 µL of 95% sulfuric acid. The plate was then read at 490 nm using a UV-Vis spectrophotometer after a 15 min incubation. The full protocol is given in the Appendix section 2.7.1.

2.4. Chlorophyll

Chlorophyll was quantified using UV-Vis spectroscopy in absorption mode following a method modified from Arnon (1949).[51] 1 mL of cell culture was centrifuged at 13,000 rpm for 10 min at 4℃ and the supernatant was removed by aspiration. The pellet was resuspended in 650 µL of 100% ethanol. The chlorophyll in the pellet was allowed to dissolve into the ethanol solution for 10 minutes, followed by centrifugation (13,000 rpm, 10 min, 4℃) to remove cellular debris. The supernatant was read using clear 96-well plates at 652 nm. 100% ethanol was used as blank. Total chlorophyll was assayed for three biological replicates with three technical replicates each. The chlorophyll concentration was calculated using the A_{652} value and excitation coefficient, taken as 36,[36] using the following formula:

$$Total\ chlorophyll\ (mg.mL^{-1}) = \frac{A_{652}}{extinction\ coefficient}$$

2.2.5. Lipids

Lipid quantification was carried out using a Nile Red fluorescence assay adapted from Cooksey et al, 1987.[52] 1 mL of harvested cells suspended in growth medium was mixed with 4 µL of Nile Red solution (250 µg/mL solution in acetone).[53] Fluorescence was measured on Costar 96-well black opaque plates using a spectrometer with an excitation wavelength of 485 nm and an emission wavelength of 590. A standard curve with a working range from 500 µg/mL to 5 µg/mL was used to create a linear regression for measuring the lipid content using coconut oil as a standard. As lipids tend to degrade quickly after harvest, all measurements were made within 2 hours after harvest and within two minutes after mixing with Nile Red. Lipid content was measured for three biological replicates for each condition tested using three technical replicates each. The full protocol is given in the Appendix section 2.7.1.

2.2.6. Amino Acids

450 µL of concentrated, lysed cells were obtained after harvest by the procedures described in Section 2.2.1 and labeled as crude lysate. These samples were used for intracellular free- and proteinaceous amino acid analysis. For proteinaceous amino acid analysis, the crude lysate samples were hydrolyzed using 1 mL vacuum hydrolysis tubes (Life Technologies, Carlsbad, CA) using 6N HCl with 0.02% (v/v) β -mercaptoethanol.[54] Briefly, 50 µL of the concentrated sample was mixed with 400 µL of 6N HCl in vacuum tubes and sealed with the provided stopper. Vacuum was pulled using a pump to evacuate the air inside the tubes. The tubes containing the samples were then placed in heating blocks set at a temperature of 105℃ for 20 hours. The samples were then recovered and the reaction stopped by adding 400 µL of 6N NaOH to neutralize the HCl. These samples were labeled acid-hydrolyzed proteinaceous amino acids and stored for further processing at -20℃. Intracellular free amino acid samples were obtained by centrifuging the crude lysate at 13,000 rpm for 10 min and collecting the supernatant. The full procedure is detailed in the Appendix in section 7.1.

These samples were derivatized with AccQ – Tag (Waters, Milford, MA) according to the manufacturer's instructions to separate and quantify amino acids using a Waters UPLC system. AccQ – Tag uses 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), which preferentially binds to primary or secondary amines, allowing for simple separation by reverse-phase HPLC and detection by a UV detector.[55] The only negative of this procedure discovered during analysis was the interference of amine group-containing media components which were also derivatized and present on the chromatogram. A major component of the media used to cultivate *Dunaliella* was Tris-HCl, which eluted at a retention time very close to serine, preventing accurate quantification of serine. An advantage of AQC is the ability to detect ammonium ions as well, which was useful to quantify presence of free ammonium efflux in the extracellular medium.

Derivatized samples were run on a Waters UPLC system in reverse phase mode. Standard curves for each amino acid and ammonium were generated using amino acid standards from Sigma Aldrich (St. Louis, MO) for a working range of 0 – 50 nmol/μL. All curves were extremely consistent and linear ($R^2 > 0.999$).

A shortcoming of this method is the loss of differentiation between glutamine and glutamic acid, and between asparagine and aspartic acid during acid hydrolysis. Glutamine and asparagine are acidified to glutamate and aspartate and elute along with the glutamate and aspartate fractions. Hence these values for the hydrolyzed samples will be reported as "Glutamine + Glutamic acid" and "Asparagine + Aspartic acid". Tryptophan is degraded during hydrolysis as well. β-mercaptoethanol was added to preserve tryptophan and methionine during the hydrolysis procedure but there was only a marginal improvement in recovery.[56] The full protocol for derivatization and UPLC sample preparation is given in the Appendix section 7.1.

2.2.7. Quantitative-PCR

RNA transcripts isolated from the cells grown in eight different treatments were isolated and reverse transcribed into cDNA. The cDNA was used as templates for qPCR to study the relative abundance of seven key nitrogen assimilation genes – DvAMT 1;1, DvAMT1;2, GOGAT-NADH, Low CO_2–Inducible protein (LCI), aspartate aminotransferase (DvAspAT), alanine aminotransferase (DvAAT) and malate dehydrogenase (DvMDH). The RNA extraction method has been previously described in Srirangan et al.(2015)[36] It is briefly summarized below along with modifications to those methods.

2.2.7.1 RNA EXTRACTION AND PURIFICATION

4 mL of the harvested cell culture, in the range of 1.5 – 4.5 million cells/mL, was centrifuged (4000 rpm, 15 min), concentrated into 1 mL of fresh media in an Eppendorf tube, and recentrifuged (13,000 rpm, 10 min, 4℃) to obtain a pellet. The pellet was then flash frozen in liquid nitrogen and stored at -80℃. RNA was extracted using a Trizol-Chloroform biphase separation kit. (Thermo Fischer Scientific, Waltham, MA) The addition of Trizol to the pellet lysed the cells, allowing for RNA to be released, and eventually collected in the aqueous layer. Further purification was performed using the Ambion PureLink RNA Mini kit according to the manufacturer's instructions to obtain purified RNA in RNAse free water. (Thermo Fischer Scientific, Waltham MA) Purity and recovery were determined using Nanodrop 1000 UV-Vis spectroscopy and found to be in the range of 15 ng/μL to 650 ng/μL depending on harvested cell density and extraction efficiency. DNAse treatment was performed to remove genomic DNA contamination using the Ambion Turbo DNAse I kit according to the manufacturer's

instructions. (Thermo Fischer Scientific, Waltham MA) The samples were then immediately processed to synthesize cDNA.

2.2.7.2. CDNA SYNTHESIS

The concentration of the mRNA before DNAse treatment was used as the basis for calculating the amount of RNA converted into cDNA.[57] 1 µg of RNA per sample was converted into cDNA using the Clontech RNA to cDNA Premix kit according to the manufacturer's instructions. (Takara, Mountain View, CA) The synthesized cDNA was stored at -20℃ until processed.

2.2.7.3. PRIMER DESIGN

Reverse translated putative protein sequences of the genes studied herein were obtained for *Chlamydomonas reinhardtii* and used as queries to BLAST against the transcriptome for *Dunaliella viridis* (published earlier by Srirangan et al and an unpublished transcriptome by Murphree et al) to obtain analogous expressed contigs from the genome which possibly contain the coding regions for these genes. These expressed contig loci were processed through Expasy to determine coding regions and putative amino acid sequences. These protein sequences were verified using pBLAST to confirm their homology with other genes in their families. Primers were synthesized using NCBI's Primer design tool based on these coding regions.[58] Primers were restricted to 18-25 bp in length and a product size of 100-200. Two sets of primers were tested for each gene, with one set of primers chosen for each gene for the final qPCR analysis. The primers chosen for the assays are tabulated in Appendix Table 2.10.

2.2.7.4. Quantitative PCR

The qPCR analysis was performed using a Stratagene Mx3005 Real-time PCR machine and a SYBR® Green PCR Master Mix (Life Technologies) based protocol adapted from S. Jali et al.[59] The Syber Green Master Mix used in the assay contained oligonucleutides, AmpliTaq Gold® DNA polymerase, and the buffer used for the reaction and was mixed with cDNA and primers in the ratio of 2:1:1 for a total volume of 20 µL per reaction. All measurements were made in triplicate. GAPDH and Actin were used as housekeeper genes for normalization of the results as controls. All qPCR assays were performed using the following run settings: Initial denaturation at 98℃ for 30s, followed by 40 cycles of denaturation at 98℃ for 10s, annealing at 60℃ for 30s, and extension at 72℃ for 30s. After 40 cycles, a final extension was performed at 72℃ for 300s.

2.8. Dry Weight and Cell Density

Cell density was measured using a BioRAD cell counter and reported as million cells/mL. 10 µL of harvested cells in the growth media were aliquoted on the BioRAD reader microchips in duplicate for each sample. Dry weight measurements were obtained after oven drying biomass pellets at 75°C.[60] 40 mL of harvested cell culture was concentrated to 5 mL, 1 mL was removed for amino acid analysis and the remaining culture was further concentrated in pre-weighed Eppendorf tubes. The pellets were dried at 75°C for 24 h, weighed, and tared for weight of the tubes. The results were adjusted for concentration factors to obtain dry cell weight (DCW) and reported as mg/mL.

2.2.9. Data Analysis and Reporting

Measurements of carbohydrates, proteins, lipids, chlorophyll, and amino acids for both experimental time points of 48h and 144h are reported normalized to a micrograms per million cells basis and micrograms per mL. The micrograms per mL measurement is refers to the total content produced of the particular macromolecule per unit volume, while the microgram per million cells measurement refers to the productivity of the culture on a per-cell basis. Transcript abundance in the qPCR study is reported as fold change with samples grown on nitrate as control. GAPDH and β-actin were chosen as the housekeeping genes for normalization of the results across the different growth conditions.[61] JMP 11.2 (SAS) was used to perform statistical analysis on the results to elucidate significant differences between the results. Tukey's HSD test was used to compare the means between groups to ascertain if the conditions significantly differed from each other.

2.3. RESULTS

The biochemical composition of *Dunaliella* grown in the presence of different nitrogen sources was quantified using the assays described above and the results are described in the sections below. Additionally, the amino acid content of the algae cells is also described, with the free amino acids quantified to analyze genomic responses in metabolic pathway genes and proteinaceous amino acids quantified to describe the composition of proteins when *Dunaliella* was fed different amino acids. Finally, the relative gene expression of key N and C metabolism genes is provided to describe the differences between cell growth in the various N sources.

2.3.1. Lipid Composition

The lipid content, as quantified by the Nile Red assay, for the *D. viridis* cultures grown on the various nitrogen sources tested is shown below (Figures 2.4 and 2.5). After 144 h, cultures grown in ammonium had the highest total lipid content at 20 µg/mL and at 3.2 µg/million cells, the second highest productivity. The samples grown with all twenty amino acids had the highest lipid productivity at 4.5 µg/million cells but the lowest total lipid content owing to a low total biomass at the end of 144 h (Figure 2.4). In the cultures harvested after 48 hours, the highest lipid accumulation was observed in samples grown with histidine, followed by glutamate and cysteine-utilizing samples. As these samples also reported low cell densities (except Cysteine), their lipid content per cell was also the highest (Figure 2.5).

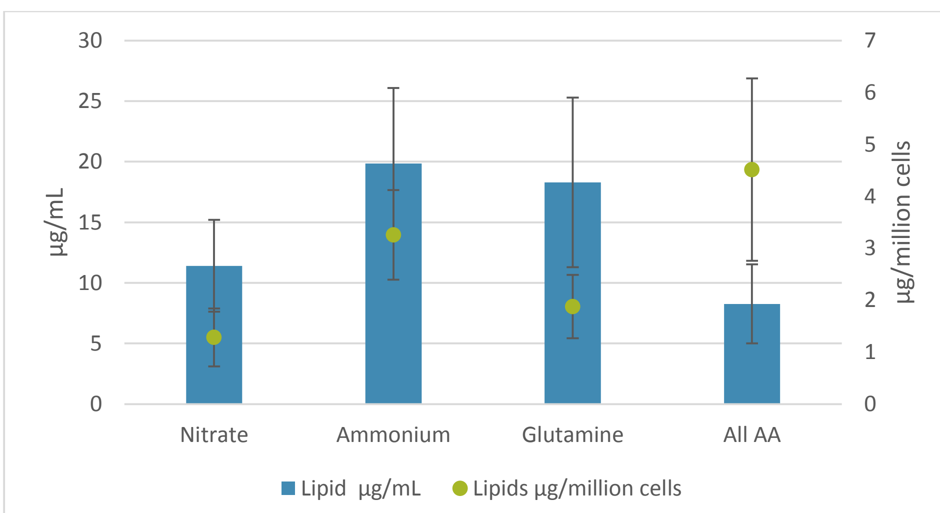

Figure 2.4. Lipid composition of *Dunaliella* cells after 144 h of growth. Bars represent µg/mL lipids and points represent µg/million cells. Error bars represent standard deviation of measurements from three biological replicates, each with three internal technical replicates.

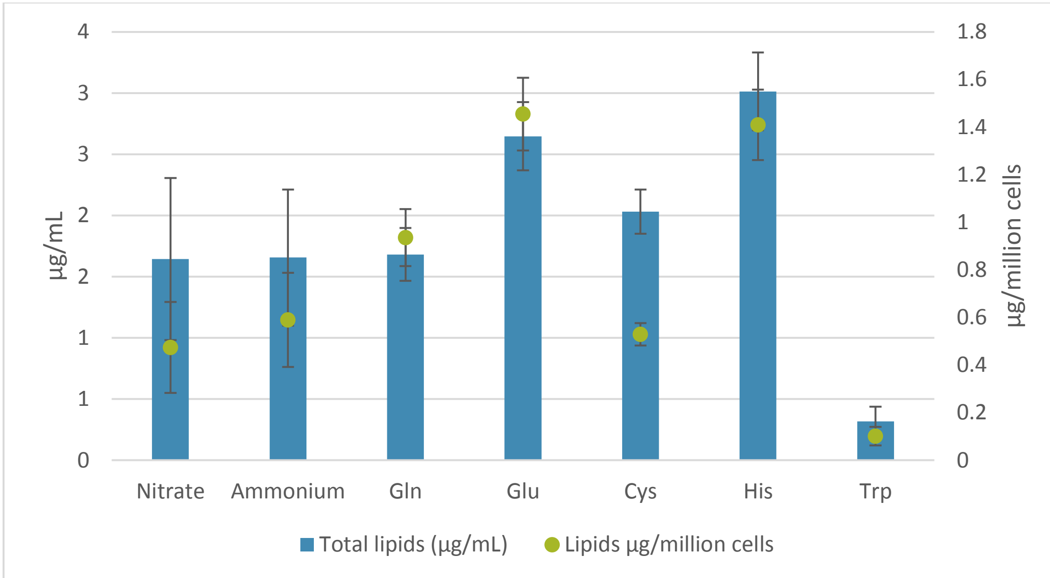

Figure 2.5. Lipid composition of *Dunaliella* cells after 48 h of growth. Bars represent total lipids in µg/mL and points represent µg lipids per million cells. Error bars represent standard deviation of measurements from three biological replicates, each with three internal technical replicates.

2.3.2. Protein Composition

The protein content, as measured using the BCA assay, of *Dunaliella* cells grown with different nitrogen sources is given below. After 144 hours, the total protein content in the samples grown in nitrate was found to be the highest, while the per cell protein content was found to be highest in samples grown with all twenty amino acids. However, just as in the case of lipids, the total protein content was low because of low cell growth. (Figure 2.6)

After 48 h, samples grown with tryptophan had the highest total protein content at 27 µg/mL and the highest protein content on a per cell basis at 8.5 µg/million cells. On a per cell basis, tryptophan was followed by cultures grown with glutamine (7.5 µg/million cells) and then cysteine (5.5 µg/million cells). When comparing total protein content, tryptophan was followed by samples grown with cysteine (21 µg/mL) and nitrate (17 µg/mL). (Figure 2.7) The lowest protein content was found in cells grown with glutamine or histidine. This is consistent with the results obtained for lipid content, which indicate that these conditions mimic nitrogen deprivation and stress conditions. Similarly, high protein content in samples grown with tryptophan coupled with low lipid productivity reveal evidence for high cellular growth under stress-free conditions.

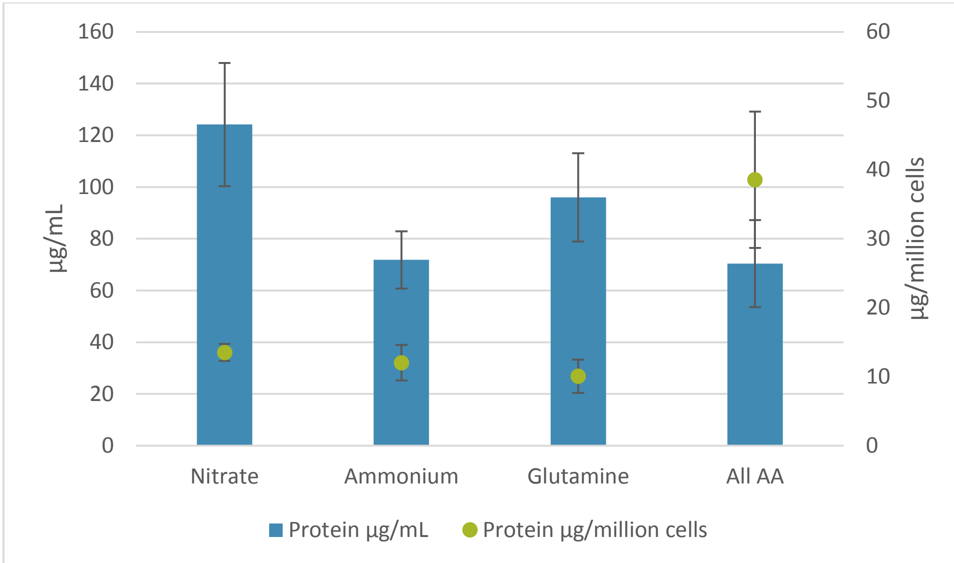

Figure 2.6. Protein composition of *Dunaliella cells* after 144 h of growth. Bars represent g proteins/g biomass and points represent mg protein/L culture. Error bars represent standard deviation of measurements from three biological replicates, each with three internal technical replicates.

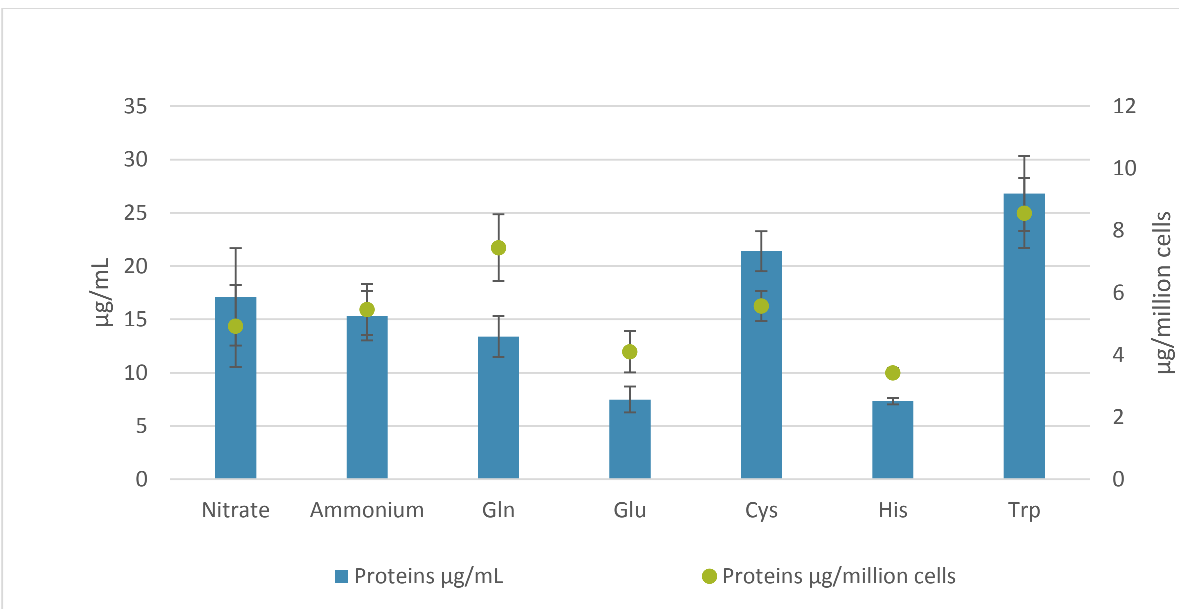

Figure 2.7. Protein composition of *Dunaliella cells* after 48 h of growth. Bars represent total protein in µg/mL and points represent µg protein per million cells. Error bars represent standard deviation of measurements from three biological replicates, each with three internal technical replicates.

The amino acid profiles of the protein content in *Dunaliella* cultures were also compared and quantified across the different nitrogen feeding schemes using reverse phase UPLC. The amino acid profile of cultures harvested at 144 h are given in Figure 2.8 and at 48 h in Figure 2.9.

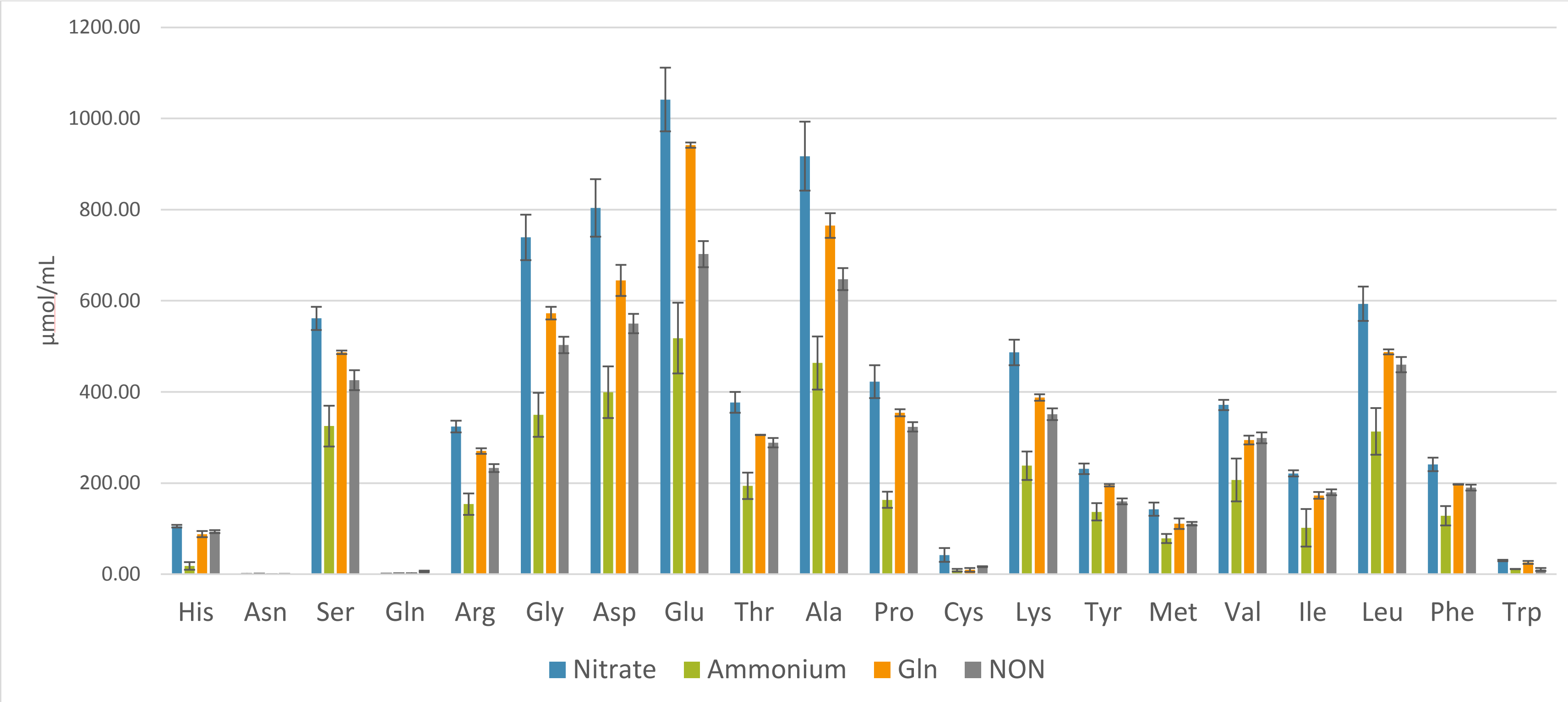

Figure 2.8. Hydrolyzed protein-based amino acid profile of *Dunaliella* cultures grown in four different nitrogen conditions obtained after 144 h of growth. Error bars represent standard deviations from three biological replicates. KNO – potassium nitrate as nitrogen source; NH – ammonium chloride; GLN – glutamine; NON – no nitrogen source in media. Asp values represent Asp + Asn concentrations, while Glu represents Glu + Gln values. Cysteine and Tryprophan are generally degraded during hydrolysis, hence are not reflective of accurate concentrations.

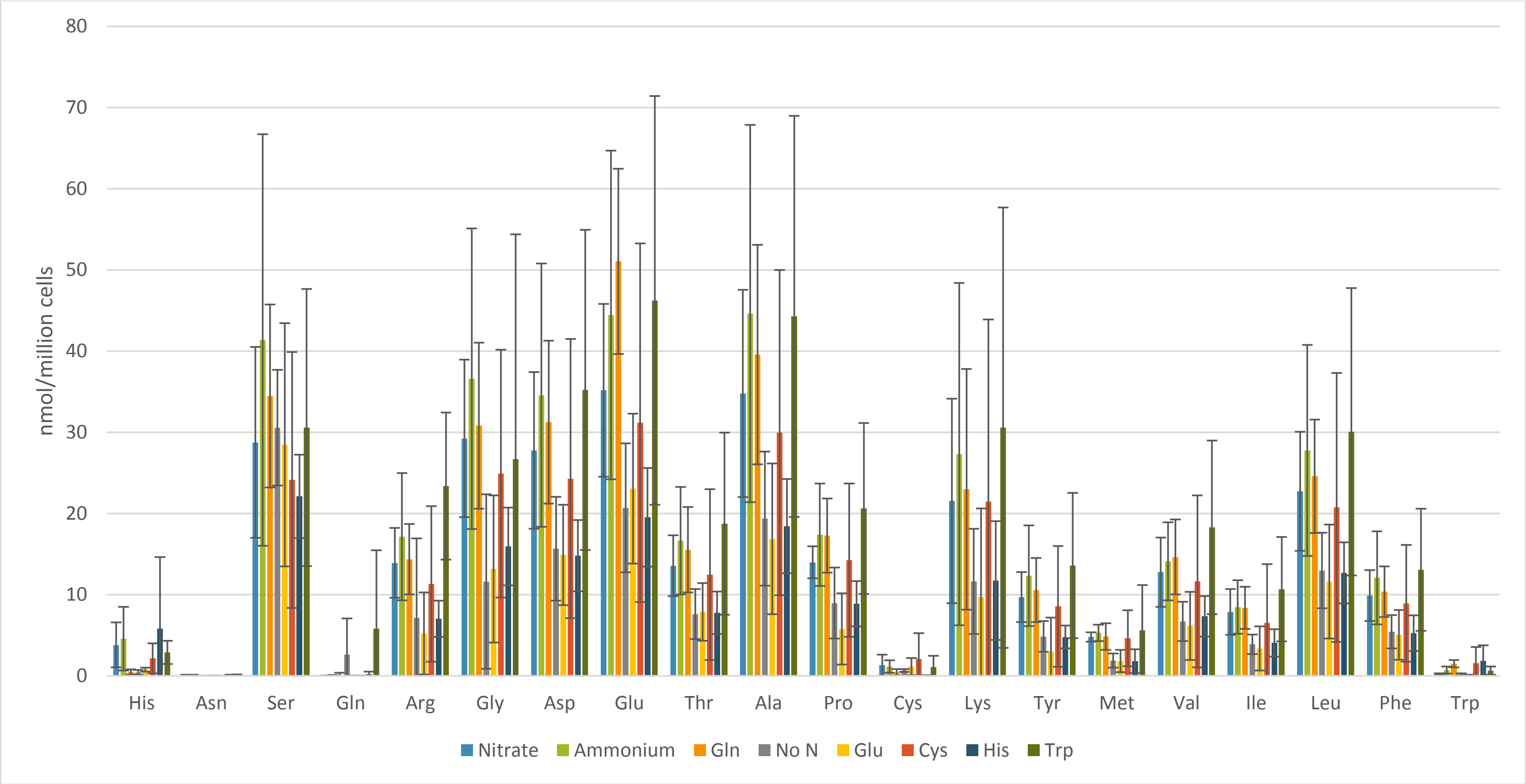

Figure 2.9. Hydrolyzed protein-based amino acid profile of *Dunaliella* cultures grown in four different nitrogen conditions obtained after 48 h of growth. Error bars represent standard deviations from three biological replicates. Columns represent nmol/million cells of the amino acid in different nitrogen conditions: KN – potassium nitrate as nitrogen source; NH – ammonium chloride; GLN – glutamine; NON – no nitrogen source; GLU – glutamic acid; CYS – cysteine; HIS – histidine; TRP – tryptophan. Asp values represent Asp + Asn concentrations, while Glu represents Glu + Gln values. Cysteine and Tryprophan are generally degraded during hydrolysis, hence are not reflective of accurate concentrations.

2.3.3. Chlorophyll Content

Chlorophyll content was found to be highest in cultures grown on nitrate for 144 h, followed by cells supplied with glutamine. Samples grown in ammonium and amino acids had the lowest chlorophyll content. After 48 hours, the chlorophyll content was highest in samples grown with nitrate (1 µg/mL), followed by tryptophan, ammonium, and cysteine. Samples grown with glutamine had comparatively high amounts of chlorophyll per cell (0.18 µg/million cells), but low total content (0.3 µg/mL) due to fewer cells.

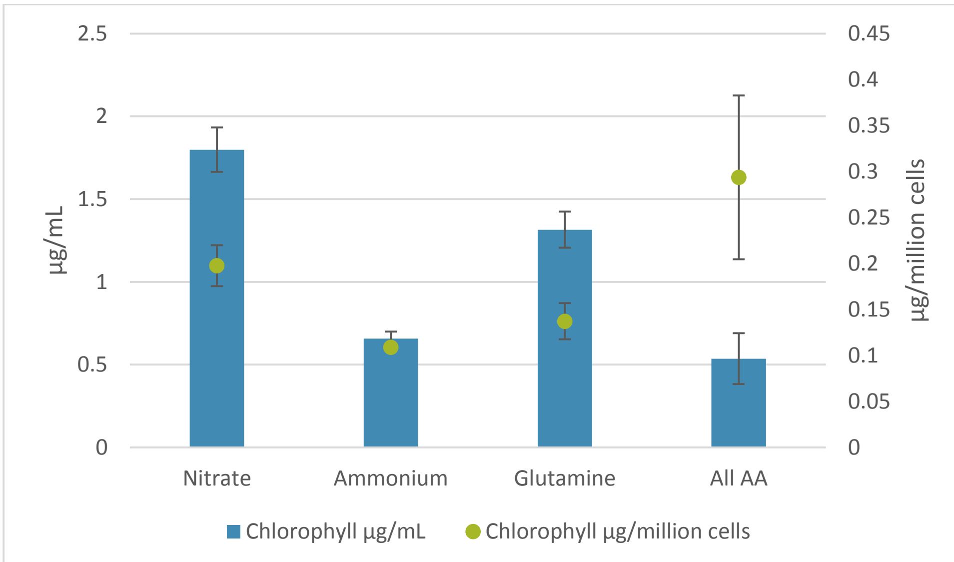

Figure 2.10. Chlorophyll composition of *Dunaliella cells* after 144 h of growth. Bars represent mg chlorophyll/g biomass and points represent mg chlorophyll/L culture. Error bars represent standard deviation of measurements from three biological replicates, each with three internal technical replicates.

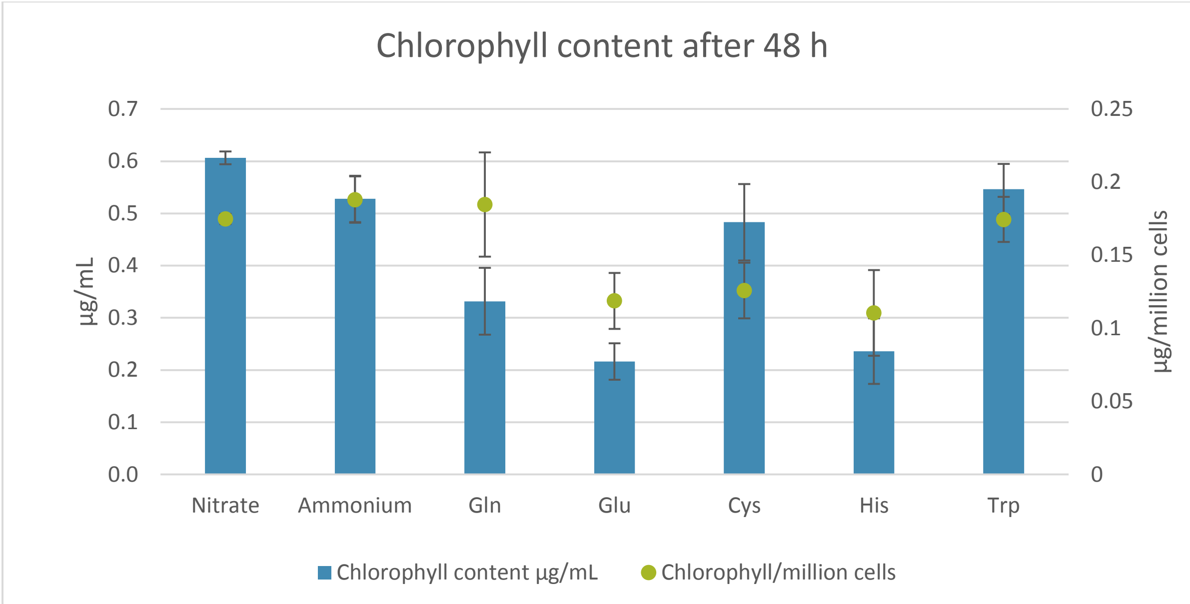

Figure 2.11. Chlorophyll composition of *Dunaliella cells* after 48 h of growth. Bars represent total chlorophyll in µg/mL and points represent µg chlorophyll per million cells. Error bars represent standard deviation of measurements from three biological replicates, each with three internal technical replicates.

2.3.4. Carbohydrate Composition

Carbohydrate content was measured using the phenol-sulfuric acid assay and was found to be the most abundant compound in *Dunaliella* in all conditions. At the end of 144 h, carbohydrate content was found to be highest in cultures grown with ammonium (571 µg/mL) and glutamine (545 µg/mL). At the end of 48 hours, cells grown with cysteine, histidine, and glutamate had the highest carbohydrate content. (Figure 2.13) The least carbohydrate content at the end of 144 hours was found in cells grown with all amino acids (114 µg/mL). (Figure 2.12) and in glutamine samples at the end of 48h.

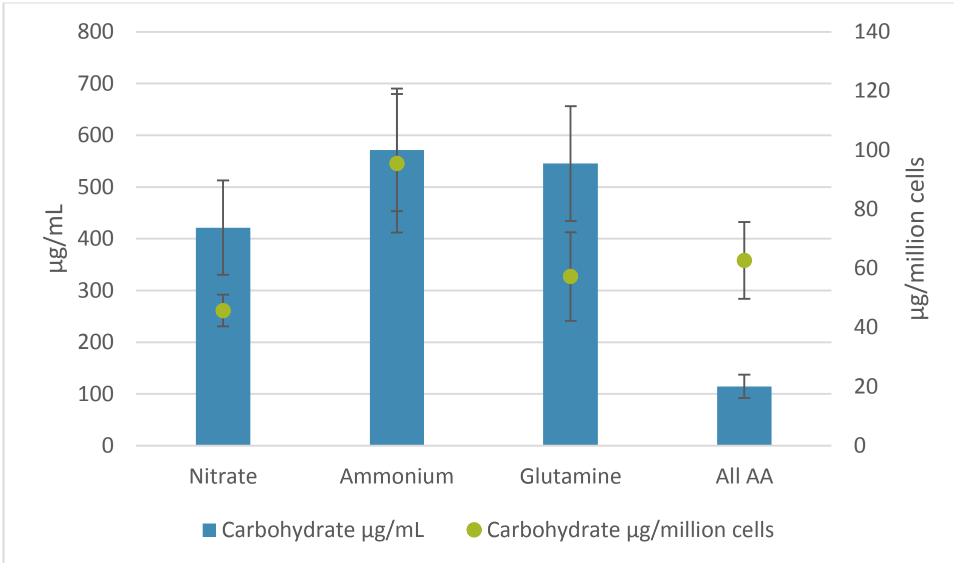

Figure 2.12. Carbohydrate composition of *Dunaliella cells* after 144 h of growth. Bars represent g carbohydrate/g biomass and points represent mg carbohydrate/L culture. The chlorophyll content shown for No nitrogen media indicates the protein amount left at the end of 288 hours of growth, with the last 144 hours in no-nitrogen media. Thus, there is no reduction in total carbohydrate in these samples, but due to the increase in biomass, there is a reduction of over 0.5 g/g biomass after transfer. Error bars represent standard deviation of measurements from three biological replicates, each with three internal technical replicates.

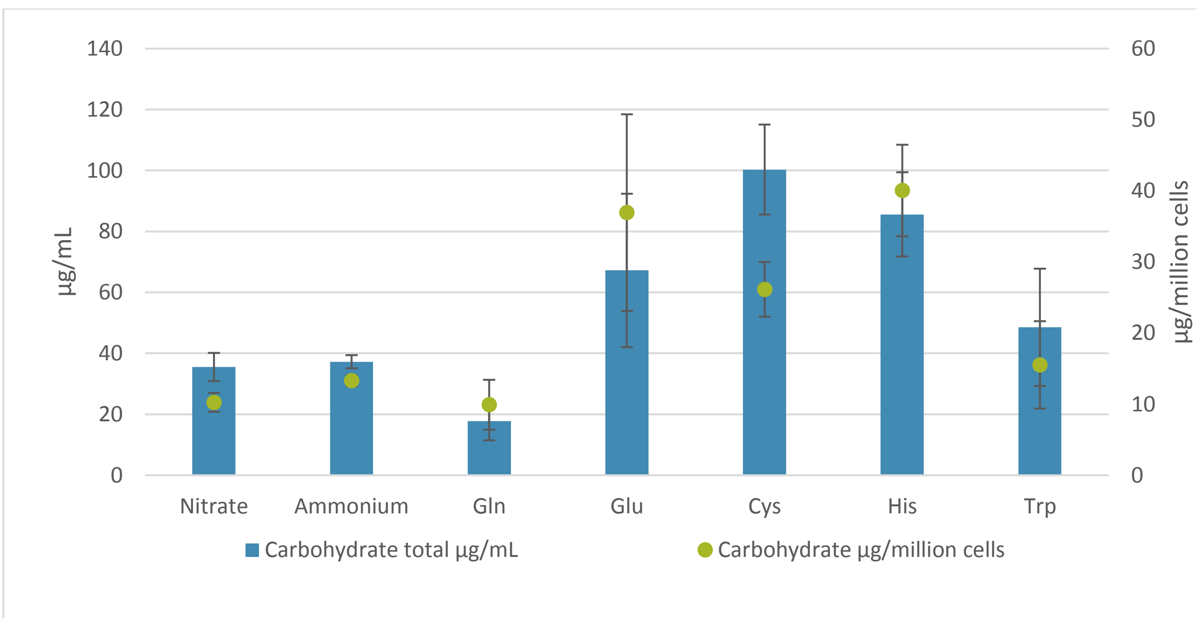

Figure 2.13. Carbohydrate composition after 48 hours. Bars represent total carbohydrate in µg/mL and points represent µg carbohydrate per million cells. Error bars represent standard deviation of measurements from three biological replicates, each with three internal technical replicates.

2.3.5. Nitrogen Starvation

To study the effects of nitrogen starvation, cultures with a starting cell density of 1 million cells/mL were grown in medium containing nitrate for 144 h and transferred to a nitrogen-free medium at a cell density of ~8 million cells/mL, and incubated for an additional 144 h. This was done to mimic a two-stage biofuel production process where the first stage is the cell growth phase to attain maximal biomass and the second stage is the lipid production phase to induce lipid accumulation.

Nitrogen starvation resulted in lipid accumulation, with the lipid content reaching 47 µg/mL at the end of the 144 h incubation in nitrogen-free medium. This was more than twice the lipid content of cells grown with nitrate or ammonium for 144 h. The carbohydrate content of N-starved cells remained unchanged after transfer to nitrogen-free medium, while the protein content decreased to 56 µg/mL from 124 µg/mL when grown in medium with nitrate. Nitrogen starved cells had the highest lipid productivity (4 µg/million cells), and the lowest protein (4.7 µg/million cells) and carbohydrate (35 µg/million cells) productivities.

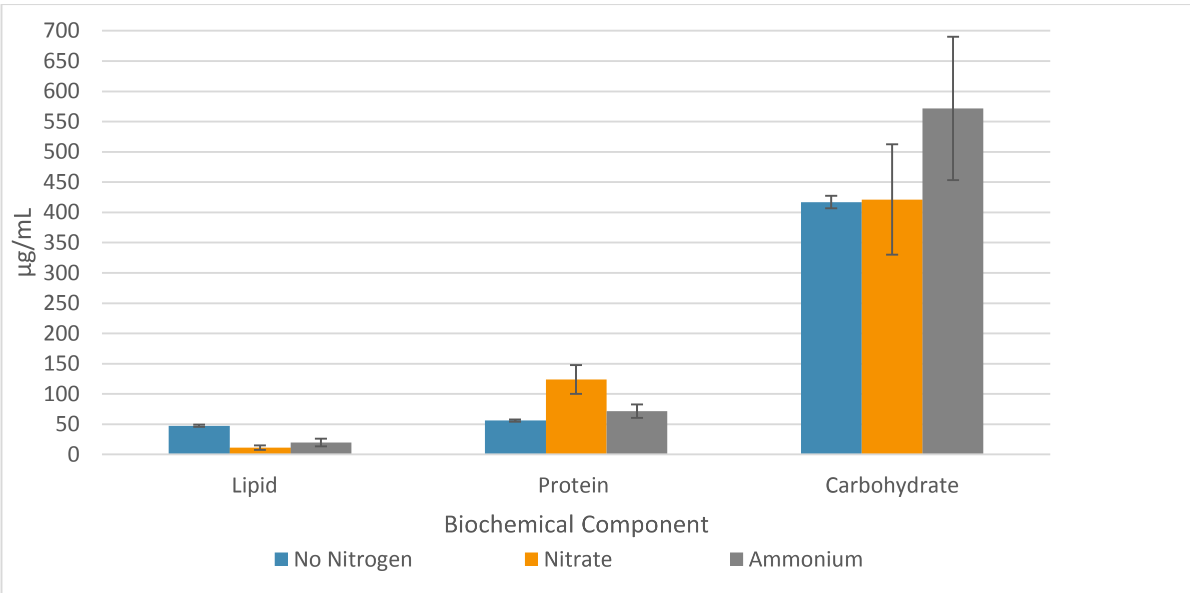

Figure 2.14 Comparison of macromolecular composition per mL of *Dunaliella* grown under nitrogen deprivation and with nitrate and ammonium after 144h.

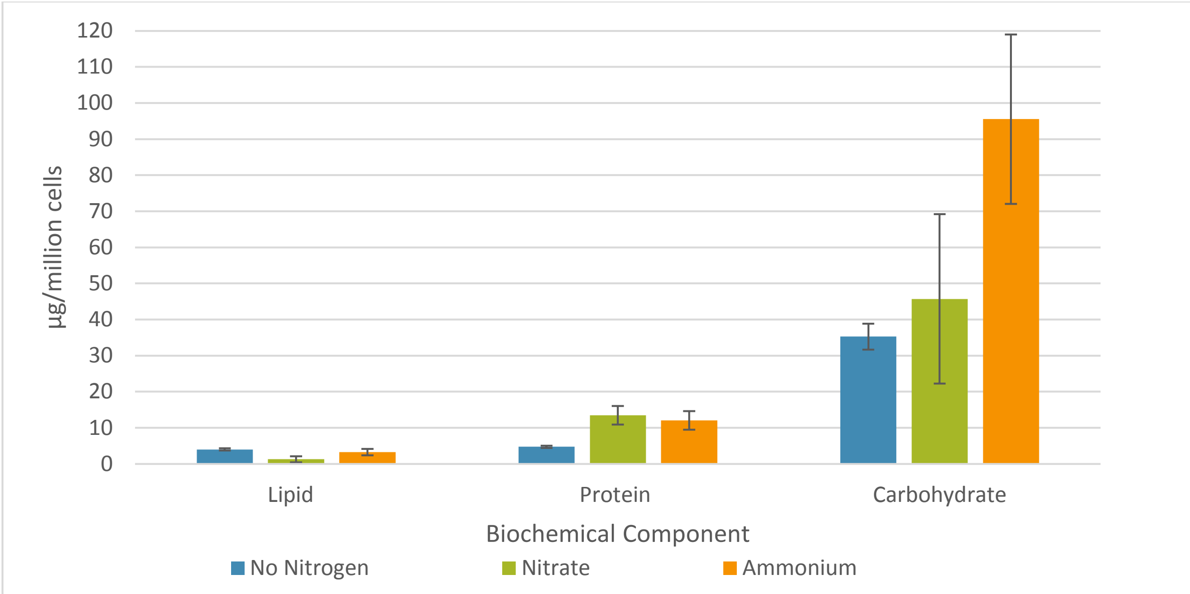

Figure 2.15. Comparison of macromolecular productivity per million cells of *Dunaliella* grown under nitrogen deprivation and nitrate and ammonium after 144h.

2.3.6. Amino Acids and Ammonium Profiles

Free amino acids were quantified using reverse phase UPLC to infer differences in the metabolite pool in *Dunaliella* grown with different nitrogen sources. After 144 hours, the most abundant free amino acids in *Dunaliella* are glutamate, alanine, and tyrosine - each around 40 – 90 µmol/mL. (Figure 2.18) In some conditions, glutamine and glycine are also present in large amounts (~30 µmol/mL). After 48 hours, the most abundant amino acids present are also glutamate, alanine, and tyrosine (~20 nmol/ million cells). Complete individual amino acid concentration tables are given in Appendix section 7.3.

The figures 2.16 and 2.17 below show the distribution of the free amino acid content arranged in their biosynthetic pathway groups. Glutamine and glutamate are products of the GS-GOGAT pathway, alanine and aspartate are the first amino acids synthesized after the GS-GOGAT pathway, glycine and serine are a part of the photorespiratory pathway with cysteine, tryptophan, tyrosine, and phenylalanine are the aromatic amino acids, arginine, threonine, lysine, and methionine are directly derived from aspartate, leucine, isoleucine, and valine are the branched chain amino acids and finally arginine, proline, and histidine are derived through unrelated biosynthetic pathways. Nitrate grown samples showed a tendency to accumulate free alanine and glycine after 144h. Taking into account relative protein content, no other major differences were shown from these results. Conditions that favored protein production also encouraged higher levels of free amino acids. Cysteine-grown samples were unusual in that they had relatively high protein content but low free amino acid content.

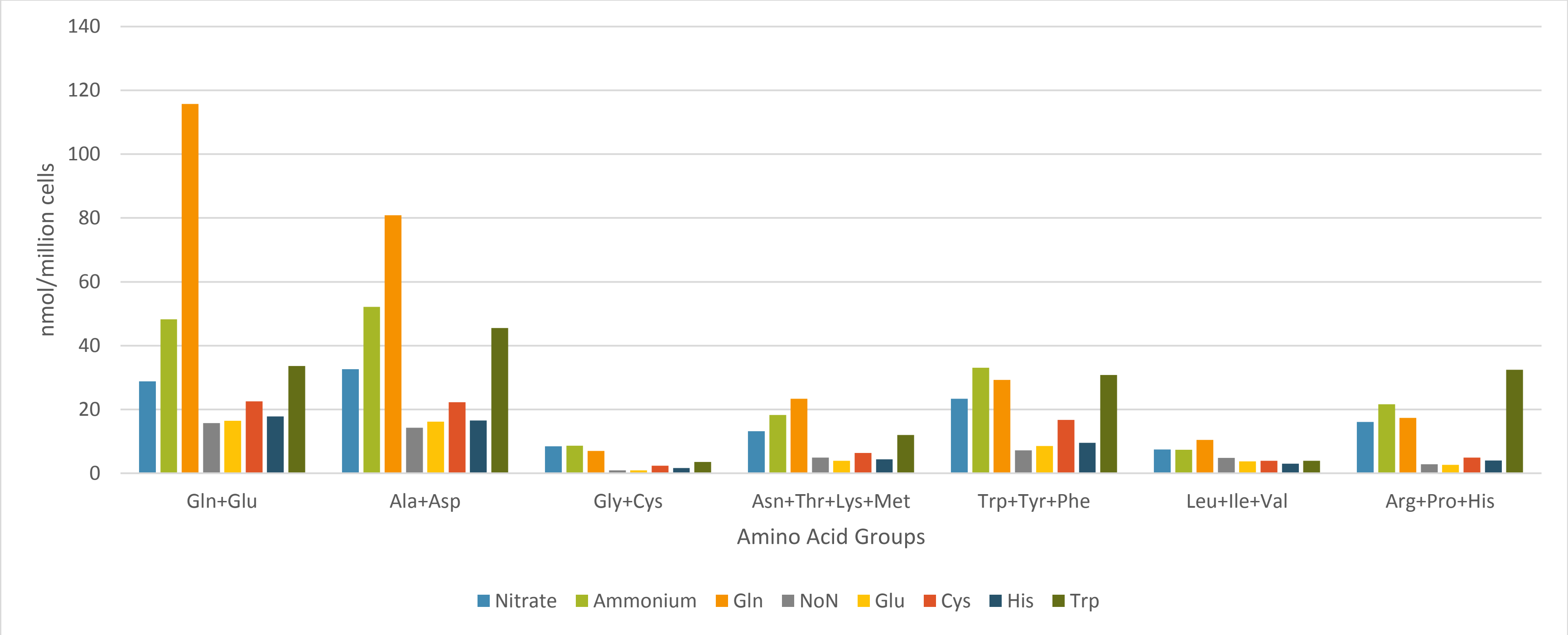

Figure 2.16. Intracellular free amino acids in *Dunaliella* cells presented in nmol/million cells and arranged in groups representing common biosynthetic pathways for cultures harvested after 48h.

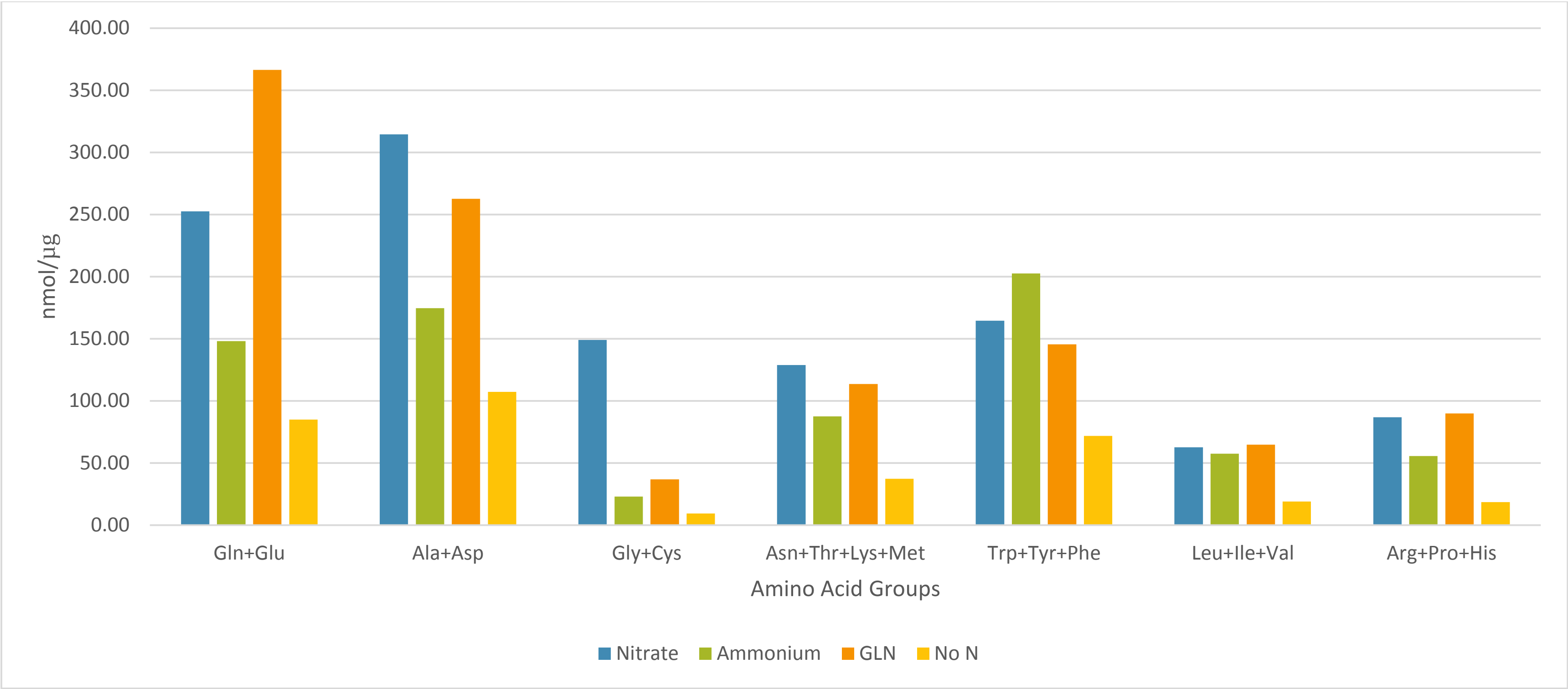

Figure 2.17. Intracellular free amino acids in *Dunaliella* cells represented in nmol/µg and arranged in groups representing common biosynthetic pathways for cultures harvested after 144h.

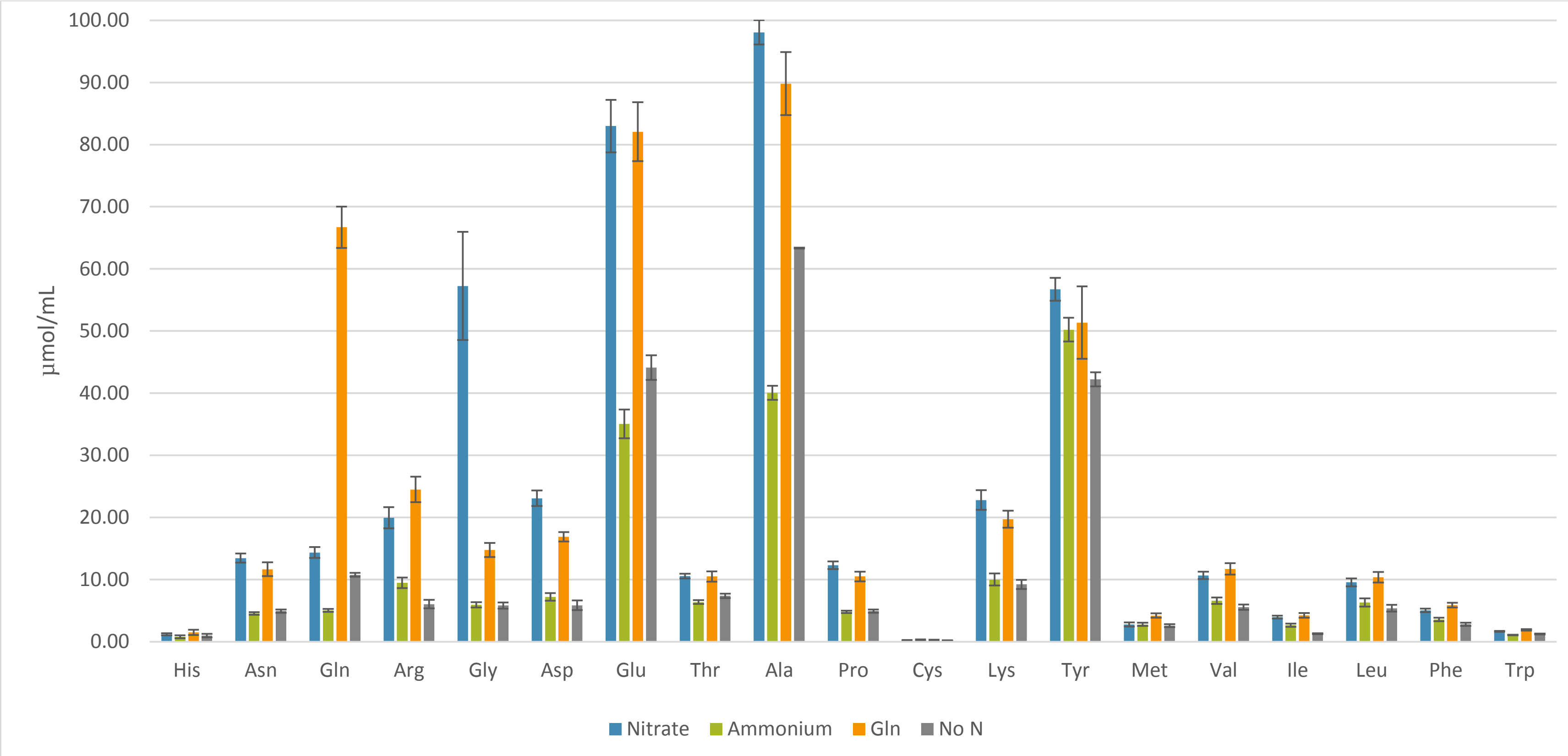

Figure 2.18. Intracellular free amino acid profiles for *Dunaliella* cells grown in four different nitrogen conditions obtained after 144 hours. Error bars represent standard deviations from three biological replicates. KNO – potassium nitrate as nitrogen source; NH – ammonium chloride; GLN – glutamine; NON – no nitrogen source in media. Ser peaks do not represent true serine concentration values as there is contamination with Tris-HCl, which elutes too close to serine and obscures the peak for serine in the UPLC chromatogram.

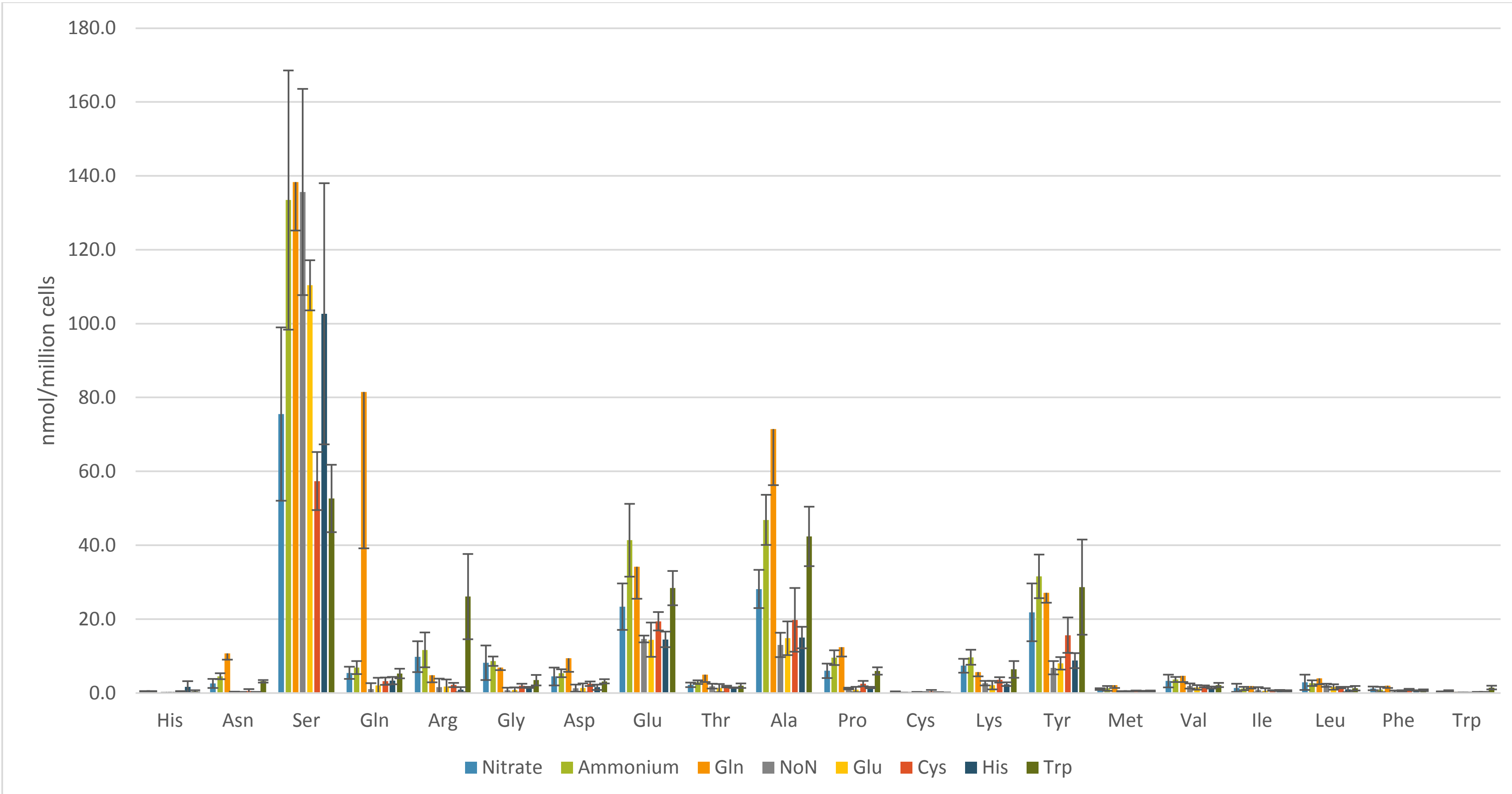

Figure 2.19. Intracellular free amino acids obtained for eight different nitrogen conditions after 48 hours. Error bars represent standard deviations from three biological replicates. Columns represent nmol/million cells of the amino acid in different nitrogen conditions: KN – potassium nitrate as nitrogen source; NH – ammonium chloride; GLN – glutamine; NON – no nitrogen source; GLU – glutamic acid; CYS – cysteine; HIS – histidine; TRP – tryptophan.

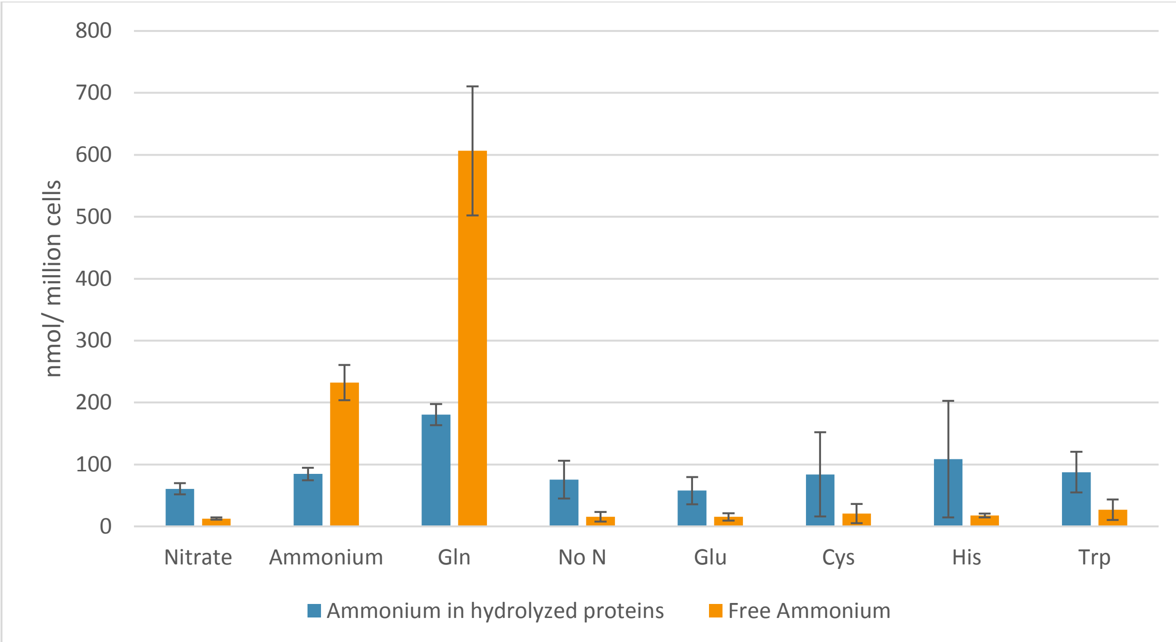

Figure 2.20. Ammonium content in *Dunaliella* cells after 48 hours. Error bars represent standard deviations from three biological replicates. Columns represent nmol/million cells of the amino acid in different nitrogen conditions: KN – potassium nitrate as nitrogen source; NH – ammonium chloride; GLN – glutamine; NON – no nitrogen source; GLU – glutamic acid; CYS – cysteine; HIS – histidine; TRP – tryptophan.

The ammonium content was also quantified during the amino acid analysis. Free ammonium highest in cells grown with glutamine and expectedly, cells grown in ammonium. However, the ammonium content in cells grown with glutamine was over three times the amount found in samples from ammonium-grown cells. This could be due to the higher concentration of glutamine (5 mM) in the media compared to ammonium (0.5 mM) and due to degradation of glutamine in the media, which has been reported previously to occur in cell culture media containing glutamine.[62] Excessive degradation of glutamine could be a concern, as it might result in ammonium concentrations tending closer towards toxicity inducing levels.

2.3.7. Transcript Abundances of Metabolic Genes

The results of the qPCR experiment have been calculated using the Δ-ΔCt method, described as follows: cycle time values for test genes were subtracted from reference genes and the result was averaged across three biological replicates. The result was then normalized to the control test condition, which was set as the nitrate-utilizing condition, by subtracting the previous results from the cycle time values obtained for samples grown with nitrate. The result was used to calculate fold change values as a measure of fold change when compared to the samples grown with nitrate. The results have been shown below in Figure 2.21.

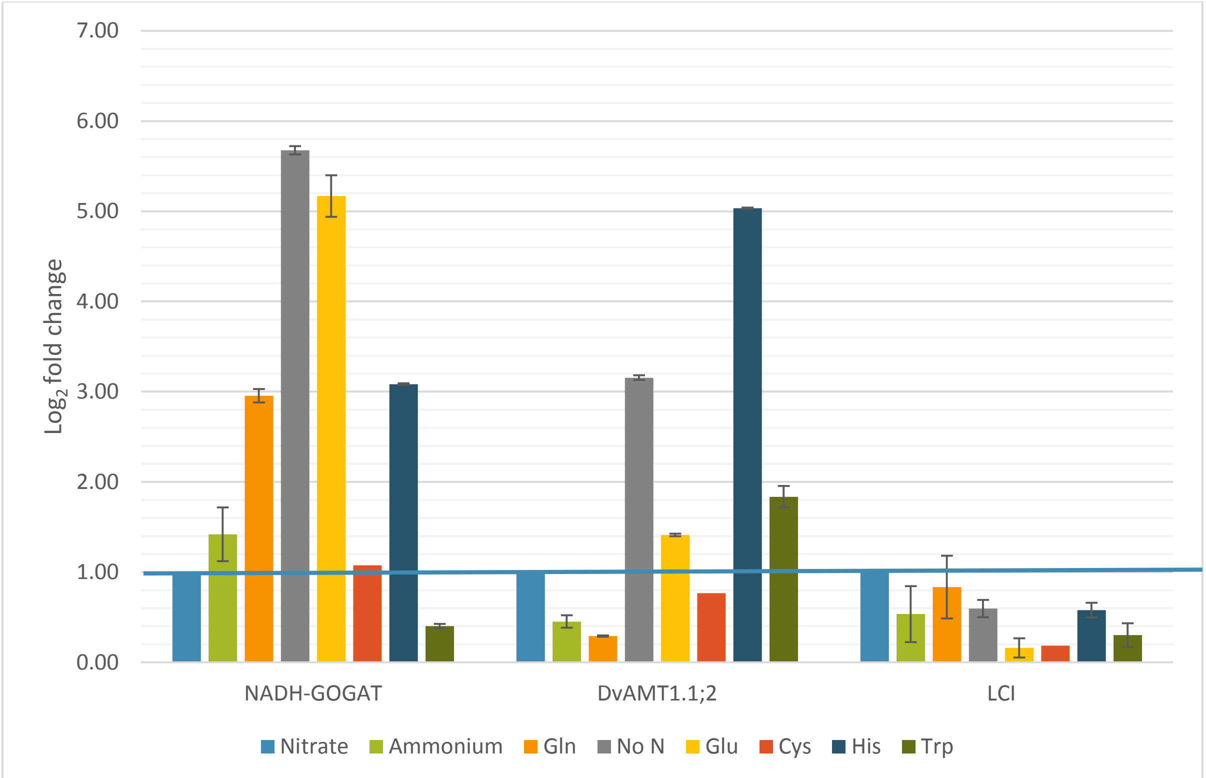

Figure 2.21. Transcript abundances of three genes in *Dunaliella* obtained from quantitative PCR of RNA. All values are fold changes in transcript levels with GAPDH as a reference gene and nitrate as the control condition. All nitrate responses have been normalized to one-fold, and the other conditions correlate to those results. Error bars represent standard deviations from three biological replicates. The horizontal line defines the control level of transcript abundance.

GOGAT was found to be expressed five-fold higher in glutamine- and glutamate-grown samples. GOGAT transcripts were also present three-fold higher in histidine-grown samples. (Figure 2.21) DvAMT1:1;1 was found to be present five-fold higher in histidine-grown samples compared to nitrate, while the levels in ammonium, glutamine, glutamate, and tryptophan were only slightly higher than in the nitrate controls. LCI was only induced in glutamine-grown samples to a level matching the control. All others showed negligible to no presence of this transcript. MDH was found to be expressed only in nitrate- and ammonium-fed samples.

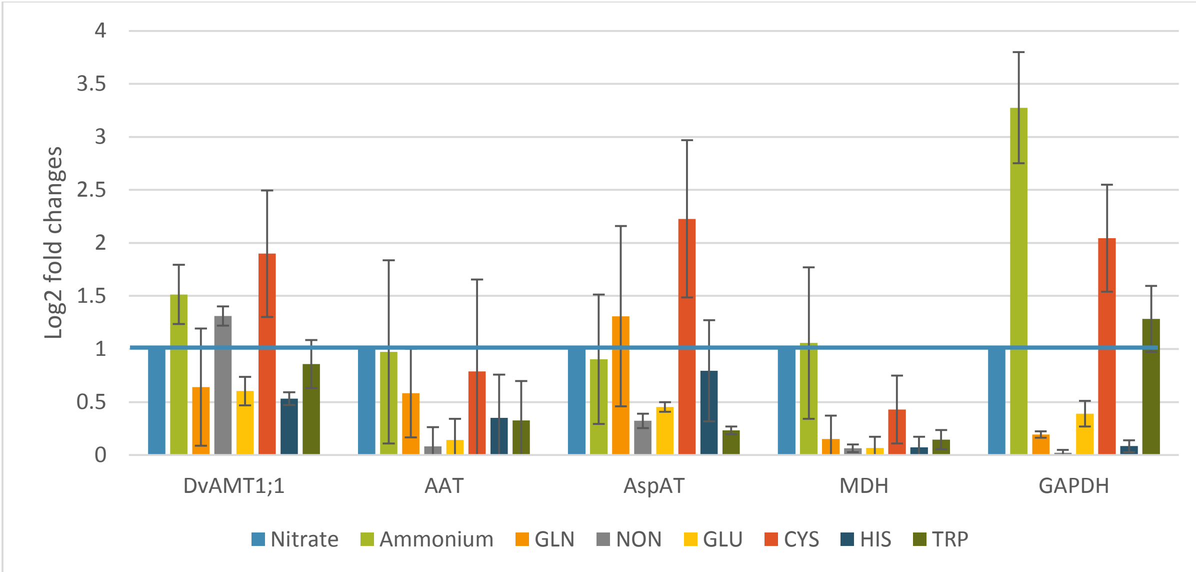

Figure 2.22. Transcript abundances of five genes in *Dunaliella* obtained from quantitative PCR of RNA. All values are fold changes in transcript levels with Actin as a reference gene and nitrate as the control condition. All nitrate responses have been normalized to one-fold, and the other conditions correlate to those results. Error bars represent standard deviations from three biological replicates except for glutamate, histidine, and tryptophan, which are biological duplicates. The horizontal line defines the control level of transcript abundance.

2.4. DISCUSSION

Based on the results detailed above, the biochemical composition of *Dunaliella* under different nitrogen source growth conditions was quantified. Further, amino acid analysis of proteinaceous amino acids provided details of protein composition changes when the alga is provided with amino acids as the N source and analysis of free amino acids provided insight into metabolic processes regulated under different nitrogen conditions. In addition to this, quantitative PCR analysis of seven transcripts for key metabolic enzymes provided additional information regarding the metabolic response of the algal cells to different nitrogen sources. These data were collated to generate the following broad observations regarding the physiology and metabolism of *Dunaliella* under these conditions.

2.4.1. *Dunaliella* Biochemical Composition Analysis

The highest protein fractions were observed in cultures grown in nitrate and amino acids after 144 h, and in nitrate, glutamine, and tryptophan after 48 h. High protein content was found to coincide with a lower carbohydrate fraction. When taking into account the cell densities, samples grown with nitrate, tryptophan, and cysteine were found to be the most actively dividing. Carbohydrate content as a percentage of total mass of the four fractions (proteins, carbohydrates, lipids, chlorophyll) was found to be the highest in samples grown in ammonium after 144 hours, and in glutamate and histidine after 48 hours. Carbohydrate content was found to generally have a higher fraction at 48 hours, compared to 144 hours, and this is consistent with what has been reported previously for 9 different photosynthetic microalgal species.[63] In *Chlamydomonas* grown under light:dark conditions, it was found to be an indication of early photosynthetic carbon fixation that maintained enough carbohydrate content, but in this study, the *D. viridis* cultures were grown in continuous light, preventing starch degradation. The chlorophyll content was found to be diminished after 48 hours in the samples which accumulated carbohydrates the most – nitrogen-deprived, glutamate, and histidine. After 144 hours, the culture that yielded the highest carbohydrate content was the one grown on ammonium, but those samples contained a low chlorophyll content – less than half the chlorophyll content compared to nitrate grown samples. Ammonium assimilation requires fewer electrons than nitrate assimilation, and nitrate assimilation utilizes a large portion of the electrons generated during photosynthesis. The cells may have a reduced demand for harvesting light when grown on ammonium, hence showing a lower chlorophyll content. Reduction of chlorophyll biosynthesis and accumulation of carbohydrates may also be signs of nitrogen deprivation[63,64], hence these conditions – glutamate, and histidine could be seen to be mimicking nitrogen deprivation-like physiology as early as 48 hours, while samples grown in ammonium might be tending towards nitrogen deprivation-like conditions later. Carbohydrates are often the main storage molecule for algae.[64] Except for cysteine-grown samples, an inverse relationship between protein and carbohydrate content was observed. It has been reported for other photosynthetic microalgae that an initial buildup of starch may occur during N-limitation, which gives way to lipid biosynthesis once nitrogen deprivation begins.[26,63]

The ecological significance of this may be that carbohydrates are more easily channeled into the metabolic pathways for protein biosynthesis once nitrogen limitation is lifted in a natural habitat, thus serving as a suitable storage molecule under stress conditions, but not true deprivation. True deprivation may then induce lipid biosynthesis as a longer term survival option.[63] Chlorophyll content has been found to correlate with protein content in plants due to the requirement for N in RuBisCO.[65] This is found to be true for samples harvested at both time points, with the protein and chlorophyll trends correlated to $R^2 > 0.70$ in both cases. No correlations were found between chlorophyll and carbohydrate levels.

Samples grown in glutamate and histidine also showed the lowest cell densities, whereas samples grown in ammonium only showed slightly lower cell densities compared to nitrate grown cells. Lower growth rates when cultured with histidine as an N source has been reported for *D. tertiolecta* as well.[10] Samples grown with cysteine and tryptophan had the highest growth rates among amino acids, comparable to the inorganic sources nitrate and ammonium.

Table 2.2. Biomass constituents as a percentage of total mass of the three fractions (proteins, lipids, carbohydrates) after 144 hours of growth. Standard deviations represent three biological replicates with three technical replicates each.

	KNO_3	NH_4Cl	Glutamine	All AA	No N
Lipid %	2.1 ±1.1%	3.1 ±1.4%	2.9 ±1.6%	4.3 ±2.0%	9.1 ±0.4%
Protein %	22.2 ±1.2%	10.9 ±0.9%	14.5 ±0.6%	36.2 ±6.9%	10.7 ±0.3%
Carbohydrate %	75.2 ±2.1%	85.8 ±1.9%	82.2 ±2.0%	59.0 ±8.7%	79.9 ±0.6%

Table 2.3. Biomass constituents as a percentage of total mass of the four fractions (proteins, lipids, carbohydrates, chlorophyll) after 48 hours of growth.

µg/million cells	KNO_3	NH_4Cl	Gln	No N	Glu	Cys	His	Trp
Protein %	31.2%	28.0%	40.3%	11.4%	9.6%	17.2%	7.6%	35.1%
Carbohydrate %	64.7%	68.0%	53.6%	83.2%	86.7%	80.7%	89.0%	63.7%
Lipids %	3.0%	3.0%	5.0%	5.0%	3.4%	1.6%	3.1%	0.4%
Chlorophyll %	1.11%	0.96%	1.00%	0.28%	0.28%	0.39%	0.25%	0.72%

2.4.2. Ammonium is the Most Efficient N Source for Growth

Experiments to ascertain the optimal ammonium concentration for this species found that ammonium levels greater than 0.5 mM restricted cell growth, while ammonium levels greater than 1 mM were severely detrimental to growth. Thus, it is plausible that the low ammonium concentration results in nitrogen depletion earlier than samples grown in other conditions which contained 5 mM of each nutrient. Finally, it can be summarized that cells grown with 0.5 mM ammonium initially behaved physiologically as healthy cells, comparable to nitrate or tryptophan, and subsequently mimic nitrogen deprivation conditions towards the end of 144 hours by accumulating lipids and carbohydrates. This is corroborated by the amino acid analysis, which quantifies dissolved ammonium as well. There is a reduction in ammonium by over 20 times from 48 hours to 144 hours in ammonium-grown samples, to near negligible levels (<0.78 µM), confirming that ammonium is practically depleted by that time point. It is proposed that ammonium, whether inorganic or organically-derived via amino acid oxidation, would be a good candidate as primary nitrogen source for the batch culturing of this microalga, as it requires the least amount to grow successfully, which allows for timely self-limitation towards the end of the log phase, which can auto-induce a lipid accumulation phase by virtue of nitrogen deprivation.

It is not surprising that a low concentration of ammonium is suitable for growth of this alga. It is a marine microalga, and marine environments generally have low, and rapidly changing ammonium concentrations.[1] However, ammonium has also widely been considered to be the more preferential nitrogen source for photosynthetic microalgae, despite the concerns arising from ammonium toxicity.[29,66,67] These concerns are heightened for *D. viridis,* as it thrives at high pH, reaching almost pH 9 towards the end of 144 hours in all shake flask cultures (grown without pH control). High pH leads to higher dissolved ammonium, and disassociation of ammonium.[68] This ammonium toxicity has been shown to be caused by futile cycling of ammonium ions in and out of the cells using active transport systems, wasting energy that could have been used for other cellular processes.[67] Amino acid analysis performed in this study showed that the ideal ammonium concentration of 0.5 mM did not promote this futile cycling, and led to a gradual decrease in extracellular ammonium.

The relative expression abundances of seven genes were analyzed using qPCR. DvAMT1;2 was found to be upregulated in ammonium, under nitrogen starvation, glutamate, histidine, and tryptophan when compared to nitrate whereas DvAMT1;1 was upregulated in ammonium, nitrogen starvation, and cysteine. The study by Song et al to characterize *D. viridis'* ammonium transporters found that the transporters were found to be higher in nitrate-grown cells. While this was not reported in this study for DvAMT1;1, it was seen in DvAMT1;2.[31] In a separate study on the marine diatom *Cylindrotheca,* it was found that mRNA levels for a sequentially similar ammonium transporter were higher in nitrogen-starved cells, followed by nitrate grown cells, and finally in ammonium grown cells.[69] Those results are mirrored in this study for DvAMT1;2. Additionally, the effects of organic nitrogen sources are reported in the present study as well wherein there is an upregulation of DvAMT1;2 transcription in glutamate, histidine, and tryptophan. This data reflects the current understanding in transporter transcription that they are induced in the absence of their substrate, to promote scavenging responses to find the most optimal nutrient[70], in this case, ammonium, as previously established. It is worth noting, that the magnitude of difference in response of AMT1;2 between the control and tests is much larger than the magnitude of differential response for AMT1;1. Thus, it is possible that one of them is a primary transporter and the other has a secondary function – such as for sensing or internal transport. Their divergent sequences, which share very little in common, also point towards either different localizations or different functions – one may be plasma membrane localized and the other to the chloroplast envelope. Song et al (2011) report that AMT1;1 is diurnally controlled while others found that AMT1;2 is responsive to salt stress.[71]

2.4.3. Nitrogen Starvation Promotes Lipogenesis

Metabolic changes due to nitrogen deprivation have been studied widely due to its promotion of economically valuable lipid and pigment accumulation.[64] Nitrogen deprivation causes carbon fluxes to be redirected to storage compounds such as lipids or starch, depending on the algal species and its environment. In *Chlorella,* both storage stratagems have been noted in various species.[53,72] In *Dunaliella tertiolecta,* lipid accumulation was observed in response to nitrogen starvation.[73,74] A study by Breuer et al noted 21 out of 96 phototrophic microalgal strains that exhibited lipid accumulation under nitrogen stress and increased the fraction of lipids in their biomass.[63] In the present study, after 144 hours, the lipid fraction was 9.1% of the total of proteins, lipids, and carbohydrates content at the cost of protein content, which was reduced to 10.7%, in cultures grown without nitrogen. (Table 4.1-1) In cultures harvested after 48 hours, the highest lipid fractions were found to be in nitrogen-starved cells and cells grown on glutamine. (Table 4.1-2) The lowest fractions were found to be cells grown on cysteine and tryptophan. Lipid accumulation has been found to be negatively correlated with cell growth at both time points, while the correlations of protein and carbohydrate fraction values with cell density are variable. At both time points, carbohydrate content remained stable when compared to nitrate as the control nitrogen source, suggesting that *Dunaliella* favors lipid accumulation and not carbohydrate accumulation as a nitrogen stress response.[74]

The highest response for NADH-GOGAT transcript accumulation was found to be in nitrogen-starved and glutamate-supplied cells, followed by histidine-grown cells. NADH-GOGAT forms a part of the GS-GOGAT pathway to convert ammonium into glutamate, more so for ammonium re-assimilated from protein degradation.[32,75] Hence, it is plausible that these conditions would increase their NADH-GOGAT transcription to scavenge and reuse amino acids from degraded proteins, as they all exhibit the signs of nitrogen starvation. NADH-GOGAT was, in any case, as or more abundantly expressed as compared to cells grown with nitrate in all conditions except for cells grown with tryptophan, where it was downregulated. No studies on tryptophan uptake have been performed on marine microalgae, hence there are no available explanations in the literature for this. However, combining this information with the findings towards healthy cellular functioning and high, stable protein content in tryptophan-grown samples allows for the hypothesis that cells grown with tryptophan do not require the services of NADH-GOGAT due to a lower rate of protein catabolism.

Cultures grown with glutamine had equivalent lipid accumulation levels compared to samples grown in inorganic nitrogen sources. This provides promising results towards the utilization of organic sources of nitrogen that can provide equivalent or even more favorable lipid yields for commercial production. Samples grown with tryptophan showed the least lipid production and an above average biomass yield. This suggests a more preferential uptake mechanism for this nitrogen source that involves fewer indicators of stress-related indicators. A comparative study of uptake rates with a mix of these amino nitrogen sources to ascertain the order of preference for uptake would be a good follow up study to confirm if tryptophan is more readily assimilated than the other amino acids. Based on the current information, glutamic acid and histidine almost mimic starvation conditions with only a single doubling of cell density occurring in 48 hours, compared to almost two doublings for all other samples.

2.4.4. Free Amino Acid Levels Indicate Photorespiration Activity

The amino acid analysis was performed on three fractions of the cell culture – extracellular free amino acids (ExAA), intracellular free amino acids (InAA), and proteinaceous amino acids (PAA). ExAA analysis yielded approximate uptake rates for the four amino acids tested. Tryptophan was found to be the most depleted from the medium, followed by cysteine, and glutamine. Glutamate and histidine were utilized the least. As mentioned previously, high ammonium levels were reported in glutamine samples, with a reduced glutamine fraction as well. Chemical degradation of glutamine over time in cell culture media has been reported[62], however, biological degradation by *D. viridis* has not been ruled out as well, unlikely as it may be due to no putative LAO1 transcripts having been found yet. Multiple unidentified peaks were observed in extracellular tryptophan samples, pointing towards photodegradation products of tryptophan. This has been implicated in 'photoyellowing' in wool which is similar to the yellow coloring obtained in the cell culture media after tryptophan addition.[76] Intracellular amino acid analysis found several amino acids with significantly different abundances among the different growth conditions. After 144 hours, cells grown in glutamine and nitrate had similar abundances for all amino acids, while ammonium-grown samples had generally lower abundances due to lower protein content per cell. An interesting result is the high glycine content in samples grown with nitrate. Free glycine is used as an intermediate for photorespiration and as a precursor for serine, hence the presence of glycine in large quantities might suggest a greater push for photorespiration as serine has been shown to act as a transcriptional signaling molecule for photorespiration.[77] Direct quantification of serine however was not reliable as there was interference with Tris-HCl, a buffer component in the medium, during the amino acid analysis which eluted at the same retention time as serine. Free tyrosine was also present at high levels in all samples. Free glutamate and alanine were also present in large quantities in all conditions, with more present in the conditions with higher protein composition. Glutamate and alanine are the first two amino acids synthesized after ammonium incorporation by the GS-GOGAT pathway. Alanine is a precursor to glycine and serine, hence it may be kept as a free amino acid for further biosynthetic purposes. Glutamate is used as an ammonium donor for almost all amino acid biosynthesis, hence it is available for those biosynthetic requirements. Proteinaceous amino acid content remained unchanged between the different nitrogen sources tested, suggesting that the presence of a particular amino acid does not increase the proportion of its use in the proteome of the cell.

Alanine aminotransferase and aspartate aminotransferase were studied to better understand the amino acid biosynthesis status of the cells. In plants, alanine aminotransferase has been targeted as a site for genetic engineering by upregulating its transcription to increase nitrogen use efficiency.[78] In *Arabidopsis,* it was found that alanine aminotransferase is linked to the photorespiratory pathway as well, specifically, linking glycolate oxidation and glycine synthesis.[79] In this study, alanine aminotransferase activity was found to be consistently downregulated in all conditions compared to nitrate. This upregulation of AAT activity in nitrate compared to the other test conditions, along with the high glycine content found in the InAA nitrate samples, provides a strong case for an upregulation in photorespiration in cells grown in nitrate. In *Arabidopsis* and C_3 plants, it has extensively been proven that high CO_2 and low O_2 levels depress nitrate assimilation.[80,81] The hypothesis linking photorespiration and nitrate assimilation is thus: Photorespiration promotes the efflux of malate out of the chloroplast into the cytoplasm, which generates NADH that powers the first stage of nitrate assimilation – the reduction of nitrate to nitrite.[80] Ammonium and glutamine samples also showed free intracellular glycine, and a comparable AAT expression to nitrate samples. In some cases in C_3

plants, ammonium supply was found to increase photorespiration as well. This hypothesis states that the NADPH which would have been utilized by nitrate assimilation would be available to use by photorespiration, thus promoting its effect.[82] It has been found that the usage of ammonium as the primary nitrogen source allows plants to utilize higher atmospheric carbon dioxide concentrations, thus presenting ammonium as a source less likely to trigger photorespiration than nitrate.[82] Aspartate aminotransferase transcripts were twice as high in cysteine as in nitrate samples, and slightly upregulated in glutamine-grown samples.

2.4.5. Nitrogen Source Affects Carbon Concentrating Mechanism Regulation

MDH and the Low CO_2-Inducible protein were evaluated to ascertain the links between nitrogen and carbon assimilation. Two processes are key in carbon assimilation in photosynthetic microalgae, the carbon-concentrating mechanism and fixation by RuBisCO. RuBisCO catalyzes the fixation of carbon dioxide via the Calvin-Benson-Bassham cycle, and makes it available to the cell. It is a slow and inefficient enzyme, which cannot reach maximum efficiency on its own in the low CO_2 environment of marine habitats. To improve CO_2 assimilation efficiency, photosynthetic algae have developed a mechanism by which inorganic carbon is assimilated into the cell and stored as bicarbonate ions, clustering close RuBisCO, enabling the enzyme to function closer to optimal efficiency.[83,84] New evidence has also suggested that RuBisCO may not be as abundant in algae as it is in vascular plants, where it often accounts for over 50% of the protein content.[85] In a recent study, it was found that RuBisCO accounted for less than 10% of the total algal protein content, which is far less than previously theorized.[86] *Chlamydomonas* has developed regulatory mechanisms to control the carbon-concentrating mechanism via proteins encoded by the LCI protein family. The putative protein from that family studied in this experiment in *Dunaliella* has the closest homology with *Chlamydomonas'* LCID and the multicellular green alga *Volvox's* LCIB protein. LCID's function is currently unknown but may be related to inorganic carbon uptake.[87] In this study, it was found that nitrate promoted the transcription of the probable LCID transcript, followed by glutamine, nitrogen starvation, and ammonium. It is difficult to estimate what effect this transcript promotes, but considering that nitrate probably promotes photorespiration, it would be strange for it to promote the carbon concentrating mechanism as well. Hence, LCID's function may be as a signaling or regulatory protein preventing the carbon concentrating mechanism from activating, or repressing its function in some way.

Malate dehydrogenase catalyzes the production of malate, a component in the malate-aspartate shuttle that provides reducing equivalents in the mitochondria. MDH transcription is downregulated in all organic samples, with the highest transcription found in nitrate and ammonium-fed samples. In *Chlamydomonas reinhardtii,* NADP-MDH is found clustered with nitrate assimilation genes.[44] This is theorized to be due to the high requirements of reducing equivalents during nitrate assimilation.[44] A hypothesis for the low MDH transcription in the case of amino acid assimilation may be due to the fact that amino acid assimilation requires oxidative equivalents more to cleave the ammonium off the amino acid backbone[44], and the reduced requirement for reducing equivalents. The activation of higher plant NADP-MDH is also known to be inhibited by oxidized cofactors.[88]

2.5. CONCLUSION & FUTURE WORK

The biochemical composition of *D. viridis* was elucidated under different nitrogen conditions. The protein, lipid, carbohydrate, chlorophyll, and amino acid contents were quantified at two time points, at the beginning and at the end of the log phase. After 144h of growth, five conditions were compared – nitrate, ammonium, glutamine, N-starvation, and on a mix of all 20 amino acids. After 48 h of growth, eight conditions were compared – nitrate, ammonium, glutamine, glutamate, N-starvation, cysteine, histidine, and tryptophan. Additionally, at the 48 hour time point, transcript abundances of seven key genes involved in nitrogen metabolism were compared – two ammonium transporters, NADH-GOGAT, alanine aminotransferase, aspartate aminotransferase, malate dehydrogenase, and a low CO_2 inducible (LCI) protein, presumably identified as LCID.

It was found that after 144 h of growth, cells supplied with nitrate or glutamine had the highest cell densities and cellular protein contents. Ammonium-grown cells had presumably depleted the medium of ammonium at 144 hours and had begun to show the signs of nitrogen starvation by decreasing the protein content, and increasing the lipid and carbohydrate content. N-starved cells induced the highest lipid production as a percentage of total metabolites and on a per gram biomass basis. Additionally, incorporating the qPCR results with the biochemical analysis yielded the following results, tabulated in Table 2.4.

Table 2.4. Consolidation of key findings from this study.

Factor	Tested By	Hypothesized Response	Actual Response
C allocation to major components	Protein/Lipid/ Carbohydrate Assay	Some nitrogen sources promote lipid accumulation at the cost of protein/carbohydrate content	His, Glu, NoN promote lipogenesis; Trp, nitrate, Gln promote protein, ammonium median, Cys promotes carbohydrate
	GOGAT activity	Protein degradation is a response to unfavorable nitrogen conditions	High GOGAT activity and low protein content in His, Glu, NoN: opposite in Trp, Cys, nitrate; median in ammonium, Gln
C Assimilation/ Photosynthesis	Chlorophyll	Higher chlorophyll in sources with more C-assimilation from photosynthesis	Sources with low growth had low chlorophyll.
	LCID activity	Low CO2 concentration regulator, unknown function, no hypothesis	Nitrate promotes LCID transcription
	Total macromolecular content	Total content of biomass reflects total C assimilated from photosynthesis and nitrogen source with C	Ammonium had the highest dry weight after 144h; Cys and Trp had highest DW after 48h
Growth rate	Cell Density/ Dry Weight	lower growth rate leads to higher lipid productivity	Conditions with lower growth had higher lipid concentration.
Photorespiration	Glycine and Alanine levels	high free glycine and alanine indicate photorespiratory pathway activated	High Glycine in nitrate, ammonium, Gln low in rest. High alanine in nitrate, ammonium, Gln, Trp
	AAT transcription	High AAT transcription shows higher free alanine generated for the same.	Downregulated in all conditions except nitrate and ammonium.
Nitrogen Limitation	Biochemical analysis without N	N limitation inhibits growth and promotes lipogenesis	N limitation promoted lipogenesis and inhibited growth

Considering the results from the preliminary assessment of ammonium concentrations on *Dunaliella* growth, it is clear that a low concentration of 0.5 mM ammonium is sufficient to achieve growth results comparable to inorganic nitrate at 5 mM and the best amino acid sources. Additionally, this low concentration enables a possible growth regime that self-induces an N-limited lipid accumulation phase, if biofuel production was the aim of a large scale cultivation. A tightly controlled feeding strategy with ammonium may lead to the ideal level of growth and lipid production.

Microalgae have to adapt their cellular functioning to their environmental conditions, and some conditions tend to be more favorable than others. This study has shown that select amino acids and ammonium can provide N for large scale cultivation of *Dunaliella.* However, concerns remain about the efficacy of mixed-amino acid feeds, as what a hydrolyzed biomass-based nutrient feed would be. The next step to study the possibility of recycled biomass as a nitrogen source for this alga would be to study the effects of a mixture of these amino acids together as a nitrogen source, to discover and delineate any cross-regulation. Although cultures fed all 20 amino acids at 5 mM was included in the 144 hour study, the results of those samples could not be traced back to the effects of the main affecting amino acids, and the results from those samples were inconclusive. Now with the understanding of the effects of the most important individual amino acids, further tests with combinations of these and pilot studies on hydrolyzed biomass-based sources can be implemented to further the path towards fully sustainable algal biofuels.

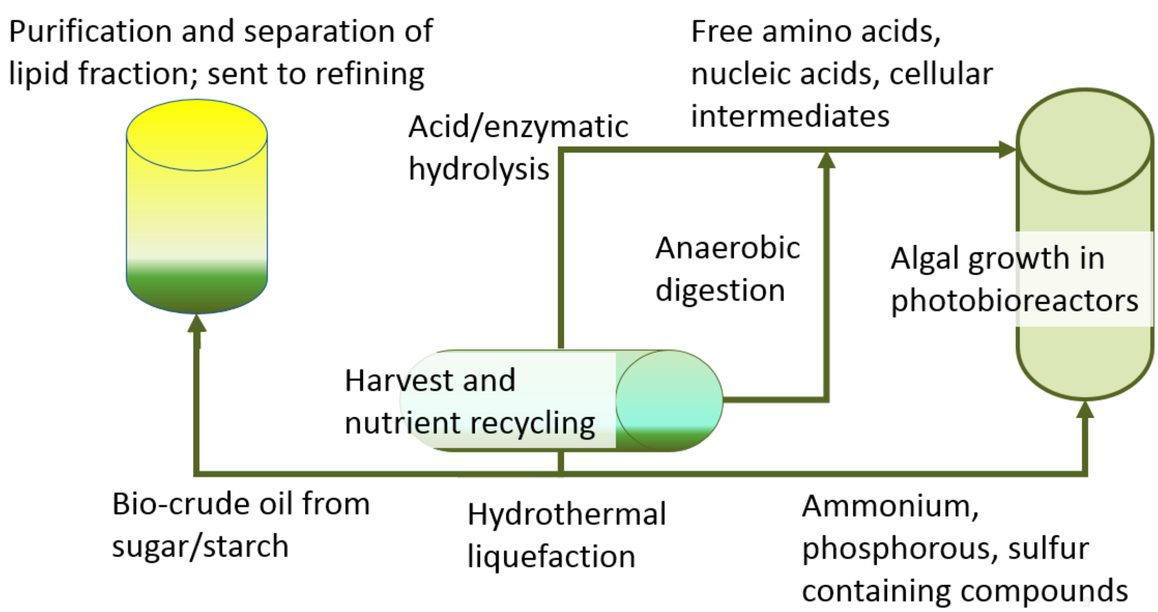

Figure 2.23. Methods of recycling algal biomass to obtain bioavailable nutrients in an algal biorefinery.

Nutrient recycling from spent algal biomass can be achieved in various ways.[12] (Figure 2.23) Once the lipid fraction is removed from the harvest culture, the remaining biomass can be passed through hydrothermal liquefaction (HTL) to produce a crude oil – like mixture of hydrocarbons that can then be processed further to produce refined oils. The HTL process produces an aqueous phase containing nitrogen, phosphorous, and other micronutrients that can then be recycled. Besides HTL, anaerobic digestion has been suggested as an energy efficient method for recycling nutrients from spent algae.[89] Enzymatic hydrolysis, either alone or in combination with solvent extraction has been researched as potential ways to recycle algal biomass.[16,90] These techniques could also potentially lead to accumulation of inhibitory products, and further work needs to be done to ascertain the extent of repeatability and efficacy of each of these techniques.

Improving amino acid utilization is another area of further development of this research. Only four amino acids are utilized by *Dunaliella viridis* naturally and a mixture of all 20 amino acids resulted in slow growth and did not promote lipid formation. As ammonium was found to be a good nitrogen source, future work on extracellular L-amino oxidases for the extracellular oxidation of amino acids into ammonium may be a potential path forward for the improvement of this technology. Keeping in mind that high levels of free ammonium causes cellular toxicity as well, the recycled N content must be regulated accordingly, such as in a fed-batch growth regime. The four amino acids currently utilized by *Dunaliella* may be taken up via active transporters, and thus future work in genetic engineering of *Dunaliella* to express more diverse amino acid transporters may also be a potential path forward. (Figure 2.24)

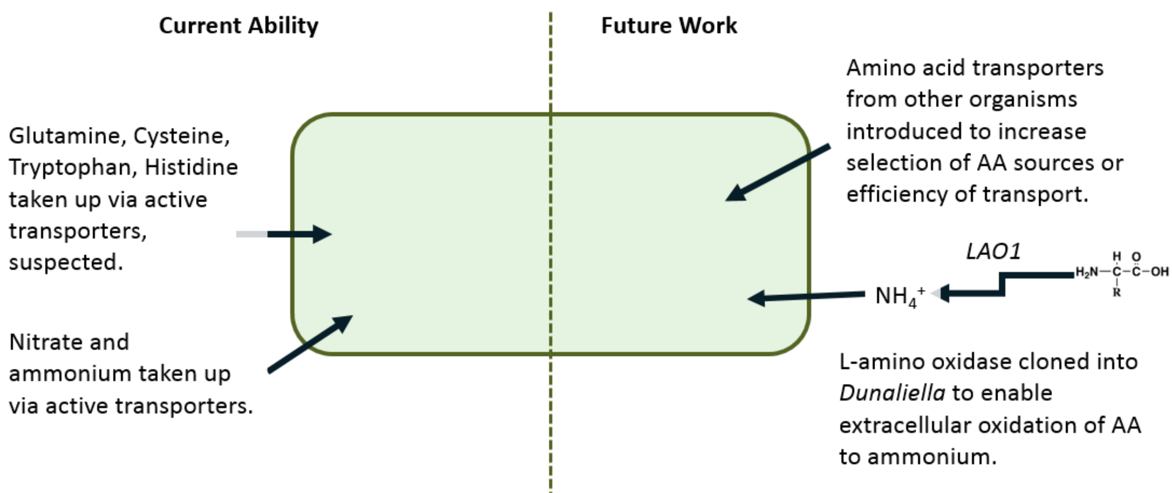

Figure 2.24. Current and future work related to *Dunaliella viridis* to be carried out.

Based on these results, it can be surmised that *Dunaliella viridis* has the potential to be grown on hydrolyzed algal biomass, due to its ability to grown on amino acids or ammonium as a sole nitrogen source along with evidence of growth on a mixture of all 20 amino acids. Combined efforts to produce a hydrolysate via one of the methods mentioned above coupled with extending the ability of *Dunaliella* to assimilate organic nitrogen will help to enable this alga to be utilized in a nutrient-recycling biorefinery in a sustainable way.

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APPENDIX

2.7.1. Protocols

2.7.1.1. Wang's Media Preparation

Table 2.5. Medium composition used for all experiments. *Provided together as a part of a metal stock solution prepared at a 1000X concentration.

Nutrient	Final Concentration
NaCl	1M
$NaHCO_3$	50mM
$MgSO_4.7H_2O$	5mM
KH_2PO_4	0.12mM
EDTA	6µM
$FeCl_3$	2µM
$CaCl_2.2H_2O$	0.12mM
$MnCl_2$	7µM
***$ZnSO_4$**	1µM
***$Co(NO_3)_2$**	1µM
***$CuSO_4$**	1µM
***$(NH_4)_6Mo_7O_{24}$**	1µM
Tris-HCl	10mM

Supplemented with a nitrogen source from the table given below.

Table 2.6. Nitrogen sources studied in the 144 hour experiment. In addition to these, a set of no nitrogen negative controls were tested as well.

Nitrogen source	Concentration
Potassium Nitrate	5 mM
Ammonium Chloride	0.5 mM
Glutamine	5 mM
All 20 amino acids	5 mM each

Table 2.7. Nitrogen sources tested in the 48 hour experiment. In addition to these, a set of no-nitrogen negative controls were tested as well.

Nitrogen source	Concentration
Potassium Nitrate	5 mM
Ammonium Chloride	0.5 mM
Glutamine	5 mM
Glutamate	5 mM
Cysteine	5 mM
Histidine	5 mM
Tryptophan	5 mM

2.7.1.2. Carbohydrate Analysis Protocol

1. 200mg/mL glucose solution was prepared in distilled water followed by 1/10 serial dilutions to obtain 200 μg/mL.
2. The 200 μg/mL solution was used to make standards in the range of 5 μg/mL to 150 μg/mL using distilled water.
3. Samples harvested after 144 hours were diluted 1:2 (v/v) with distilled water to obtain values within this standard curve's range.
4. 50μL of control or sample was aliquoted into a clear polystyrene microplate under a fume hood.
5. 50μL of 5% (w/v) phenol was added to each well using a multichannel pipette. The plate was incubated at room temperature for 10 minutes.
6. 250 μL of 94% sulphuric acid was added and immediately mixed 10 times upon addition to induce color generation. Proper and rapid mixing at this step is crucial for consistent results.
7. The previous reaction is highly exothermic, so the plate was subsequently cooled for 15 min at room temperature.
8. After cooling, the plate was measured on a spectrophotometric plate reader with absorbance wavelength set at 490 nm.
9. The Absorbance values for the standard curve samples were plotted and used to measure the carbohydrate content of the unknown samples.

2.7.1.3. Protein Analysis Protocol

1. 1 mL of culture was aliquoted for protein analysis and centrifuged at 13000 rpm for 5 min.
2. The pellet was frozen at -80°C until protein extraction.
3. 300 μL of deionized water was added for osmotic lysis of the cells. The pellet were resuspended in the water by repeated pipetting.
4. After resuspension of the cells, they were recentrifuged at 13000 rpm for 5 minutes at 4°C.
5. The supernatant was collected and stored at 4°C for further processing as the aqueous protein fraction.
6. 300 μL of 0.1 N NaOH was added to the pellet to leach out protein from the remaining cellular debris.
7. After resuspension, the solution was incubated for 1 hour at room temperature with intermittent vortexing.
8. After 1h the solution was recentrifuged at 13000 rpm for 5 minutes at 4°C.
9. The supernatant was collected and combined with the supernatant stored in step 4, and store at 4°C until assay. This is then labeled "crude extract". Generally, the assay is performed on the same day.
10. Protein assay was carried out using the Micro BCA^{TM} protein assay kit (Thermo scientific, catalog number: 23235) by measuring absorbance at 562 nm.
11. In a microplate, 150μL of standard or unknown sample was added in triplicate.
12. 150μL of working reagent prepared using the instructions provided by the manufacturer, was added to each well and mixed thoroughly for 10 seconds.
13. Cover plate using sealing tape for 96 well plates ad incubate at 37°C for 2 hours. The palte was cooled to room temperature before being measured using a plate reader.
14. Measure the absorbance at 562 nm on a Synergy HT multi-mode microplate reader.

15. To obtain the readings for the samples, the average absorbance reading of the blank was subtracted from the average standard/unknown sample absorbance using the three technical replicates.
16. A standard curve was prepared by plotting the average blank corrected 562 nm reading for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of the unknown sample.
17. *Standard curve preparation:* Stock BSA concentration: 2mg/mL. Different BSA concentrations from the stock for standard curve generation using 1X PBS – pH 7.4 [137 mM NaCl, 2.7mM KCl, 8mM Na_2HPO_4 and 2mM KH_2PO_4]. The following concentrations were prepared used for standard curve generation: 0, 2.5, 5, 10, 20, 40, 80, 120, 160, 200 (µg/mL)
18. *Working Reagent:* working reagent was prepared by mixing 25 parts of reagent MA and 24 parts reagent MB with 1 part of reagent MC (25:24:1).

2.7.1.4. Lipid Analysis Protocol

1. Standard Preparation

Tween-20 Preparation

Different percentages of fresh Tween-20 were prepared before the start of the experiment according to the recipes given below:

Table 2.8. Tween solutions preparation.

Tween-20 (%)	Volume of Tween 20 used	Volume of 1M Wang media used (mL)
1% (25 mL total)	0.25 mL Tween 20 from bottle (Fisher)	24.75
0.2% (20 mL total)	4 mL of 1% Tween 20	16
0.05% (10 mL total)	2.5 mL of 0.2% Tween 20	7.5
0.01% (50 mL total)	2.5 mL of 0.2% Tween 20	47.5

Coconut oil was used as triacyl glyceride standard because of its similarity to *Dunaliella's* TAG composition. The following table was used to generate the standards for curve generation using coconut oil and tween-20.

Table 2.9. Coconut oil emulsion with tween-20 preparation for lipid assay standard curve.

Oil concentration (µg/mL)	Preparation	Sonication needed	Observation before sonication	Observation after sonication
2000	0.01g/5 mL using 0.2% Tween-20	Yes (20 mins, setting 5)	Clear	Milky
500	1.25 mL from 2mg/mL + 3.75 mL from 0.05% Tween-20	Yes (20 mins, setting 5)	Clear	Milky
100	1.2 mL from 500 µg/mL + 4.8 mL from 0.01% Tween-20	No		

80	4.8 mL from 100 µg/mL + 1.2 mL from 0.01% Tween-20	No
60	4.5 mL from 80 µg/mL + 1.5 mL from 0.01% Tween-20	No
40	4 mL from 60 µg/mL + 2 mL from 0.01% Tween-20	No
20	2 mL from 40 µg/mL + 2 mL from 0.01% Tween-20	No
10	2 mL from 20 µg/mL + 2 mL from 0.01% Tween-20	No
5	2 mL from 10 µg/mL + 2 mL from 0.01% Tween-20	No

2. Sample Preparation

1 mL of the prepared oil in detergent solution (5 µg/mL through 100 µg/mL) or harvested cell culture sample was taken and mixed with 4µL of Nile Red. For the blank, 1 mL of 0.01% tween-20 was mixed with 4µL of Nile Red**.** Vortex briefly.

3. Plate Preparation

200 µL of prepared standard/sample solution was added to each well. Each sample/standard was plated in triplicate.

Plate type	**Costar 96 black opaque**
Read	Fluorescence end point
Filter set	Excitation: 485/20, Emission: 590/35
Gain	35
Optics	Top

2.7.1.4. Amino Acid Analysis Protocol

1. Sample Harvest

1. Harvest 40 mL of cell culture and spin at 3500 rpm for 15 min.

2. Remove 35 mL of media and resuspend the cells in the remaining ~5 mL.

3. Transfer 1 mL to an Eppendorf tube and spin at max speed for 10 min.

4. Transfer supernatant to a fresh tube and label as ExAA (extracellular amino acids).

5. Add 400 µL distilled water to lyse cells, resuspend and freeze at -20/-80°Ctill further processing.

2. Acid Hydrolysis

[For Intracellular free amino acids, skip to part C.]

To get hydrolyzed proteinaceous amino acids, thaw and resuspend the sample stored above and:

1. Turn on a heating block to 105℃.

1. Add 50 µL of the stored sample to 200 µL of 6N HCl w/ 0.02% B-mercaptoethanol in the glass hydrolysis tube.

2. Close the cap on the hydrolysis tube until it just starts getting tight, then pull a vacuum on it using a pump.

3. Place tube on heating block for 20 hours.

4. Remove tube, open cap and neutralize with 200 µL 6N NaOH.

5. Decant contents into an Eppendorf tube and spin for 10 minutes to settle the debris

6. Pipette the supernatant into a new tube and store at -20℃ until sample preparation.

3a. UPLC Sample Preparation

For intracellular AA: spin down the 400 µL lysed cells sample stored above and use the supernatant directly. *For PAA:* Use the hydrolyzed sample (volume should be ~450 µL).

1. Dilute samples with distilled water. For the intracellular AA and ExAA samples, a 1:2 dilution and for the PAA samples a 1:4 dilution was done. For low cell densities (0-3 million cells/mL originally) 1:2 dilution is sufficient for hydrolyzed samples if they are clear enough.

3b. Standard Preparation

Generate standards for the assay using amino acid standards Sigma Aldrich AAS18 or AA88212. The AAS18 contains standards for 17 AA + ammonium. Also prepare 10 mM standards for Asparagine, Glutamine, and tryptophan. The AAS18 can be stored in the fridge, store the other three in the freezer.

The standards used are in the range of 50 pmol/µL to 0.78 pmol/µL in their final form. So the peak areas obtained will be plotted against 50-0.78 and used to calculate the unknowns.

1. Thaw the standards and mix before use.

2. Label eight 0.5 mL tubes 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, and Blank.

3. Add 162.5 µL water to the tube labeled 50 and 100 µL to the rest.

4. To the “50” tube, add 50 µL of AAS18 and 12.5 µL each of Gln,As, and Trp standards for a total volume of 250 µL.

5. Perform a two-fold dilution series down to 0.78. Blank contains 100 µL distilled water.

3c. AccQ Tag Derivitization

Protocol retrieved from: http://www.waters.com/webassets/cms/support/docs/715001331.pdf

Follow the protocol above to reconstitute the derivatization reagent AccQ. Take care to not heat the vial for longer than 15 min during the reconstitution. Vortex every five minutes. Make sure the powder is completely dissolved. This reagent must be used up in a week or it starts giving failed reactions. The reagent is reconstituted with 1 mL of the solvent, and 20 µL is needed for each reaction so one vial has enough material for 50 samples/standards.

1. Add 70 µL borate buffer to the glass vials.

2. Add 10 µL of sample or standard to the glass vials. Vortex.

3. Add 20 µL of reconstituted derivatization reagent. Immediately pipette up and down 10 times.

4. Check vials for bubbles. Tap vials to remove bubbles; there shouldn't be any inside the liquid, especially in the well at the bottom of the vial. The liquid should completely fill the thin well at the bottom.

5. Check vials for debris, particles, and thin crystals. Debris or particles come from the sample, so the samples may have to be cleaned up by centrifugation some more. Thin wispy crystals are from the derivatization reagent not being mixed properly. Vials must contain clear - bubble and debris free – liquid.

6. Run samples on Waters UPLC system in reverse phase mode.

2.7.1.5. Quantitative PCR Data

Table 2.10. List of primers used for qPCR analysis. GOI - Gene of interest; REF - reference genes (GAPDH and Actin)

GOI/REF		Primer Sequence
DvAMT1;1	Forward	5' GATCTAACCCAGGAGTACAA 3'
	Reverse	3' ATTTGTCCCATTCTCCTT 5'
DvAMT1;2	Forward	5' GATTTTCACTGGCTTCTTC 3'
	Reverse	3' CAGCAGAAAGAAGTTGATG 5'
NADH-GOGAT	Forward	5' AAGGACTTCTACATGATGGT 3'
	Reverse	3' ATCCTTGGTCACATAGTAGC 5'
MDH	Forward	5' AGTGGTTCAAGGAGGAGT 3'
	Reverse	3' GCCAGAAGAGAAGCAGTC 5'
DvAAT	Forward	5' AGAACATCTACCAGGATGAG 3'
	Reverse	3' CACTTGTAAATCTCCTCGAT 5'
DvAspAT	Forward	5' TACGATGAGGTCAAGATGA 3'
	Reverse	3' ATGTAGATGCTGTGGTTGT 5'
Low CO_2	Forward	5' CTCTGTCTGTCGATGACTAC 3'
	Reverse	3' CACACTCTCAATCTTATCCTC 5'
GAPDH	Forward	5' ATGGCCACCTCAATGGCGAAG 3'
	Reverse	3' GCCACCACCTCCATCTTCAC 5'
Actin	Forward	5' CCCTGTTGCTGTGGCGGTGG 3'
	Reverse	3' GCGAGAGTTTGTGCTGCCTGATGG 5'

2.7.2. Preliminary Experiments

2.7.2.1. Investigation on Optimal Ammonium Concentration

The optimal ammonium concentration for *Dunaliella viridis* was determined. Increasing concentrations of ammonium were tested in a range of 0 – 5 mM ammonium in the form of ammonium chloride supplemented in the regular Wang's media used for the cultivation of this alga. The recipe for the media is provided in the Appendix Section 7.1 and is identical to the one adapted from the media described by Wang et al[91] in the protocol described by Srirangan et al.[36] 5mM ammonium chloride was found to repress growth completely, and even induce cell lysis with cell density reducing drastically in 96 hours after inoculation. To find the optimal growth concentration for ammonium, a concentration gradient experiment was set up with five ammonium concentrations tested from 0.1 mM to 2 mM. 4 mL cultures were grown with three technical replicates in 12-well plates with a starting cell density of 1 million cells/mL (other conditions consistent with the description provided below in section 2.2). This experimented was repeated twice more to obtain three biological replicates to ensure statistical validity. The results obtained are given below in Figure 2.25.

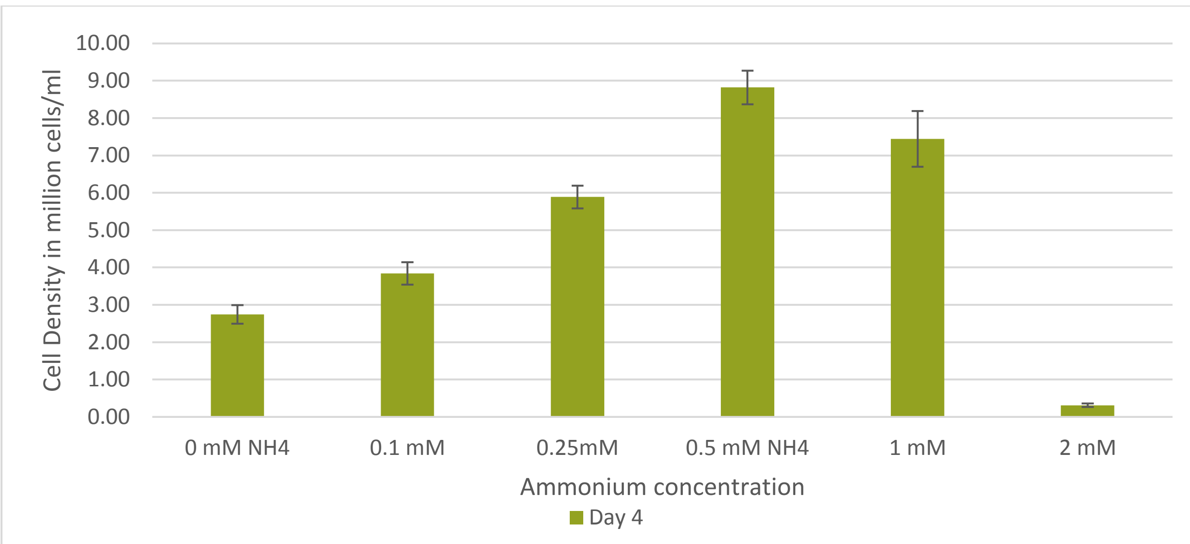

Figure 2.25. Growth of Dunaliella under different ammonia concentrations at the end of 96 h.

The results indicate an optimal ammonium concentration beyond which higher ammonium concentrations appear to be toxic to *Dunaliella.* The reasons for this toxicity have been discussed previously in Chapter 1 of this thesis in section 3.2.5. Thus, 0.5 mM ammonium chloride was selected as the concentration for ammonium to be implemented for further use in these experiments.

2.7.2.2. Amino Acid Analysis Method Development

Prior to beginning the experiments, different sample preparation methods for amino acid analysis were assessed to develop a robust amino acid analysis protocol. Initially, the sample preparation method utilized was similar to protein extraction (described in section 2.4), utilizing 0.1N NaOH to extract proteins to be used for amino acid analysis. This was found to lead to a reduced recovery of amino acids, hence alternative extraction protocols were tested to obtain the best sample extraction method for this analysis. Besides, extraction with NaOH, ethanol was tested, mimicking extraction protocol for chlorophyll, and finally extraction with just distilled water. The results of the tests is provided below in table 2.7-1. All values are averages of four independent samples of *Dunaliella* cultures grown in Wang's media supplemented with nitrate. Extraction with distilled water was found to give the highest extraction efficiency and least interference with the downstream amino acid analysis protocol, hence was implemented for the experiments described herein.

Table 2.11. Comparison of three extraction protocols for amino acid analysis. All values are in nmol/mL.

Name	*H2O*	*NaOH*	*Ethanol*
His	6.48	6.56	6.57
Ser	11.85	11.19	10.92
Arg	14.51	14.41	14.50
Gly	19.28	19.98	21.31
Asp+Asn	17.88	16.50	17.64
Glu+Gln	22.57	20.52	21.47
Thr	8.21	7.93	8.27
Ala	19.22	17.78	17.96
Pro	9.08	8.98	9.19
Cys	1.39	1.86	1.57
Lys	12.55	7.67	12.06
Tyr	12.69	12.65	11.83
Met	3.45	3.69	4.86
Val	9.99	9.33	8.90
Ile	7.33	7.25	7.12
Leu	14.02	13.76	13.79
Phe	12.37	12.31	12.16
Total	202.88	192.35	200.11

2.7.3.3. Amino Acid Analys Data

Table 2.12. Proteinaceous amino acid content after 144 hours in pmol/µg. All values are averages of three biological replicates. Tukey's HSD test was used to delineate significant sets of conditions for each amino acid. Conditions not connected by the same letter indicate significant differences (p > 0.05).

	KNO_3	NH_4^+	GLN	NON
His	273^{A}	67	216	144
Asn	1.54	2.85	0.09	0.66
Ser	1457	1200	1198	659
Gln	2.96	5.93	4.69	10
Arg	841^{A}	$567^{A,B}$	$665^{A,B}$	$360^{A,B}$
Gly	1917^{A}	1290	1409	778
Asp	2085^{A}	$1474^{A,B}$	$1587^{A,B}$	852^{B}
Glu	2702^{A}	$1912^{A,B}$	$2318^{A,B}$	1087^{B}
Thr	978^{A}	$715^{A,B}$	$752^{A,B}$	447^{B}
Ala	2379^{A}	$1711^{A,B}$	$1882^{A,B}$	1002^{B}
Pro	1096^{A}	603	872	500^{B}
Cys	109	31	23	25
Lys	1263^{A}	$879^{A,B}$	$954^{A,B}$	543^{B}
Tyr	600^{A}	505	480	247^{B}
Met	370^{A}	290	272	171^{B}
Val	964	763	724	463
Ile	574	376	425	278
Leu	1539	1156	1200	712
Phe	625	473	485	294
Trp	77^{A}	$41^{B,C}$	$63^{A,B}$	16^{C}

Table 2.13. Intracellular free amino acid content after 144 hours in pmol/μg. All values are averages of three biological replicates. Tukey's HSD test was used to delineate significant sets of conditions for each amino acid. Conditions not connected by the same letter indicate significant differences (p > 0.05).

	KNO	NH	GLN	NON
His	3.06	2.88	3.64	1.51
Asn	34^{A}	$16^{A,B}$	28^{A}	7.64^{B}
Ser	250	284	243	109
Gln	37	18	164^{A}	16
Arg	51^{A}	$34^{A,B}$	$60^{A,B}$	9.35^{B}
Gly	148^{A}	21	36	9.01
Asp	59^{A}	$26^{B,C}$	$41^{A,B}$	9.07^{C}
Glu	215^{A}	$129^{A,B}$	202^{A}	68^{B}
Thr	27^{A}	23	25	11^{B}
Ala	254^{A}	$147^{A,B}$	221^{A}	98^{B}
Pro	31^{A}	$17^{A,B}$	25^{A}	7.62^{B}
Cys	0.50	0.90	0.57	0.26
Lys	59^{A}	36^{A}	48^{A}	14
Tyr	147	185^{A}	126	65^{B}
Met	7.22	10	10	4.02
Val	27^{A}	$24^{A,B}$	28^{A}	8.60^{B}
Ile	$10^{A,B}$	$9.79^{A,B}$	10^{A}	2.00^{B}
Leu	24	23	25	8.33
Phe	13	13	14^{A}	4.34^{B}
Trp	4.31	3.95	4.66	1.89

Table 2.14. Proteinaceous amino acid and ammonium content in nmol/million cells after 48 hours. All values are averages of three biological replicates. Tukey's HSD test was used to delineate significant sets of conditions for each amino acid. Conditions not connected by the same letter indicate significant differences ($p > 0.05$). No significant differences were found for any amino acids between the different growth conditions at that level, as well as at ($p > 0.10$).

Name	KN	NH	GLN	NON	GLU	CYS	HIS	TRP
NH_3	60	84	180	75	57	84	108	87
His	3.81	4.56	0.49	0.41	0.75	2.14	5.79	2.87
Asn	0.06	0.05	0.00	0.00	0.00	0.00	0.07	0.06
Ser	28	41	34	30	28	24	22	30
Gln	0.01	0.05	0.13	2.62	0.02	0.01	0.20	5.80
Arg	13	17	14	7.14	5.22	11	7.02	23
Gly	29	36	30	11	13	24	15	26
Asp	27	34	31	15	14	24	14	35
Glu	35	44	51	20	23	31	19	46
Thr	13	16	15	7.60	7.86	12	7.76	18
Ala	34	44	39	19	16	29	18	44
Pro	13	17	17	8.95	5.76	14	8.88	20
Cys	1.30	1.14	0.42	0.65	1.13	2.07	0.06	1.07
Lys	21	27	22	11	9.73	21	11	30
Tyr	9.69	12	10	4.85	3.00	8.55	4.77	13
Met	4.79	5.30	4.84	1.87	1.82	4.63	1.80	5.59
Val	12	14	14	6.69	6.15	11	7.32	18
Ile	7.85	8.47	8.37	3.89	3.37	6.50	4.05	10
Leu	22	27	24	12	11	20	12	30
Phe	9.88	12	10	5.43	5.03	8.92	5.26	13
Trp	0.26	0.70	1.48	0.18	0.09	1.56	1.84	0.63

Table 2.15. Free amino acid and ammonium content in nmol/million cells after 48 hours. All values are averages of three biological replicates. Tukey's HSD test was used to delineate significant sets of conditions for each amino acid. Conditions not connected by the same letter indicate significant differences ($p > 0.05$).

Name	KN	NH	GLN	NON	GLU	CYS	HIS	TRP
NH_3	12	232^{B}	606^{A}	15	15	20	17	26
His	0.26	0.43	0.20	0.03	0.02	0.26	1.65	0.40
Asn	$2.57^{B,C}$	4.50^{B}	10^{A}	0.20	0.14	0.52^{C}	0.13	3.16^{B}
Ser	75	133	138	135	110	57	102	52
Gln	5.43	6.87	81	1.05	2.02	3.15	3.33	5.28
Arg	9.82	11	4.76	1.58	1.74	2.17	0.91	26^{A}
Gly	8.21	8.60	6.90	0.72	0.82	1.99	1.50	3.46
Asp	$4.48^{A,B}$	$5.31^{A,B}$	9.43^{A}	1.30^{B}	1.35^{B}	2.49^{B}	1.55^{B}	3.14^{B}
Glu	23	41	34	14	14	19	14	28
Thr	2.21	2.90^{A}	4.92^{A}	1.84	1.32	1.75	1.44	1.97
Ala	$28^{B,C}$	46^{B}	71^{A}	13^{C}	14^{C}	19^{C}	14^{C}	42^{B}
Pro	$5.99^{B,C}$	$9.58^{A,B}$	12^{A}	1.20	0.91	2.53^{C}	1.47	$5.95^{B,C}$
Cys	0.23^{A}	0.06^{A}	0.14^{A}	0.17	0.08	0.41	0.12	0.08^{A}
Lys	$7.38^{A,B}$	9.65^{A}	$5.66^{A,B}$	2.62^{C}	2.11	3.55^{B}	2.37^{C}	$6.38^{A,B,C}$
Tyr	$21^{A,B}$	31^{A}	27^{A}	6.82^{B}	8.03^{B}	$15^{A,B}$	8.76^{B}	28^{A}
Met	1.03	1.23^{A}	2.11^{A}	0.29	0.36	0.59	0.42	0.46
Val	3.23	3.62	4.67^{A}	1.88	1.52	1.77	1.45	2.15
Ile	1.37	1.18	1.92	0.84	0.59	0.65	0.64	0.53
Leu	2.90	2.62	3.92	2.08	1.63	1.47	0.94	1.28
Phe	$1.21^{A,B}$	$0.91^{A,B}$	1.94^{A}	0.33^{B}	0.51^{B}	$0.90^{A,B}$	0.68^{B}	0.74^{B}
Trp	0.30	0.55	0.25	0.09	0.04	0.20	0.15	1.44

Table 2.16. Standard deviations for proteinaceous amino acid content after 48h in nmol/million cells.

Name	Nitrate	Ammonium	Gln	No N	Glu	Cys	His	Trp
NH_3	8.97	10.06	16.98	30.49	22.11	67.73	93.94	32.93
His	2.78	3.92	0.31	0.30	0.25	1.84	8.82	1.43
Asn	0.06	0.09	0.00	0.00	0.01	0.00	0.06	0.11
Ser	11.77	25.34	11.29	7.13	14.98	15.76	5.15	17.08
Gln	0.02	0.06	0.23	4.43	0.02	0.01	0.32	9.65
Arg	4.31	7.83	4.34	9.77	5.06	9.58	2.23	9.06
Gly	9.71	18.51	10.21	10.74	9.05	15.27	4.79	27.69
Asp	9.65	16.21	10.04	6.38	6.18	17.20	4.39	19.72
Glu	10.63	20.25	11.41	7.95	9.22	22.08	6.08	25.17
Thr	3.74	6.62	5.26	3.09	3.55	10.52	2.62	11.22
Ala	12.76	23.24	13.53	8.26	9.29	20.03	5.79	24.70
Pro	1.97	6.31	4.57	4.37	4.39	9.44	2.80	10.52
Cys	1.31	0.78	0.41	0.16	1.06	3.19	0.02	1.41
Lys	12.60	21.07	14.84	6.48	10.90	22.44	7.32	27.12
Tyr	3.10	6.21	3.96	1.89	4.17	7.44	1.40	8.95
Met	0.57	1.01	1.63	0.87	1.37	3.44	1.46	5.59
Val	4.27	4.80	4.61	2.41	4.19	10.60	2.49	10.70
Ile	2.83	3.28	2.61	1.20	2.73	7.25	1.68	6.44
Leu	7.33	13.00	6.99	4.66	7.03	16.57	3.75	17.71
Phe	3.13	5.74	3.12	2.05	3.07	7.19	2.19	7.53
Trp	0.06	0.44	0.45	0.11	0.02	1.97	1.91	0.50

Table 2.17. Standard deviations for free amino acid content after 48h in nmol/million cells.

Name	Nitrate	Ammonium	Gln	No N	Glu	Cys	His	Trp
NH_3	2.04	28.34	104.11	7.82	6.07	15.65	2.94	16.62
His	0.24	0.09	0.33	0.04	0.03	0.24	1.56	0.32
Asn	1.25	0.80	1.67	0.14	0.15	0.55	0.11	0.35
Ser	23.49	35.07	13.15	27.92	6.76	7.86	35.38	9.14
Gln	1.64	1.76	42.33	1.61	2.12	0.99	0.98	1.31
Arg	4.16	4.72	1.89	2.27	1.87	0.59	0.68	11.51
Gly	4.68	1.23	0.74	0.68	0.68	0.55	0.13	1.45
Asp	2.41	1.09	3.74	0.95	1.26	0.62	0.74	0.59
Glu	6.29	9.87	8.69	0.93	4.64	2.49	2.11	4.67
Thr	0.63	0.49	1.94	0.64	1.12	0.23	0.08	0.64
Ala	5.17	6.79	15.18	3.32	4.54	8.61	2.92	8.08
Pro	1.96	1.99	2.54	0.21	0.72	0.72	0.17	0.99
Cys	0.20	0.02	0.07	0.07	0.09	0.42	0.10	0.07
Lys	1.90	2.04	1.18	0.61	1.18	0.71	0.52	2.27
Tyr	7.81	5.91	2.67	1.79	1.71	4.81	2.02	12.89
Met	0.25	0.63	0.73	0.25	0.16	0.10	0.14	0.16
Val	1.75	0.69	1.73	0.65	0.61	0.28	0.26	0.55
Ile	1.13	0.45	0.75	0.72	0.71	0.08	0.17	0.20
Leu	2.07	0.76	1.58	0.40	0.74	0.18	0.73	0.60
Phe	0.49	0.74	0.57	0.30	0.21	0.19	0.08	0.25
Trp	0.13	0.18	0.17	0.06	0.04	0.05	0.12	0.50