Understanding nitrate assimilation by eukaryotic green microalgae

PhD Thesis

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
ACCase	Acetyl-CoA carboxylase
ALMT	Aluminum-activated malate transporters
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BCAA	Branched-chain amino acid
CAH	Carbonic anhydrase
CD	Chemical denitrification
cGMP	Cyclic guanosine monophosphate
CIPK23	Calcineurin B-like protein-interacting serine/threonine-protein kinase 23
CLC	Chloride channel
CNX	Cofactor for nitrate reductase and xanthine dehydrogenase
Cytb5-R	Cytochrome b5 reductase
DCF	2′,7′-dichlorofluorescein
DCFH	2′,7′-dichlorodihydrofluorescein
DCFH-DA	2′,7′-dichlorodihydrofluorescein diacetate
DHAP	Dihydroxyacetone phosphate
ED	Electrodialysis
FAD	Flavine adenine dinucleotide
FAS	Fatty acid synthase
FNT	Formate nitrite transporter
FW	Fresh weight
G3P	Glycerol-3-phosphate
GAF	cGMP phosphodiesterase/adenylate cyclase/FhlA
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Guanylate cyclase
GLRs	Glutamate-like receptors
GO	Gene ontology
GOGAT	Glutamine oxoglutarate amino transferase
GPDH	Glycerol-3-phosphate dehydrogenase
GPP	G3P phosphatase
GS	Glutamine synthetase
GTP	Guanosine triphosphate
HNOB	Heme nitric oxide binding
HRT	Hydraulic retention time
iGluRs	Ionotropic glutamate receptors
LCIA	Low CO_2 Component A
MACC	Mosonmagyaróvár Algae Culture Collection

MBP	Moco binding protein
MCP	Moco carrier protein
MFS	Major facilitator superfamily
Moco	Molybdenum cofactor
Mo-MPT	Molybdenum-molybdoprotein
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NAR1	Nitrate assimilation-related component 1
NAR2	Nitrate assimilation-related component 2
NCBI	National Center for Biotechnology Information
NED	N-(1-naphthyl)ethylenediamine dihydrochloride
NiR	Nitrite reductase
NNP	Nitrate nitrite porter
NO	Nitric oxide
NOFNiR	Nitric oxide-forming nitrite reductase
NPF	Nitrate peptide transporter family
NR	Nitrate reductase
NRT1	Nitrate transporter 1
NRT2	Nitrate transporter 2
NZF1	Nitrate zinc finger 1
PBS	Phosphate-buffered saline
PAS	Per/Arnt/Sim
PCA	Principal component analysis
PHY	Phytochrome
PTR	Peptide transporter
RO	Reverse osmosis
ROS	Reactive oxygen species
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SiR	Sulfite reductase
SLAC1/SLAH	Slow anion channel-associated 1 homolog 3
SWW	Synthetic wastewater
TAP	Tris-acetate-phosphate
THB1	Truncated hemoglobin 1
TM	Transmembrane

1 INTRODUCTION

Increasing anthropogenic pressure on water bodies has resulted in the worldwide problem of eutrophication, in which nitrate has emerged as one of the principal pollutants (Mohensi-Bandpi et al., 2013). The widespread use of fertilizers in agricultural fields and the improper disposal of wastewater in water bodies are the primary causes of eutrophication. This eutrophication, resulting from nutrient enrichment of nitrogen and phosphorus, poses a major threat to the aquatic ecosystem. Multiple nations have associated rising levels of nitrogen and phosphorus in water with significant environmental problems. The primary inorganic nitrogen compounds are nitrate, nitrite, and ammonia. Nitrite and ammonia are unstable, whereas nitrate is extremely stable, making it one of the most prevalent water contaminants (Yu et al., 2020). The consequences of eutrophication are a decrease in macrophyte abundance, an increase in the growth of algae and plankton, algal blooms, and deoxygenation (Moss et al., 2011; Maberly et al., 2020). The World Health Organization and the European Drinking Water Directive have established a limit of 50 mg NO_3^- L^{-1} for nitrate in drinking water to protect human health (Grizzetti et al., 2011). Various technologies are utilized to eliminate nitrate from water based on scientific developments.

1.1 Methods for nitrate removal

In general, there are two main methods for removing nitrate from water: physicochemical and biological. A brief overview of these methods is as follows:

1.1.1 Physicochemical methods

1.1.1.1 Reverse Osmosis

In reverse osmosis (RO), water is pushed through a semi-permeable membrane under a certain pressure, allowing the water to pass through while the pollutants are blocked by the membrane without any selection (Rezvani et al., 2019). The RO membranes can be made of polyamides, or cellulose triacetate (Archna et al., 2012). The effectiveness of pre-treatment methods and the quality of the water are directly related to the lifespan of the RO membranes (Jensen et al., 2014). Fouling, compaction, and deterioration over time are issues related to RO membranes. Consequently, membrane fouling may reduce the effectiveness of treatment (Jensen et al., 2014). Though RO can be utilized to remove nitrate from nitrate-concentrated water with a nitrate removal efficiency of 59–95%, the resulting filtered water is demineralized which further requires a remineralization step (Rezvani et al., 2019). By integrating RO systems with biological,

chemical, or catalytic denitrification, it is possible to remove nitrate from waste concentrate and convert it to nitrogen gas (Jensen et al., 2014).

1.1.1.2 Ion exchange

The ion exchange method employs a strong base anion exchange resin for nitrate removal. There are several resins that are selective for nitrate and have an affinity for the following ions: $NO_3^- > SO_4^- > Cl^- > HCO_3^-$ (Nujić et al., 2017). In the ion exchange method, nitrate-concentrated water is passed through a resin bed composed of strong base anion exchange resins, where nitrate ions are exchanged for chloride until the resin is depleted (Nujić et al., 2017). The regeneration of the exhausted resin is carried out using a concentrated solution of sodium bicarbonate or sodium chloride (Kapoor and Viraraghavan, 1997). Operating ion exchange equipment entails substantial long-term expenses. Although ion exchange beds can be regenerated, during this process, salt water is directly released into the environment.

1.1.1.3 Electrodialysis

In electrodialysis (ED), nitrate removal is achieved by supplying an electrical current through a series or stack of anion and cation exchange membranes, which results in the transfer of ions from the source solution to a concentrated waste stream (Jensen et al., 2014). The anion exchange membrane shuttles nitrate ions and other anions to the anode. As nitrate continues toward the anode, the anion-impermeable cation exchange membrane rejects it and traps it in the recycled waste stream. Nitrate-selective membranes facilitate water purification without substantially changing the equilibrium of other ions (Jensen et al., 2014). Even though ED can be utilized to convert nitrate-rich water into potable water, the disposal stream at the end of the process ends up with a high concentration of nitrate-nitrogen (Rezvani et al., 2019).

1.1.1.4 Chemical denitrification

In this particular method, nitrate is reduced by using metals such as iron and aluminium. Nitrate is chemically denitrified when an electron is donated from an electron-donating metal to the nitrate via a catalyst (Rezvani et al., 2019). Chemical denitrification (CD) has an advantage over other above-mentioned technologies as it converts nitrate to other nitrogen species instead of just moving it to a more concentrated waste stream (Rezvani et al., 2019). Yet, the main limitation of CD is that it leads to the formation of ammonia in the treated water, which further requires the removal of ammonia by air stripping (Archna et al., 2012).

1.1.2 Biological denitrification

Biological denitrification uses biological organisms for the reduction of nitrate. In comparison to physicochemical decontamination approaches, biological methods for denitrification are significantly more environment friendly (Shirmali and Singh, 2001; Taziki et al., 2015). This method is largely applied for nitrate removal because of its selective reduction of nitrate to harmless nitrogen gas without any requirement for a remineralization step. Biological denitrification can be conducted in an anaerobic environment with a very small amount of biomass produced as waste sludge (Lew et al., 2012). The majority of denitrifying bacteria are heterotrophic, which means that they use oxidizable substrates such as methanol, ethanol, acetic acid, and carbon monoxide to convert nitrate into nitrogen, whereas some bacteria can grow anaerobically by reducing ionic nitrogenous oxides to gaseous products (Archna et al., 2012). Bacteria belonging to the genera *Paracoccus*, *Thiosphaera*, *Thiobacillus*, etc. use hydrogen or various reduced sulphur compounds as energy sources for the purpose of autotrophic denitrification (Archna et al., 2012). Moreover, ferrous ions can be utilized by bacteria from the genera *Sphaerotilus*, *Ferrobacillus*, *Leptothrix*, and *Gallionella* as an energy source for autotrophic denitrification (Archna et al., 2012). The disadvantages of the biological denitrification method include a longer hydraulic retention time (HRT) for denitrification and the potential contamination of the treated water with the microbes used for biological denitrification and their metabolic by-products (Rezvani et al., 2019). HRT is the average amount of time wastewater spends inside a biological reactor, indicating the amount of time the pollutant and microorganisms are in contact and it typically ranges from 5 to 24 hours for conventional operations (Reif et al., 2013). Furthermore, both autotrophic and heterotrophic denitrification generates excessive biomass of released bacterial cells and residual carbon source that must be removed carefully from the treated water. Although the HRT of denitrification by microalgae can be longer than conventional operations, the use of microalgae has become a viable approach for the treatment of wastewater, as the algal biomass obtained after the wastewater treatment can be used as feedstock in biorefineries or other applications (Cabanelas et al., 2013; Jia and Yuan, 2016; Gupta et al., 2017; Li et al., 2019; Ru et al., 2020; Ansari et al., 2021; Mohsenpour et al., 2021). Inorganic nutrients like nitrogen and phosphorus, which microalgae require for their photosynthetic/photoheterotrophic/fermentative growth, can be absorbed by them from wastewater.

1.2 Microalgae-based biological denitrification

Algae are the ecosystem's primary photosynthesizers and can be unicellular or multicellular. They can be found in common environments, such as marine and freshwater, as well as extreme environments, including deserts, arctic, hypersaline habitats, etc. (Lewis and Lewis, 2005; Foflonker et al., 2015). Nitrogen is one of the most essential nutrients for microalgal growth, and it can be derived from organic (urea and amino acids) or inorganic (nitrate, nitrite, and ammonia) sources. Microalgae have the ability to elevate the level of dissolved oxygen in the culture as well as utilize nutrients and carbon dioxide to produce microalgal biomass rich in protein, carbohydrates, and lipids that can then be used to produce biofuels, agricultural fertilizers, animal feedstock, etc. (Su et al., 2012). Domestic and industrial sewage contain considerable amounts of nitrogen, phosphorus, and organic matter in both soluble and particulate forms. Due to their ability to utilize nitrogen and phosphorus, microalgae are gaining recognition for the treatment of wastewater. This environmentally friendly treatment uses less energy, reduces carbon emissions significantly, and can lead to the production of biofuels (Prasad et al., 2017). Moreover, recovered microalgal biomass rich in nitrogen and phosphorus can be utilized as animal feed or low-cost fertilizer (Wilkie and Mulbry, 2002; Muñoz and Guieysse, 2006). Several microalgae, for example, *Scenedesmus* sp., *Nannochloropsis oceanica, Neochloris oleoabundans, Desmodesmus abundans, Chlorella sorokiniana,* and *Isochrysis galbana,* etc. have been studied for their nitrate removal capability, where species belonging to genera *Chlorella* and *Scenedesmus* have found to be suitable for nitrate removal studies. (Xin et al., 2010, Wang and Lan, 2011; Wan et al., 2013; Prasad et al., 2017; Li et al., 2019; Mollamohammada et al., 2020; Nguyen et al., 2022).

1.3 Effect of nitrate on morphological and biochemical changes in microalgae

Nitrogen is one of the most essential elements for microalgae, as it is a key component of numerous biological macromolecules, such as DNA, protein, chlorophyll, etc. Nitrogen is also one of the most vital nutrients for microalgae, and changes in its concentration can affect their growth rate, carbohydrate content, protein content, and lipid content. Nitrogen/nitrate limitation or starvation results in a decrease in growth rate, photosynthesis, and protein synthesis, as well as an increase in lipid and carbohydrate content (Li et al., 2012; Philipps et al., 2012; Simionato et al., 2013; Pancha et al., 2014). Furthermore, some studies have shown a change in the morphology of microalgal cells grown under nitrate limitation (Gavis et al., 1979; Pancha et al., 2014). One such study observed that the nitrate-limited cells of *Scenedesmus* sp. CCNM 1077 were changed from

unicell to 2- and 4-cell coenobia with spines or skies at the terminal cells (Pancha et al., 2014). Nitrogen limitation increases the production of lipids and carbohydrates in microalgae at the expense of low biomass productivity and a reduced growth rate (Li et al., 2012; Philipps et al., 2012; Pancha et al., 2014). This increased lipid production under nitrogen deprivation in microalgae may be due to neutral lipid accumulation in microalgal cells, primarily in the form of triacylglycerols, which produce lipid droplets as a carbon and energy storage form. Triacylglycerol is a glycerolipid with a three-carbon glycerol backbone esterified with three fatty acids. Gour et al. (2018) demonstrated that lower nitrate concentrations result in increased lipid content and lipid productivity in *Scenedesmus dimorphus*, whereas a few studies (Kiran et al., 2016; Zarrinmehr et al., 2020) have also shown that even high nitrate concentration can lead to a high amount of lipids in *Chlorella* sp. and *Isochrysis galbana* microalgae. One such example is *Chlorella minutissima* in which lipid content increased from 22.7% to 36% when the nitrate concentration was increased from 57 mg L^{-1} to 225 mg L^{-1} (Sánchez-García et al., 2013). Nitrogen plays an important part in the structure of proteins, as every amino acid has one or more than one nitrogen atom in its structure or composition. Protein levels have shown to be increased from 16.87% to 47.75% due to an increase in the concentration of nitrate from 0 mg L^{-1} to 247 mg L^{-1} in *Scenedesmus* sp. CCNM 1077 (Pancha et al., 2014). Similarly, in *Isochrysis galbana*, protein content increased when nitrate concentration was increased from 0 mg L^{-1} to 144 mg L^{-1} (Zarrinmehr et al., 2020). Levels of photosynthetic pigments in algae also appear to vary with nitrate concentration (Cabello-Pasini et al., 2005; Li et al., 2008; Pancha et al., 2014; Kiran et al., 2016; Zarrinmehr et al., 2020). In *Ulva rigida* (a macroalga), *Neochloris oleoabundans*, and *Chlorella* sp., amount of chlorophyll increased as the concentration of nitrate was increased (Cabello-Pasini et al., 2005; Li et al., 2008; Kiran et al., 2016). Furthermore, in *Scenedesmus* sp. CCNM 1077 and *Isochrysis galbana*, nitrate limitation or starvation led to a decrease in the photosynthetic pigments (Pancha et al., 2014; Zarrinmehr et al., 2020). Moreover, it has been shown that high nitrate concentration induces the production of sulfated polysaccharides with potent bioactive properties in *Chlamydomonas reinhardtii* (Vishwakarma et al., 2019).

1.4 Factors influencing nitrate uptake in microalgae

The ability of microalgae for nitrate uptake from a certain medium can be influenced by multiple factors, including nitrate concentration, light conditions, pH, temperature, salinity, etc. Light is crucial to the life cycles of cyanobacteria, algae, and higher plants; the colour or

wavelength of the light has a significant impact on their growth (Sæbø et al., 1995; Maltsev et al., 2021). Light is essential to microalgae because it facilitates the synthesis of important molecules needed for growth via the production of adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) through photosynthesis. Photosynthesis is a process through which light energy and inorganic compounds are converted to organic matter by microalgae. This process takes place in thylakoid membranes inside the chloroplast. Traditionally, photosynthesis is divided into two stages: light reactions and dark reactions. In the light reactions that occur in thylakoid membranes, light energy is converted into chemical energy, yielding the biochemical reductant NADPH and the high-energy molecule ATP (Masojídek et al., 2013). NADPH and ATP are utilized in the sequential biochemical reduction of carbon dioxide to carbohydrates (Calvin-Benson cycle) during the stromal dark reactions (Masojídek et al., 2013).

The photosynthetic light wavelengths utilized by microalgae for photosynthesis range from 400–700 nm. The absorption bands of the photosynthetic pigments chlorophyll-a and chlorophyll-b are blue or blue-green (450–475 nm) and red (630–675 nm) (Masojídek et al., 2013). Carotenoids are a class of biological chromophores that play a crucial role in photosynthesis and have an absorption range of 400–550 nm (Masojídek et al., 2013). As the absorption bands of photosynthetic pigments chlorophyll-a and chlorophyll-b lie in the blue and red ranges of visible light, numerous studies have been conducted to characterize the effects of these two colours on the growth of microalgae. Several studies have demonstrated that red or blue light, or both, increases the biomass yields of *Nannochloropsis* sp., *Scenedesmus* sp., and *Chlamydomonas reinhardtii* (Das et al., 2011; Kim et al., 2013; Li et al., 2021). In *Nannochloropsis* sp., *Tetraselmis* sp., and *Porphyra haitanensis*, blue light has been shown to increase the growth (Das et al., 2011; Teo et al., 2014; Wu, 2016), whereas in *Chlorella kessleri* (UTEX 398), and *Porphyra umbilicalis* (macroalga), it is red light which promoted faster growth (Figueroa et al., 1995; Koc et al., 2013). Das et al. (2011) noted that *Nannochloropsis* sp. shows best growth under blue light and least growth under red light. On the other hand, *Chlorella kessleri* (UTEX 398) showed higher growth under red light in comparison to blue light (Koc et al., 2013). Algae can regulate their photosynthetic apparatus in response to a light colour (Lehmuskero et al., 2018). In *Porphyra haitanensis* (macroalga), maximum F_v/F_m (photosynthetic efficiency) was observed under blue light and least under red light (Wu, 2016). Interestingly, red light exposure led to the expression of a red-shifted antenna complex (F710) in *Phaeodactylum tricornutum* (Herbstová et al., 2017).

So, it turns out that the effect of light colour on growth varies with the respective algae. There is a data scarcity related to the effect of light colour on the nitrate removal capacity of the microalgae. Kim et al. (2013) observed no significant difference in the nitrate removal amount by *Scenedesmus* sp. under either blue or red light. When it comes to the combination of red and blue light, *Scenedesmus* sp. exhibited a higher nitrogen removal rate when exposed to a 7:3 ratio of blue to red light compared to exposure to white, red, or blue light exclusively (Kim et al., 2013). The vast majority of studies have focused solely on red or blue light but not their combination; as a result, there is a lack of data regarding the effect of this particular light combination on various parameters in microalgae.

Photoreceptors are known to play a role in the metabolism of nitrate under various light colours (Azuara and Aparicio, 1983; López-Figueroa and Rüdiger, 1991; Figueroa, 1993). In green macroalga *Ulva rigida* and red macroalga *Corallina elongate*, phytochrome (red-light photoreceptor) and a blue-light photoreceptor were proposed as receptors involved in nitrate metabolism under red light and blue light, respectively (López-Figueroa and Rüdiger, 1991; Figueroa, 1993). In *Chlamydomonas reinhardtii*, blue light increased the levels of nitrate reductase (Azuara and Aparicio, 1983). A plasma-membrane bound flavoprotein has been shown to help with the nitrate anion uptake in *Monoraphidium braunii* under blue light (Quiñones et al., 1997). Recent studies have identified two blue light photoreceptors in algae: phototropins and cryptochromes (Kianianmomeni and Hallmann, 2014; Jaubert et al., 2017). Phototropins contain a photosensory region containing two light-oxygen-voltage (LOV) domains at the N-terminal and serine/threonine kinase domain at C-terminal, whereas cryptochromes are flavoproteins consisting of highly conserved photolyase homology region (PHR) at their N-terminal domain including a flavin adenine dinucleotide (FAD)- binding domain, and an extension of variable length at the C-terminal (Kianianmomeni and Hallmann, 2014). Both phototropins and cryptochromes have been identified in the genomes of Chlorophytes *Chlamydomonas reinhardtii* and *Volvox carteri* (Kianianmomeni and Hallmann, 2014). Phytochromes were originally defined as reversible photoreceptors for red/far-red light (Jaubert et al., 2017). Their photosensory domain consists of conserved Per/Arnt/Sim (PAS), cGMP phosphodiesterase/adenylate cyclase/FhlA (GAF), and phytochrome (PHY) domains in a sequential manner on the N-terminal (Jaubert et al., 2017). Unlike phototropins and crytochromes, phytochromes have not been identified in Chlorophytes but they have been identified in Charophytes (Kianianmomeni and Hallmann, 2014).

In addition to light colour, light intensity has also been shown to play a role in the life cycle of microalgae. There were differences in the growth rate and nutrient removal rate of microalgae grown under different light intensities (Hempel et al., 2012; Atta et al., 2013; He et al., 2015; Zhang et al., 2015). Microalgal growth rates are maximized by increasing light intensity to the point of light saturation, where photosynthetic activity reaches its peak; however, above the saturation point, photoinhibition occurs, the photosynthetic capacity decreases, and growth is suppressed (Taziki et al., 2015). Since photoinhibition can occur at high light intensities and slow growth can occur at low intensities, research has been conducted to determine the optimal light intensity at which maximum growth of the particular microalga can be achieved. Maximum growth rates for various species of microalgae have been recorded in the light intensity range of 26–400 µmol m^{-2} s^{-1}, as reviewed by Maltsev et al. (2021). Certain algal species, such as *Chlamydomonas reinhardtii* cc124 and *Chlorella ohadii*, are resistant to photoinhibition and can grow at 3000 µmol m^{-2} s^{-1} and 3500 µmol m^{-2} s^{-1}, respectively (Treves et al., 2013; Virtanen et al., 2021). Increasing the light intensity from 400 µmol m m^{-2} s^{-1} to 2000 µmol m^{-2} s^{-1} resulted in a rising trend in total nitrogen and total phosphorus removal efficiency in *Chlorella vulgaris* (Yan et al., 2013). Nitrate uptake by *Chlamydomonas reinhardtii* increased from 2.2 mg L^{-1} d^{-1} to 6.3 mg L^{-1} d^{-1} when the light intensity was raised from 400 µmol m m^{-2} s^{-1} to 1000 µmol m m^{-2} s^{-1} (Garbayo et al., 2000).

1.5 Nitrate assimilation pathway in microalgae

Nitrate has been defined as a preferred nitrogen source for the synthesis of nucleic acids, amino acids, proteins, and biomass (Calatrava et al., 2017). Even though many species prefer ammonium over nitrate and that ammonium has a lower energy cost than nitrate (Bloom et al., 1992), nitrate is the most preferred nitrogen source because it is significantly more abundant (roughly 10–1,000 times more available) in natural soils, except for some ecosystems like coniferous forests (Stark and Hart, 1977).

Understanding the mechanism behind the regulation of the nitrate assimilation pathway is crucial to improve nitrogen use efficiency and avoiding the negative effects of Nitrogen-fertilization, such as environmental contamination and waste nitrogen. Nitrate assimilation pathway in microalgae or photosynthetic eukaryotes consists of two transport steps (nitrate and nitrite transport) and two reduction steps. First nitrate is transported into the cell, where a cytosolic nitrate reductase (NR) catalyzes the reduction of nitrate into nitrite, which is subsequently

transported into the chloroplast. Nitrite is further reduced to ammonium by the action of the enzyme nitrite reductase (NiR) in the chloroplast. Chloroplast is the main site for ammonium incorporation into carbon skeletons by glutamine synthetase/glutamine oxoglutarate amino transferase (GS/GOGAT) or glutamate synthase cycle. Figure 1.1 represents the nitrate assimilation pathway in *Chlamydomonas reinhardtii*.

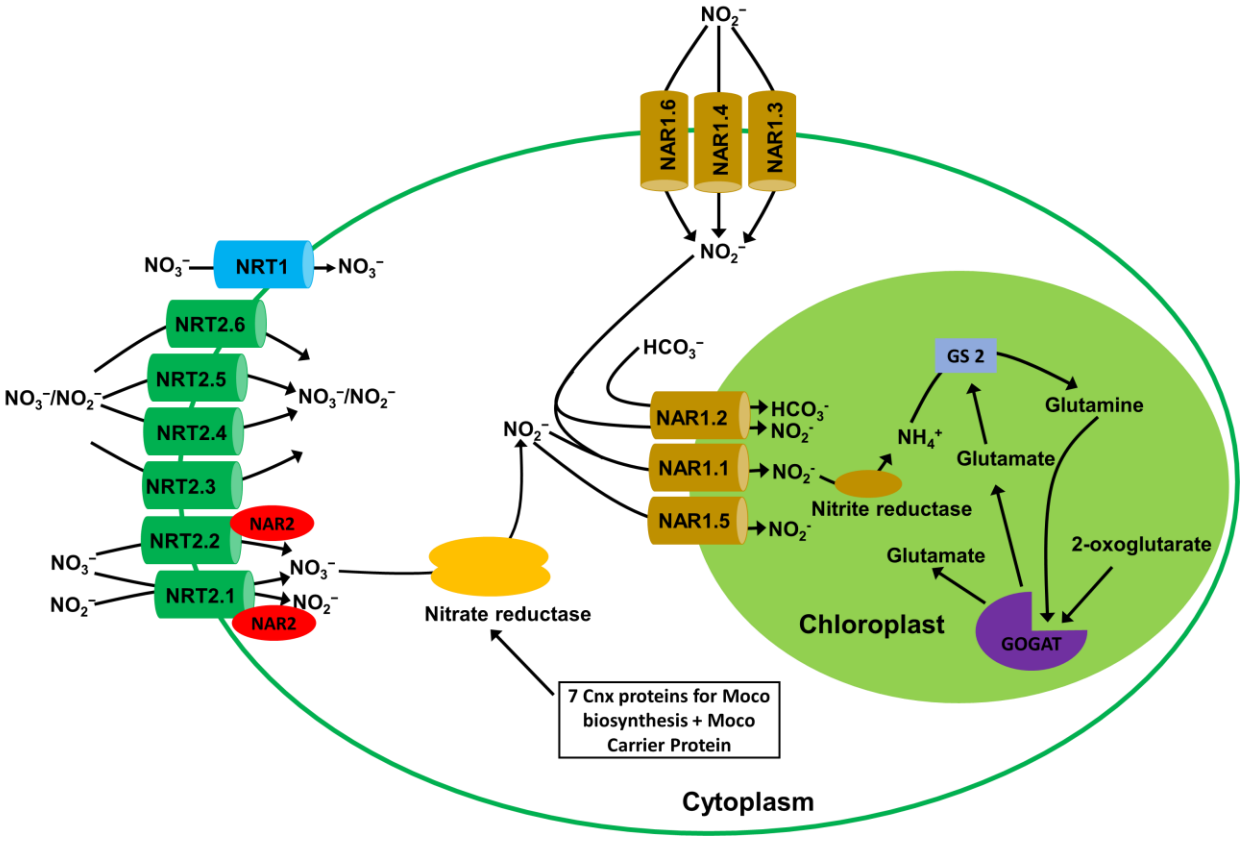


Figure 1.1. Schematic representation of nitrate assimilation pathway in *Chlamydomonas reinhardtii* (Sources: Fernandez and Galvan, 2007; Sanz-Luque et al., 2015a).

1.5.1 Nitrate Transporters

Nitrate transporters are the first door that allows the entry of nitrate inside the cell. In plants, a complex network of membrane proteins that mediate the sensing, absorption, storage, and distribution of nitrate among the various tissues carry out nitrate transport. There are at least four main families of plant nitrate transporters: chloride channels (CLC), slow anion channel-associated 1 homolog 3 (SLAC1/SLAH), aluminium-activated malate transporters (ALMT), nitrate

transporter 1/peptide transporter/nitrate peptide transporter family (NRT1/PTR/NPF), and NRT2/ nitrate nitrite porter (NRT2/NNP) (O' Brien et al., 2016; Calatrava et al., 2017). In *Chlamydomonas*, genes showing homology to SLAC1/SLAH are absent; instead, six CLC homologues (*CLV1*, *CLV2*, *CLV3*, *CLV4*, *CLV5*, and *CLV6*) are present, the role of which is still unknown (Calatrava et al., 2017). *Chlamydomonas* consists of one NRT1 and six NRT2 transporters.

1.5.1.1 Nitrate Transporter 1

Nitrate transporter 1 (NRT1) belongs to the peptide transporter family now known as the nitrate peptide transporter family. These transporters are abundant and well known in plants, but in *Chlamydomonas*, only one gene has been found to code for putative NRT1 (Fernandez and Galvan, 2007). Furthermore, the functional and molecular characterization of this *NRT1* gene is unknown in *Chlamydomonas*. The first nitrate transporter to be identified in *Arabidopsis* was NRT1, which is also known as CHL1/NRT1.1/NPF6.3 (Tsay et al., 1993). NRT1.1 of *Arabidopsis*, encoded by the *AtNRT1.1* gene, is a dual affinity nitrate transporter, meaning that its phosphorylation state determines whether it has high or low affinity for nitrate transport (Wang et al., 1998; Liu et al., 1999). NRT1.1 in *Arabidopsis* is a high-affinity transport system when it is phosphorylated by CIPK23 (calcineurin B-like protein-interacting serine/threonine-protein kinase 23) at Thr101 residue under low nitrate concentration, whereas unphosphorylated NRT1.1 is a low-affinity transport system under high nitrate concentration (Wang et al., 1998; Liu et al., 1999; Ho et al., 2009). Furthermore, NRT1.1 has been shown to be involved in the influx and efflux of nitrate, and therefore, is a bidirectional nitrate transporter (Léran et al., 2014). Additionally, this transporter has also been shown to play a role as an auxin transporter regulated by nitrate (Krouk et al., 2010).

1.5.1.2 Nitrate Transporter 2

Nitrate transporter 2 (NRT2) proteins belong to the NNP family and major facilitator superfamily (MFS) (Pao et al., 1998). This transporter was first identified in *Aspergillus nidulans* (Unkles et al., 1991). Later, it was also discovered in *Chlamydomonas* and barley (Quesada et al., 1994; Trueman et al., 1996). Although NRT2 proteins typically have 12 putative transmembrane (TM) domains and typical carrier-type structures, some of them need an additional component made up of a TM domain known as nitrate assimilation related-component 2 (NAR2) in order to

function (Fernandez and Galvan, 2007). As a result, NRT2 transporters are categorized as either single-component (NRT2) or two-component (NRT2/NAR2) systems (Fernandez and Galvan, 2007). In *Arabidopsis*, most of the NRT2s require NAR2, except AtNRT2.7, which seems to be an NAR2-independent transporter (Chopin et al., 2007). In *Chlamydomonas*, six genes (*NRT2.1–2.6*) code for NRT2s, where NAR2 is only required for the functionality of NRT2.1 and NRT2.2. NRT2.1, along with NAR2, is a bispecific, high-affinity nitrate and nitrite transporter that also plays a role in nitrate signalling, whereas NRT2.2, along with NAR2, is specific for nitrate only (Galván et al., 1996; Rexach et al., 2002; Calatrava et al., 2017). The remaining transporters are independent of NAR2 and are very likely involved in nitrite transport. NRT2.3 has been associated with the high-affinity transport of nitrite and the low-affinity transport of nitrate (Rexach et al., 1999). NRT2.4 and NRT2.5 were recently characterized as atypical NRT2 proteins due to the fact that they are half-size transporters with six transmembrane domains, with NRT2.4 exhibiting high-affinity nitrite transport activity (Higuera et al., 2016). Although *Chlamydomonas* supposedly has six NRT2s, only one, NRT2.2, is highly specific for nitrate. The other NRT2s either transport both nitrate and nitrite or only nitrite (Calatrava et al., 2017). This highlights the significance of controlling nitrite entry into the cell to prevent toxicity. *Chlamydomonas* does not store nitrite inside the cell but rather excretes it when faced with stressful environments (Calatrava et al., 2017).

1.5.2 Nitrite Transporters

Nitrate assimilation-related component 1 (NAR1) transporters belong to the family of formate nitrite transporters (FNT) (Calatrava et al., 2017). In *Chlamydomonas*, these transporters are encoded by six genes (*NAR1.1–1.6*). Transporters NAR1.1, NAR1.2, and NAR1.5 are present on the membrane of the chloroplast, whereas NAR1.3, NAR1.4, and NAR1.6 are present on the plasma membrane (Marsical et al., 2006; Fernandez and Galvan, 2007). *NAR1.1* and *NAR1.6* are regulated by the nitrogen status and nitrate assimilation regulatory gene *NIT2*, whereas expression of *NAR1.2* is strongly carbon-regulated (Mariscal et al., 2006). Expression of *NAR1.3*, *NAR1.4*, and *NAR1.5* is modulated by nitrogen or carbon sources (Mariscal et al., 2006). NAR1.1 is co-regulated with other essential proteins for nitrate assimilation (NR, NiR, NRT2.1, NRT2.2, and NAR2) (Rexach et al., 2000). First, it is required for cell growth under nitrate-limiting conditions, and second, it regulates the amount of nitrate taken up by cells under carbon dioxide-limiting conditions (Rexach et al., 2000, Mariscal et al., 2006). NAR1.2, also known as LCIA (Low CO_2

Component A), is a bispecific chloroplast envelope transporter for nitrite and bicarbonate when overexpressed under low CO_2 conditions (Mariscal et al., 2006).

1.5.3 Nitrate Reductase

In the cytoplasm, nitrate is reduced to nitrite by nitrate reductase, which is encoded by the *NIA1*/*NIT1* gene. Nitrate reductase (NR) is a homodimeric protein in eukaryotes, with each subunit being 100–120 kDa in size and containing five structurally distinct domains: Molybdenum-molybdoprotein (Mo-MPT), dimer interface, cytochrome b, flavine adenine dinucleotide (FAD) binding domain, and NADPH binding domain (Campbell, 1999). Only one gene for nitrate reductase is present in the genome of *Chlamydomonas* (Fernandez and Galvan, 2007; Sanz-Luque et al., 2015a). Nitrate reductase enzyme requires molybdenum cofactor for its functioning. Biosynthesis and transfer of the molybdenum cofactor to the nitrate reductase enzyme require the action of seven *CNX* (cofactors for nitrate reductase and xanthine dehydrogenase) genes and one moco carrier protein (*MCP1*) gene (Fernandez et al., 1989; Ataya et al., 2003; Fischer et al., 2006, Sanz-Luque et al., 2015a).

1.5.4 Nitrite Reductase

Algal nitrite reductase (NiR) is a monomer of about 63 kDa with a [4Fe-4S] cluster and a siroheme as prosthetic groups and is encoded by a single gene *NII1* in *Chlamydomonas* (Fernandez and Galvan, 2007; Sanz-Luque et al., 2015a). It catalyzes the reduction of nitrite to ammonium in the stroma of the chloroplast. In *Chlamydomonas*, NiR is encoded by a single gene that is grouped with other genes necessary for nitrate/nitrite assimilation (Figure 1.2) (Sanz-Luque et al., 2015a; Calatrava et al., 2017).

1.5.5 GS/GOGAT Cycle

GS/GOGAT cycle helps with the assimilation of ammonium obtained after the reduction of nitrite by nitrite reductase. *Chlamydomonas* genome contains four GS genes (*GLN1*, *GLN2*, *GLN3*, and *GLN4*); *GLN1* and *GLN4* code for cytosolic GS1, whereas *GLN2* and *GLN3* code for chloroplastic GS2 (Vallon and Spalding, 2009). Furthermore, two GOGATs, one of which is NADH dependent encoded by *GSN1*, and another which is ferredoxin-dependent encoded by *GSF1* are also present in the chloroplast of *Chlamydomonas* (Fernandez and Galvan, 2007; Vallon and Spalding, 2009). Unlike the form of GS found in photosynthetic prokaryotes, both GS isoforms of *Chlamydomonas* have been characterized as large, octameric proteins similar to the plant type

of GS (Florencio and Vega, 1983). In the first step of the GS/GOGAT cycle, ammonia and glutamate combine to form the amide group of glutamine in a reaction that is ATP-dependent and catalyzed by GS2 (Florencio and Vega, 1983; Chen and Silflow, 1996). In a reaction catalyzed by chloroplastic GOGAT isoform, the incorporation of ammonium into glutamine is followed by the reductive transfer of the amide group from glutamine to α-ketoglutarate, yielding two glutamates (Vallon and Spalding, 2009).

1.6 Nitrate assimilatory genes in various microalgae

Table 1.1 presents the name and number of genes participating in nitrate assimilation in Chlorophytes, Rhodophytes, and Glaucophytes. Chlorophytes are green algae, Rhodophytes are red algae, and Glaucophytes are freshwater unicellular microalgae which are assumed to resemble ancestor of plants. The *NRT1* genes are present in large numbers in the genomes of plants, whereas genomes of algae contain either only a few or none (Sanz-Luque et al., 2015a). *Chlamydomonas reinhardtii* has only one *NRT1* gene, while genomes of *Coccomyxa subellipsoidea* and *Chlorella* NC64A contain two (Table 1.1). Furthermore, this gene is not present in the microalgae *Micromonas pusilla* and *Chlorella paradoxa* (Table 1.1). From Table 1.1, it can be seen that all of the microalgae contain the NRT2 proteins which carry out high-affinity nitrate/nitrite transport. However, NAR2-dependent high-affinity nitrate/nitrite transport by NRT2 seems to be absent from Rhodophytes and Glaucophytes as the *NAR2* gene is not present in either of these microalgae. *NAR2* shows a lower level of conservation among other Chlorophytes, but it is highly conserved between *Chlamydomonas* and *Volvox* (Sanz-Luque et al., 2015a). *Chlamydomonas reinhardtii* has the highest number of *NRT2* and *NAR1* genes (six of each) in comparison to other microalgae. Like *NAR2* genes, *NAR1* genes are also absent in Rhodophytes and Glaucophytes. The fact that *Chlamydomonas reinhardtii* has lots of *NRT2* and *NAR1* genes, this might suggest that it has found the best way to utilize nutrients in a wide range of environments.

In microalgae, NR and NiR are encoded by single genes except for *Coccomyxa subellipsoidea,* which has two genes for NR in its genome (Table 1.1). Interestingly, *Cyanidioschyzon merolae* lacks the gene coding for NiR (*NII*). *CmSiRA* and *CmSiRB* are two of the many sulfite reductase (SiR) homologues encoded by the genome of the unicellular red alga *Cyanidioschyzon merolae,* which lives in acidic hot springs. In terms of structure and function, sulfite reductase and nitrite reductase are very similar (Crane and Getzoff, 1996). One of the two

SiR homologues, *CmSiRB*, has been found to function as nitrite reductase in the presence of nitrate in the growth medium (Sekine et al., 2009). Moreover, the *CmSiRB* gene is located in the genome of *Cyanidioschyzon merolae* between the NR genes and a nitrate transporter (Sekine et al., 2009). GLN genes coding for GS are present in equal numbers in *Chlamydomonas reinhardtii* and *Volvox carteri* (Vallon and Spalding et al., 2009). One *GLN* gene in *Ostreococcus* RCC809 and *Cyanidioschyzon merolae* is more closely related to bacterial *GLN* than to the plant-type *GLN*s found in other algae (Sanz-Luque et al., 2015a). Chlorophytes have both chloroplastic NADH-GOGAT (*GSN1*) and ferredoxin-GOGAT (*GSF1*) genes, whereas Rhodophytes and Glaucophytes only have the ferredoxin-GOGAT gene (Table 1.1).

Table 1.1. Nitrate assimilatory proteins encoding genes in various microalgae (Sanz-Luque et al., 2015a).

	Chlorophytes										*Rhodophytes*		*Glauco-phytes*
Gene	***Chlamydomonas reinhardtii***	***Volvox carteri***	***Coccomyxa subellipsoidea***	***Micromonas pusilla***	***Micromonas* RCC299**	***Chlorella* NC64A**	***Ostreococcus lucimarinus***	***Ostreococcus* RCC809**	***Ostrecoccus tauri***	***Bathycoccus prasinos***	***Cyanidioschyzon merolae***	***Galdieria sulphuraria***	***Cyanophora paradoxa***
NRT1	1	1	2	NF	1	2	1	1	1	1	1	1	NF
NRT2	6	3	1	1	3	2	1	1	1	1	1	1	2
NAR1	6	5	2	1	1	3	1	1	1	1	NF	NF	NF
NAR2	1	1	1	1	2	1	1	1	1	1	NF	NF	NF
NIA/NIT	1	1	2	1	1	1	1	1	1	1	1	1	1
NII	1	1	1	1	1	1	1	1	1	1	1*	1	1
GLN	4	4	3	1	1	2	1	1	1	1	1	1	2
GSN/GSF	2	2	2	2	2	2	1	1	1	1	1	1	1
MCP	1	1	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
CNX2	1	1	1	1	1	1	1	1	1	1	1	1	1
CNX3	1	1	1	1	1	1	2	1	1	1	1	1	1
CNX5	1	1	1	1	1	1	1	1	1	1	1	1	1
CNX6	1	1	1	1	1	1	1	1	1	1	1	1	1
CNX7	1	1	1	1	1	1	1	1	1	1	NF	NF	NF
CNX1G	1	1	1	1	1	1	1	1#	1#	1	1	1	NF
CNX1E	1	1	1	1	1	1	1	1#	1#	1	1	1	1

*Sulfite reductase B function as NiR.
#Single chimeric gene is formed by the fusion of *CNX1G* and *CNX1E*.

Molybdenum cofactor (Moco) storage and protection appear to be effectively mediated by the MCP (Fischer et al., 2006). As it can been seen from Table 1.1, MCP is only found in Volvocales and not in the other analyzed algal genomes, despite having a significant amount of

conservation with the plant-derived moco binding protein (MBP) (Kruse et al., 2010). 7 *CNX* genes playing role in the biosynthesis and transport of moco to the NR enzyme are present as a single copy in most of the microalgae analyzed by Sanz-Luque et al. (2015a), except for Rhodophytes and Glaucophyte where *CNX7* gene is absent (Table 1.1). Furthermore, *CNX1G* is also absent in Glaucophyte, *Cyanophora paradoxa*. In plants, the *CNX1* gene is split into two genes, *CNX1E* and *CNX1G*, each of which codes for one of the two domains (E and G) of the protein it encodes (Sanz-Luque et al., 2015a). However, these domains are combined in a single gene in *Ostrecoccus tauri*, and *Ostreococcus* RCC809 (Table 1.1). By placing these two genes from *Chlamydomonas reinhardtii* together in *CNX1E-G* or *CNX1G-E* chimeric constructions, it has been shown that the orientation of the CNX1E and CNXG domains does not change the way the chimeric CNX1 proteins work (Llamas et al., 2007).

In various organisms like fungi, cyanobacteria, and algae, major nitrate assimilatory genes are present in a cluster (Fernandez and Galvan, 2007). The reason behind this clustering of genes in algae is not well understood, but it is likely that the close proximity of genes helps coordinate their regulation and inheritance. Though the role, order, and orientation of the clustered genes vary from species to species, it is interesting to note that the nitrate-cluster of most Chlorophytes contains a complete set of genes for nitrate assimilation (*NRT2*/*NAR2*/*NIA1*/*NII1*/*NAR1*) (Sanz-Luque et al., 2015a). Figure 1.2 shows the nitrate-cluster of *Chlamydomonas reinhardtii* which is present on chromosome 9. However, the known regulatory gene *NIT2* for nitrate assimilation is present on chromosome 3.

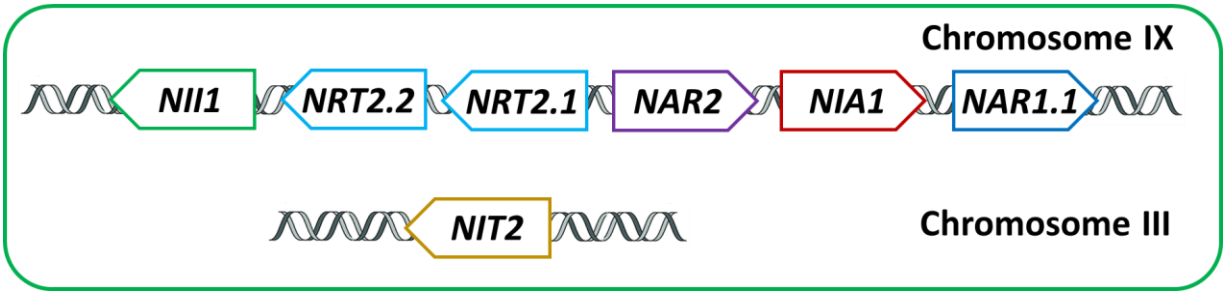


Figure 1.2. The cluster of nitrate assimilating genes in *Chlamydomonas reinhardtii*.

1.7 Regulation of nitrate assimilation

There is a vast network of proteins involved in regulating nitrate acquisition, and many of them are poorly understood. Nitrate and ammonium, in general, have opposing effects on nitrate

assimilation genes, which are referred to as positive (nitrate) and negative (ammonium) signals (Fernandez and Galvan, 2007). Ammonium strongly inhibits the expression of the NR gene (*NIA1*), while nitrate induces it (Cannons and Shiflett, 2001; Llamas et al., 2002). When both positive and negative signals are present at once in *Chlamydomonas*, repression manifests as a quantitative process that is more sensitive to the nitrate/ammonium (N/A) balance than the ammonium concentration (Llamas et al., 2002).

Nitrate is both a nitrogen source and a signalling molecule in photosynthetic organisms (Krapp et al., 2014). Nitrate is a signalling molecule because it activates the transcription of genes necessary for its uptake. In plants, fungi, yeast, and some green and red algae, several major transcription factors linked to nitrate assimilation have been identified (Sanz-Luque et al., 2015a). *Chlamydomonas* relies on the NIT2 transcription factor to upregulate the expression of primary genes; *NII1*, *NRT2.1*, *NRT2.2*, *NRT2.3*, *NAR2*, *NIA1*, *NAR1.1*, and *NAR1.6* involved in nitrate assimilation (Galván and Fernández, 2001; Mariscal et al., 2006; Camargo et al., 2007). Indeed, in the presence of nitrate, nit2 mutants are not able to grow (Quesada et al., 1993). The *NIT2* gene is activated in nitrate and nitrogen-free media and repressed by ammonium, demonstrating that *NIT2* is regulated by additional unknown transcription factors (Camargo et al., 2007). Recently, a regulatory protein, nitrate zinc finger 1 (NZF1), participating in nitrate assimilation was identified in *Chlamydomonas*. The expression of *NIT2*, a regulatory gene, and other genes (*NRT2.1*, *NRT2.2*, and *NIA1*) involved in nitrate assimilation has been shown to be reduced in the nzf1 mutant (Higuera et al., 2014). Since the expression of important genes for nitrate assimilation, including the regulatory gene *NIT2*, is affected in the nzf1 mutant, the *Chlamydomonas* NZF1 protein appears to play a role in primary nitrate signalling.

Ammonium and nitric oxide are regarded as negative signals that influence nitrate assimilation genes at both the transcriptional and post-translational levels in *Chlamydomonas* (Calatrava et al., 2017). Ammonium inhibits the expression of the NR and high-affinity nitrate/nitrite transporter genes (Sanz-Luque et al., 2015a). In *Chlamydomonas reinhardtii*, repression of *NIA1* in response to ammonium was found to be defective in the cyg56 mutant, and it was discovered that a *CYG56* gene encoding a nitric oxide (NO)-dependent guanylate cyclase (GC) was disrupted in this mutant (de Montaigu et al., 2010). This enzyme converts guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP), and it becomes active when

nitric oxide (NO) binds to its heme group, which is located in the HNOB (Heme Nitric Oxide Binding) domain (Iyer et al., 2003; Poulos, 2006). It is possible that this converted cGMP binds to the GAF domain of NIT2 and controls the structure and binding of NIT2 to particular promoters; however, further research is necessary to address and clarify this mechanism (Camargo et al., 2007; Sanz-Luque et al., 2015a).

THB1, a truncated form of hemoglobin found in the cytosol, has been shown to play an important role in controlling NR activity in *Chlamydomonas reinhardtii* (Sanz-Luque et al., 2015b). A link between NO metabolism and nitrate assimilation has been established via THB1. Under oxygenic conditions, dioxygenase activity of reduced THB1 (bonded with Fe^{2+}) can convert nitric oxide to nitrate ($NO + O_2 + e^- \rightarrow NO_3^-$). The diaphorase activity of NR is more effective at reducing THB1 than that of free cofactors (NADH and FAD) or cytochrome b5 reductase (Cytb5-R), which is highly homologous to NR (Sanz-Luque et al., 2015c). The NR/THB1 partnership appears to have two outcomes: i) nitric oxide is scavenged to generate nitrate, which is a positive signal; and (ii) electron flux is redirected from NR-diaphorase to THB1, which inhibits NR activity and thereby moderates the production of nitrite (Calatrava et al., 2017). Additionally, PII signalling proteins have been found in organisms across the evolutionary tree, from bacteria to plants, and more recently in green microalgae such as *Chlamydomonas*, *Chlorella*, and *Micromonas* (Sanz-Luque et al., 2015a). The majority of the cyanobacteria, microalgae, and higher plants code for one PII protein encoded by the *GLB1* gene. *Chlamydomonas* PII expression has been shown to be under the complex control of both positive signals (i.e., nitrate and nitrite) and negative signals (NO) (Zalutskaya et al., 2018).

2 AIM

As a result of population growth, industrialization, and rapid urbanization, nitrogen pollution, typically in the form of nitrate, has emerged as a major threat to water quality. Therefore, the main aim of the present thesis work was to identify a microalga with great potential for the purpose of nitrate removal studies. For this aim, studies were carried out with three major objectives, which are as follows:

Objective 1: To assess the nitrate removal capacity of two eukaryotic green microalgae *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360.

The motive behind the first objective was to examine the growth and nitrate removal efficiency of *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360 under various concentrations of nitrate. Additionally, protein, carbohydrate, and lipid accumulation in microalgae were also determined in response to different nitrate concentrations.

Objective 2: To assess the effect of various light conditions on the nitrate removal capacity of *Chlamydomonas* sp. MACC-216.

The second objective was utilized to observe whether the combinations of various light colours and intensities would affect the nitrate removal efficiency of *Chlamydomonas* sp. MACC-216. *Chlamydomonas* sp. MACC-216 was grown under various light conditions consisting of combinations of three light colours (blue, red, and white) and three light intensities (50 μmol m^{-2} s^{-1}, 100 μmol m^{-2} s^{-1}, and 250 μmol m^{-2} s^{-1}).

Objective 3: To analyze differential gene expression in *Chlamydomonas* sp. MACC-216 and *Chlamydomonas reinhardtii* cc124 under nitrate-replete and nitrate-deplete conditions.

For this objective, transcriptome analysis was performed to investigate differentially expressed genes in *Chlamydomonas* sp. MACC-216 and *C. reinhardtii* cc124 grown in the presence (nitrate-replete) and absence (nitrate-deplete) of nitrate to observe how nitrate assimilation differs between two strains of *Chlamydomonas*.

3 MATERIALS AND METHODS

3.1 Objective 1

3.1.1 Microalgae strains and growth media

Two genera of microalgae were selected, namely, *Chlamydomonas* sp. MACC-216, and *Chlorella* sp. MACC-360. These microalgae were provided by the Mosonmagyaróvár Algae Culture Collection (MACC). The TAP (tris-acetate-phosphate) medium was prepared with the components mentioned in Table 3.1, with the pH maintained at 7. The final concentration of CH_3COOH in the TAP medium was 16.8 mM.

Table 3.1. List of components for TAP medium.

Component	Final amount in 1L
Tris base	2.42 g L^{-1}
NH_4Cl	0.374 g L^{-1}
$MgSO_4 \cdot 7H_2O$	0.204 g L^{-1}
$CaCl_2 \cdot 2H_2O$	0.066 g L^{-1}
K_2HPO_4	0.287 g L^{-1}
KH_2PO_4	0.142 g L^{-1}
$Na_2EDTA \cdot 2H_2O$	0.049 g L^{-1}
$ZnSO_4 \cdot 7H_2O$	0.039 g L^{-1}
H_3BO_3	0.011 g L^{-1}
$MnCl_2 \cdot 4H_2O$	0.007 g L^{-1}
$FeSO_4 \cdot 7H_2O$	0.008 g L^{-1}
$CoCl_2 \cdot 6H_2O$	0.002 g L^{-1}
$CuSO_4 \cdot 5H_2O$	0.002 g L^{-1}
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.001 g L^{-1}
CH_3COOH	1 mL L^{-1}

TAP-N was prepared by substituting sodium nitrate as the sole nitrogen source instead of ammonium chloride. In addition, 0.001 g L^{-1} of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ was replaced with 0.006 g L^{-1} of $Na_2MoO_4 \cdot 2H_2O$ in the TAP-N medium. First, screening was performed for the selection of nitrate concentrations to be used for the first objective. The growth of *Chlamydomonas* sp. MACC-216, and *Chlorella* sp. MACC-360 was tested in TAP-N containing 1 mM (84.99 mg L^{-1}), 5 mM

(424.97 mg L^{-1}), 10 mM (849.94 mg L^{-1}), 15 mM (1.27 g L^{-1}), 20 mM (1.69 g L^{-1}), 40 mM (3.39 g L^{-1}), 50 mM (4.24 g L^{-1}), 75 mM (6.37 g L^{-1}) and 100 mM (8.49 g L^{-1}) sodium nitrate. Three concentrations of sodium nitrate (5 mM, 10 mM, and 15 mM) were selected for further experiments. Both microalgae were cultivated in TAP, TAP-N5 (5 mM nitrate), TAP-N10 (10 mM nitrate), and TAP-N15 (15 mM nitrate) media at 25 °C under a light intensity of 50 µmol m^{-2} s^{-1} with continuous shaking at 180 rpm in a regime of 16:8 light–dark periods.

3.1.2 Growth parameters

In two separate 24-well plates, *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360 were grown in TAP, TAP-N5, TAP-N10, and TAP-N15 media. 0.1 absorbance reading was maintained as the initial absorbance at 720 nm (day 0) for both microalgae in all four media. Using a Hidex microplate reader, the absorbance of both microalgae was measured every day for six days at 720 nm. For cell counting, a LUNA cell counter was employed, which determined the number of cells based on the autofluorescence emitted by microalgae. Three-day-old cultures of both microalgae were sampled to determine cell size, and the microalgae were observed using an Olympus Fluoview FV1000 confocal laser scanning microscope. With a 60X magnification objective, images were captured, and ImageJ was used to calculate the cell perimeter.

The growth patterns of both microalgae were determined by their number of generations (n) and mean generation time per day (g) in the logarithmic growth phase according to the following equations (Patel et al., 2018):

$$n = \frac{\log N - \log N_0}{\log 2} \quad (1)$$

$$g = \frac{t}{n} \quad (2)$$

where ‘n’ is the number of generations in a given time period, ‘N_0′ and ‘N’ are the initial and final cell number of microalgae, ‘g’ is the mean generation time, and ‘t’ is the duration of the exponential growth phase. The specific growth rate (day^{-1}) ‘µ’ was also calculated for both microalgae.

$$\mu = \frac{\ln 2}{g} \quad (3)$$

3.1.3 Nitrate determination by the salicylic acid method

Chlamydomonas sp. MACC-216 and *Chlorella* sp. MACC-360 were grown in TAP-N5, TAP-N10, and TAP-N15 media in two separate 24-well plates for nitrate removal determination. The initial absorbance at 720 nm (day 0) for both microalgae was maintained at 0.1 in all three media. From day 0 to day 6, the total nitrate removal and nitrate removal efficiency by both microalgae were determined in TAP-N5, TAP-N10, and TAP-N15 media. The nitrate removal efficiency was calculated using the following equation:

$$R = \left(1 - \frac{Cf}{Ci}\right) \times 100 \quad (4)$$

where R is the nitrate removal efficiency (%), Ci and Cf are the initial and final concentrations of nitrate (mM) in the growth medium.

To determine the nitrate removal rate, both microalgae were cultivated in 20 mL of TAP medium for three days; on the third day, the cultures were centrifuged at 3220 g for 10 minutes and then washed with fresh TAP-N0 medium (TAP medium without any nitrogen source). After washing, both cultures were divided and re-suspended into TAP-N5, TAP-N10 and TAP-N15 media. The nitrate removal rate was measured every three hours (h) for up to nine hours. The nitrate removal rate was calculated using the following equation as mentioned by Wang and Lan (2011):

$$R_{rate} = \frac{Ci - Cf}{t0 - t} \quad (5)$$

where R_{rate} is the nitrate removal rate, Ci is the initial nitrate concentration and Cf is the corresponding nitrate concentration at time t.

For the evaluation of nitrate removal, a nitrate assay as described by Cataldo et al. (1975) was conducted. Briefly, 10 µL of the sample was placed in a microcentrifuge tube, followed by the addition of 40 µL of 5% (w/v) salicylic acid in concentrated sulfuric acid. After 20 minutes of incubation at room temperature, 950 µL of 2M sodium hydroxide was slowly added to the tube and thoroughly mixed. The sample was cooled down to room temperature and the absorbance was measured at 410 nm using a Hidex microplate reader.

3.1.4 Determination of reactive oxygen species (ROS)

ROS production was measured by 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) as described by Wang et al. (2011). The stock solution of DCFH-DA was prepared in DMSO at a final concentration of 10 mM and stored at −20 °C until further use. For the determination of ROS, 3-day-old cultures of both microalgae grown in TAP medium were harvested by centrifugation at 3220 g for 10 minutes. The pellets were washed once with 1X phosphate-buffered saline (PBS) (pH of 7.0) followed by resuspension in 1X PBS. Both microalgae cultures were incubated at 25 °C in a shaker incubator for one hour in the dark. After 1 hour, both cultures were centrifuged and washed, followed by division and resuspension into TAP, TAP-N5, TAP-N10 and TAP-N15 media, each containing 5 µM DCFH-DA. Resuspension was carried out in 48-well plates. Separate plates were used for *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360. For blank, only respective media with 5 µM DCFH-DA were used and the blank measurement was carried out in a separate 48-well plate. All of the plates were incubated at 25 °C in a shaker incubator under constant white light with a light intensity of 50 µmol m^{-2} s^{-1}. The measurements for ROS production were conducted every hour for up to 4 hours using a Hidex microplate reader with excitation and emission filters set at 490 nm and 520 nm, respectively.

3.1.5 Total pigments extraction and quantification

Chlamydomonas sp. MACC-216 and *Chlorella* sp. MACC-360 were grown in 10 mL TAP, TAP-N5, TAP-N10 and TAP-N15 media for three days. For chlorophyll extraction, 10 mL culture of each 3-day-old culture was centrifuged at 3220 g for 10 minutes. The supernatants were discarded and then 5 mL of methanol was added to the pellets and mixed with pipetting. Incubation was carried out at 45 °C in the dark for 30 minutes. Afterwards, the samples were centrifuged at 6200 g for 10 minutes and supernatants were collected for absorbance. Absorbance was taken at 653 nm, 666 nm and 470 nm in a Hidex microplate reader. Calculations for chlorophyll-a, chlorophyll-b, and total carotenoids were performed as described by Lichtenthaler and Wellburn (1983).

$$C_a = 15.65A_{666} - 7.34A_{653} \quad (6)$$

$$C_b = 27.05A_{653} - 11.21A_{666} \quad (7)$$

$$C_{x+c} = 1000A_{470} - 2.86C_a - 129.2C_b/245 \quad (8)$$

where C_a, Cb, Cx_{+c} are the amounts of chlorophyll-a, chlorophyll-b and total carotenoids, respectively, in µg mL^{-1}.

3.1.6 Extraction and quantification of carbohydrates

For the extraction of total carbohydrates, pellets obtained following the extraction of total pigments were utilized. After washing the pellets with Milli-Q water, they were dissolved in 10 mL of Milli-Q. In a new glass tube, 5 mL of anthrone reagent was added to 1 mL of each dissolved pellet. The anthrone reagent was freshly prepared by dissolving 0.5 g of anthrone in 250 mL of concentrated sulphuric acid. After the addition of anthrone reagent, tubes were cooled and then incubated in a water bath at 90°C for 17 minutes. After incubation, the tubes were cooled to room temperature and the absorbance was measured using a Hidex microplate reader at 620 nm.

3.1.7 Extraction and quantification of proteins

Chlamydomonas sp. MACC-216 and *Chlorella* sp. MACC-360 were cultivated for three days in 10 mL of TAP, TAP-N5, TAP-N10, and TAP-N15 media. Each 3-day-old culture was centrifuged for 10 minutes at 3220 g, and the pellets were resuspended in 1 mL of lysis buffer. The lysis buffer consisted of 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1 mM EDTA (pH 8.0), 10% Glycerol, and 1X cOmpleteTM EDTA-free protease inhibitor cocktail (Roche). After resuspension, sonication was carried out at 0.8 cycle, 90% amplitude for 10 minutes. After sonication, centrifugation was performed at 13300 g for 20 minutes at 4 °C. The supernatants were collected in new microcentrifuge tubes and then utilized for the Bradford assay. For the Bradford assay, samples were diluted 10 times; 50 µL of each diluted sample was added to a clean microcentrifuge tube, followed by the addition of 1.5 mL of Bradford reagent. After 10 minutes of incubation at room temperature, the absorbance of the samples was measured at 595 nm using a Hidex microplate reader.

3.1.8 Extraction and quantification of lipids

Chlamydomonas sp. MACC-216 and *Chlorella* sp. MACC-360 were grown in 10 mL of TAP, TAP-N5, TAP-N10 and TAP-N15 media for three days. After three days, cultures of both microalgae were centrifuged at 3220 g for 10 minutes, and the supernatants were discarded. 3 mL of chloroform: methanol (2:1) was used to dissolve each obtained pellet. Dissolved pellets were sonicated at 0.8 cycle, 90% amplitude for 2 minutes. After sonication, 2 mL of chloroform: methanol (2:1) was added to the mixture and incubation was carried out at room temperature for

1 hour with constant shaking. After 1 hour, centrifugation was performed at 3220 g rpm for 10 minutes. Supernatants were collected in fresh glass tubes and pellets were re-dissolved in 2 mL of chloroform: methanol (2:1) followed by further incubation at room temperature for half an hour with constant shaking. After half an hour, centrifugation was carried out at 3220 g for 10 minutes. Supernatants were collected in the tubes with previously isolated supernatants. To the supernatants, 0.9% NaCl (1/5th volume of supernatant) was added, and centrifugation was carried out at 3220 g for 10 minutes. Lower phases were collected in fresh glass tubes and were evaporated to collect total lipids. For total lipids quantification, sulpho-phospho-vanillin reagent was prepared by dissolving 0.6 g of vanillin in 100 mL of hot water, followed by the addition of 400 mL of 85% phosphoric acid. This reagent can be stored in the dark for several months until it turns dark. Collected total lipids fractions were evaporated at 90 ℃ and further dissolved in 1 mL of chloroform; 100 µL from each total lipid fraction was taken in a fresh glass tube and evaporated at 90 ℃ in the water bath. A volume of 100 µL of concentrated sulphuric acid was added to each tube and tubes were incubated at 90 ℃ for 15 minutes in a water bath. Tubes were cooled down on the ice for 5 minutes and 2.4 mL of sulpho-phospho-vanillin reagent was added to all the lipid samples, followed by incubation at room temperature for 5 minutes. Absorbance was measured at 530 nm in a Hidex microplate reader.

3.1.9 Confocal microscopy with BODIPY dye

Chlamydomonas sp. MACC-216 and *Chlorella* sp. MACC-360 were grown in TAP, TAP-N5, TAP-N10 and TAP-N15 media in 24-well plates for three days. For the localization of lipids inside microalgae cells, BODIPY dye (Sigma-Aldrich) was used. The stock solution of 4 mM BODIPY was prepared in 100% methanol. To 50 µL of 3-day-old microalgae cells, 0.25 µL of 4 mM BODIPY dye was added and then microalgae were observed under an Olympus Fluoview FV1000 confocal laser scanning microscope. For BODIPY, the emission range was selected from 500 nm to 515 nm and images were taken by a 60X magnification objective at 6X zoom for *Chlamydomonas* sp. MACC-216 and 8X zoom for *Chlorella* sp. MACC-360.

3.1.10 Synthetic wastewater treatment

Synthetic wastewater (SWW) was prepared according to the procedure mentioned in "OECD guidelines for testing chemicals" (OECD, 2010). 16 g of peptone, 11 g of meat extract, 4.25 g of $NaNO_3$, 0.7 g of NaCl, 0.4 g of $CaCl_2 \cdot 2H_2O$, 0.2 g of $MgSO_4 \cdot 7H_2O$ and 2.8 g of K_2HPO_4

were added to 1 L of distilled water and the pH of this medium was set at 7.5. The initial concentration of sodium nitrate ($NaNO_3$) in synthetic wastewater was 50 mM; therefore, three more dilutions were made, i.e., 5 mM, 10 mM, and 25 mM. *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360 were grown in SWW (with the above-mentioned dilutions) for six days. The cultivation of both microalgae was carried out in a Multi-Cultivator MC-1000-OD (Photon Systems Instruments) at a continuous illumination of 50 µmol m^{-2} s^{-1} intensity. For six days, growth and nitrate removal capacity were observed for both microalgae.

3.1.11 Statistical analysis

The statistical analyses were performed using Rstudio version 1.2.5019. All statistical analyses in the current study were conducted using one-way analysis of variance (ANOVA) followed by Tukey's test to detect statistically significant differences between treatments, where p-value < 0.05 was considered statistically significant.

3.2 Objective 2

3.2.1 Cultivation under various light conditions

Chlamydomonas sp. MACC-216 was cultivated in TAP-N5 and TAP-N10 media under various combinations of light colours (or wavelengths) and light intensities to select the best light condition for the cultivation of *Chlamydomonas* sp. MACC-216 in SWW. In total, 12 light conditions made up of combinations of three light colours (blue, red, and white) and three light intensities (50 µmol m^{-2} s^{-1}, 100 µmol m^{-2} s^{-1}, and 250 µmol m^{-2} s^{-1}) were selected to cultivate *Chlamydomonas* sp. MACC-216 (Table 3.2). *Chlamydomonas* sp. MACC-216 was grown under each light condition in Multi-Cultivator MC-1000-OD (Photon Systems Instruments) with a continuous light source for 70 hours. The optical density was automatically measured at 720 nm by the multi-cultivator at a gap of 10 hours each.

Table 3.2. Light conditions for the growth of *Chlamydomonas* sp. MACC-216 in TAP-N5, TAP-N10, and SWW.

Light Condition	Growth Medium	Description
Blue 50	TAP-N5 and TAP-N10	Blue light with 50 µmol m^{-2} s^{-1} light intensity
Blue 25 + Red 25	TAP-N5 and TAP-N10	Blue light with 25 µmol m^{-2} s^{-1} light intensity + Red light with 25 µmol m^{-2} s^{-1} light intensity
Red 50	TAP-N5 and TAP-N10	Red light with 50 µmol m^{-2} s^{-1} light intensity
White 50	TAP-N5 and TAP-N10	White light with 50 µmol m^{-2} s^{-1} light intensity

Blue 100	TAP-N5 and TAP-N10	Blue light with 100 µmol m^{-2} s^{-1} light intensity
Blue 50 + Red 50	TAP-N5 and TAP-N10	Blue light with 50 µmol m^{-2} s^{-1} light intensity + Red light with 50 µmol m^{-2} s^{-1} light intensity
Red 100	TAP-N5 and TAP-N10	Red light with 100 µmol m^{-2} s^{-1} light intensity
White 100	TAP-N5 and TAP-N10	White light with 100 µmol m^{-2} s^{-1} light intensity
Blue 250	TAP-N5, TAP-N10, and SWW	Blue light with 250 µmol m^{-2} s^{-1} light intensity
Blue 125 + Red 125	TAP-N5, TAP-N10, and SWW	Blue light with 125 µmol m^{-2} s^{-1} light intensity + Red light with 125 µmol m^{-2} s^{-1} light intensity
Red 250	TAP-N5, TAP-N10, and SWW	Red light with 250 µmol m^{-2} s^{-1} light intensity
White 250	TAP-N5, TAP-N10, and SWW	White light with 250 µmol m^{-2} s^{-1} light intensity

After screening in TAP-N5 and TAP-N10 media, Blue 250, Blue 125 + Red 125, Red 250, and White 250 light conditions were selected for the cultivation of *Chlamydomonas* sp. MACC-216 in SWW. SWW consisted of 1.6 g peptone, 1.1g meat extract, 0.425 g $NaNO_3$ (5 mM), 0.07 g NaCl, 0.04 g $CaCl_2 \cdot 2H_2O$, 0.02 g $MgSO_4 \cdot 7H_2O$, and 0.28 g K_2HPO_4 in 1 L of distilled water, and the pH was set at 7.5 (OECD, 2010). The cultivation of *Chlamydomonas* sp. MACC-216 in all kinds of media was carried out in a Multi-Cultivator MC-1000-OD (Photon Systems Instruments) under the light conditions mentioned in Table 3.2 and the optical density was automatically measured at 720 nm by the multi-cultivator at a gap of 10 hours each. Furthermore, *Chlorella* sp. MACC-38 and *Chlorella* sp. MACC-360 were also grown under Blue 250, Blue 125 + Red 125, Red 250, and White 250 to examine their growth and nitrate removal efficiency in SWW.

3.2.2 Nitrate estimation

Nitrate estimation was performed at 0 and 70 hours as mentioned in section 3.1.3.

3.2.3 Nitrate reductase activity under various light conditions

For nitrate reductase activity, *Chlamydomonas* sp. MACC-216 was grown for 48 hours under Blue 250, Blue 125 + Red 125, Red 250, and White 250 light conditions in SWW. After 48 hours, 10 ml of culture was collected from each light condition and centrifuged at 3220 g for 5 minutes at 4°C. Afterwards, supernatants were discarded, and pellets were dissolved in ice-cold 0.1 M phosphate buffer (pH 7.0) followed by sonication at 0.8 cycle, 90 % amplitude for 30 seconds on ice. After sonication, centrifugation was done at 13300 g for 10 minutes at 4°C and supernatants were collected. In a fresh microcentrifuge tube, 500 µL of 10 mM potassium nitrate,

40 µL of 5mM β-Nicotinamide adenine dinucleotide, reduced disodium salt hydrate (Sigma-Aldrich), and 60 µL of Milli-Q water were added to each 400 µL of supernatant followed by incubation at 25°C for 30 minutes. Afterwards, 500 µL of 10 mM zinc acetate was added to stop the reaction followed by centrifugation at 9600 g for 5 minutes. The supernatants were collected for nitrite estimation by Griess reagent assay as described by Giovannoni et al. (1998). For the Griess reagent, two solutions were prepared namely, 1% (w/v) sulphanilamide (Sigma-Aldrich) in 5% (v/v) phosphoric acid and 0.1% (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride (NED) (Sigma-Aldrich) in water. Both solutions are stable for several months at 4°C in dark. To 100 µL of each supernatant, first, 100 µL sulphanilamide solution was added followed by incubation at room temperature for 10 minutes. Then, 100 µL of NED solution was added followed by further incubation at room temperature for 10 minutes. After incubation, absorbance was measured at 540 nm in a Hidex microplate reader.

3.2.4 Quantification of gene expression under various light conditions

For RNA isolation, *Chlamydomonas* sp. MACC-216 was grown for 70 hours under Blue 250, Blue 125 + Red 125, Red 250, and White 250 light conditions in SWW. 1 mL of microalgae sample was collected from each light condition for RNA isolation. RNA isolation was performed using TRIzol™ reagent (Thermo Fisher Scientific) by following manual instructions. RNA quantity was determined using Nanodrop spectrophotometer. cDNA was prepared from isolated RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) by following manual instructions.

To perform quantitative reverse transcription polymerase chain reaction (RT-qPCR), primers for two reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-1 tubulin, as well as five genes involved in nitrate transport and reduction, were designed (Table 3.3). Accession numbers for these genes were obtained from Fernandez and Galvan (2007) and Sanz-Luque et al. (2015a) and sequences were obtained from the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). RT-qPCR was performed with 50 ng of cDNA, 500 nM each of forward and reverse primer, 5 µL of SYBR green PCR master mix (Applied Biosystems™) and nuclease-free water to a final volume of 10 µL.

Table 3.3. List of genes and their primers used for RT-qPCR.

Gene name	NCBI Accession number	Primer (5'→3')
Nitrate Transporter (*NRT1*)	XM_043061965	F: AGGCTCTGCCCCTGATAGA R: CCTCCCATCACATTGCAGA
Nitrate Transporter (*NRT2.1*)	Z25438	F: TGAGAAGCCAGCCACAGTAA R: AAGCAAATCCAGGACAGGTG
Nitrate Transporter (*NRT2.2*)	Z25439	F: CCATCTTCGGCCTTATGAAC R: CGTTAGCGAGTTGCTGACCT
Nitrate reductase (*NIA1*/*NIT1*)	AF203033	F: AGCCGTTGACTTTGACCATG R: GCATGTTCTCCTCCTTGCG
Molybdenum cofactor carrier protein (*MCP1*)	AY039706	F: CATGGCTGGATCTTGCTGAC R: CAGGAAGGACACCGATCGT
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	L27669	F: ATTGGCCGCCTGGTTATG R: GGTCTTGTGGACCGAGTCAT
Beta 1 tubulin	M10064	F: CGCATGATGCTGACCTTCT R: GTCCAGGACCATGCACTCAT

F and R denote forward and reverse primers, respectively.

Analysis of the results obtained from RT-qPCR was done using the Pfaffl method (Pfaffl, 2001). For this, primer efficiency was calculated for all primer pairs. White light condition (White 250) was selected as a control for the analysis. The expression of each gene is provided in terms of the relative gene expression which is based on the expression ratio of a target gene versus the reference genes.

3.2.5 Statistical analysis

The statistical analyses were performed as mentioned in section 3.1.11.

3.3 Objective 3

3.3.1 Growth conditions for transcriptome analysis

Two different kinds of modified TAP media were used for the third objective: TAP-N0 and TAP-N15. TAP-N0 did not consist of any nitrogen source. *Chlamydomonas sp.* MACC-216 and *Chlamydomonas reinhardtii* cc124 were grown in TAP medium for three days. After three days, both algae cultures were centrifuged at 3220 g for 5 minutes. After centrifugation, each microalga was resuspended in 10 ml of TAP-N0 and TAP-N15 media to a final cell density of 6×10^6 cells mL^{-1} followed by further cultivation for six hours. At the end of this period, samples were collected from each culture for nitrate estimation and RNA isolation. Nitrate estimation was performed as mentioned in section 3.1.3.

3.3.2 RNA isolation and sequencing

RNA isolation was performed using TRIzol™ reagent (Thermo Fisher Scientific) by following manual instructions. The purified RNA was quantified using the Qubit RNA Assay. Zymo-Seq RiboFree Total RNA-Seq Library Kit was used for the depletion of ribosomal RNAs. Illumina Nextseq550 was used to generate 150 bp paired end reads.

3.3.3 Transcriptome analysis

Sequenced reads were first processed using rcorrector v1.0.5 (Song and Florea, 2015), a kmer-based error correction method for RNAseq data. The error-corrected reads were then trimmed using Trimmomatic v0.39 (Bolger et al., 2014) to remove adapter sequences while keeping a quality score of 25 over a 5 bp sliding window. Reads shorter than 50 bp were discarded from further analysis. The trimmed reads were then mapped to reference *Chlamydomonas reinhardtii* v5.6 transcripts downloaded from Phytozome using Kallisto v0.46.1. Genes with a log2 fold change of 1 and adjusted p-value < 0.05 were identified as differentially expressed genes. Gene ontology (GO) enrichment was carried out using TopGO v2.46.0 package in Rstudio using ontology ids retrieved from biomart v2.50.3 package. Only terms with Benjamini and Hochberg adjusted p-value < 0.05 or smaller were considered enriched terms.

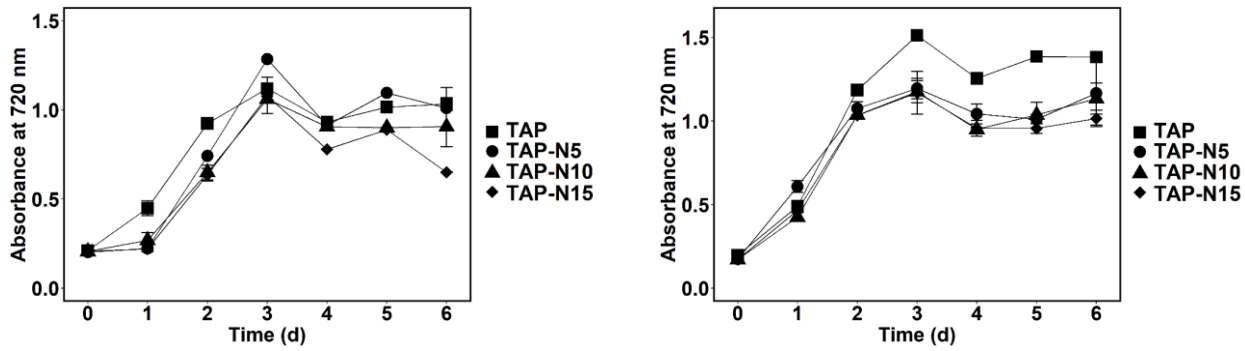
4 RESULTS

4.1 Objective 1

4.1.1 Influence of nitrate on the growth parameters

Both *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360 were first screened in modified TAP supplemented with various concentrations of nitrate as the sole nitrogen source for the growth, i.e., 1 mM, 5 mM, 10 mM, 15 mM, 20 mM, 40 mM, 50 mM, 75 mM and 100 mM (Figure S1a, b). After the screening, three concentrations of nitrate i.e., 5 mM, 10 mM, and 15 mM were selected for further experiments.

For both microalgae, maximum absorbance at 720 nm was observed on the third day in TAP, TAP-N5, TAP-N10, and TAP-N15 media (Figure 4.1a, b). TAP-N15 medium resulted in the least growth for both microalgae. Furthermore, there was a big difference between the growth of *Chlorella* sp. MACC-360 cultivated in TAP and TAP-N5/TAP-N10/TAP-N15 media. Based on the growth curve, it was determined that the high nitrate concentration affected the growth of *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360.



(a) **(b)**

Figure 4.1. Growth of *Chlamydomonas* sp. MACC-216 (a) and *Chlorella* sp. MACC-360 (b) in TAP, TAP-N5, TAP-N10, and TAP-N15 media. Error bars represent standard deviations.

Table 4.1 depicts the growth characteristics of *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360. No significant results were obtained for specific growth rates in both microalgae. The specific growth rate of *Chlamydomonas* sp. MACC-216 was highest in TAP-N5 and TAP-N10 media, whereas *Chlorella* sp. MACC-360 exhibited the highest specific growth rate in TAP. Both microalgae exhibited the least specific growth rate in TAP-N15 media. Additionally, in the presence of nitrate, there was an increase in the cell size of *Chlorella* sp. MACC-360 (Table 4.1).

Table 4.1. Growth parameters of *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360 in TAP, TAP-N5, TAP-N10 and TAP-N15 media. Values are represented as mean ± standard deviation.

Medium	*Chlamydomonas* sp. MACC-216				*Chlorella* sp. MACC-360			
	Number of generations (n)	Mean generation time (g)	Specific growth rate day^{-1} (μ)	Cell size (μm)	Number of generations (n)	Mean generation time (g)	Specific growth rate day^{-1} (μ)	Cell size (μm)
TAP	3.2 ± 0.0^{a}	0.6 ± 0.0^{a}	1.1 ± 0.0^{a}	24.2 ± 2.1^{a}	2.3 ± 0.1^{a}	0.9 ± 0.0^{a}	0.8 ± 0.0^{a}	12.9 ± 1.2^{c}
TAP-N5	3.5 ± 0.01^{a}	0.6 ± 0.0^{a}	1.2 ± 0.0^{a}	25.5 ± 2.8^{a}	2.1 ± 0.5^{a}	1 ± 0.2^{a}	0.7 ± 0.2^{a}	13.9 ± 2^{b}
TAP-N10	3.4 ± 0.5^{a}	0.6 ± 0.1^{a}	1.2 ± 0.1^{a}	25.1 ± 2.9^{a}	1.9 ± 0.0^{a}	1.1 ± 0.0^{a}	0.7 ± 0.0^{a}	15.2 ± 2.5^{a}
TAP-N15	2.9 ± 0.3^{a}	0.7 ± 0.1^{a}	1 ± 0.1^{a}	25.3 ± 3.3^{a}	1.7 ± 0.1^{a}	1.2 ± 0.1^{a}	0.6 ± 0.1^{a}	14.5 ± 1.7^{ab}

Superscript lowercase letters signify statistical differences (p-value < 0.05) as determined by Tukey's-test. Tukey's-test was done for each microalga separately to determine the statistical significance among various media.

4.1.2 Nitrate removal by Chlamydomonas sp. MACC-216 and Chlorella sp. MACC-360

Nitrate removal was determined on a daily basis by *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360 in TAP-N5, TAP-N10, and TAP-N15 media for six days. The removal of nitrate by both microalgae was quick during the first three days but subsequently slowed down (Figure 4.2a, b). Furthermore, it was observed that *Chlamydomonas* sp. MACC-216 was better compared to *Chlorella* sp. MACC-360 at nitrate removal (Table 4.2).

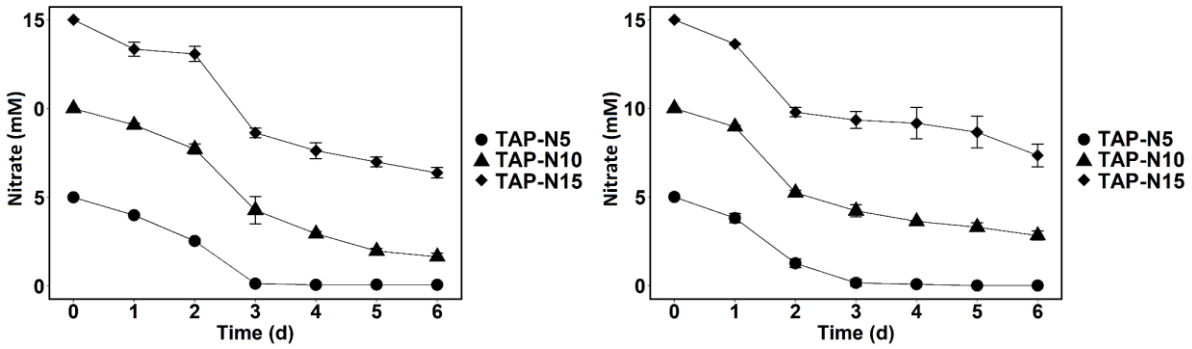


Figure 4.2. Nitrate removal by *Chlamydomonas* sp. MACC-216 (a) and *Chlorella* sp. MACC-360 (b) from TAP-N5, TAP-N10, and TAP-N15 media. Error bars represent standard deviations.

Table 4.2. Total nitrate removal by *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360 after six days. Values are represented as mean ± standard deviation.

Medium	Nitrate removal efficiency (%)	
	Chlamydomonas sp. MACC-216	*Chlorella* sp. MACC-360
TAP-N5	100 ± 0.0^{c}	100 ± 0.0^{c}
TAP-N10	84.1 ± 2.1^{b}	72.2 ± 2.5^{b}
TAP-N15	53.6 ± 1.8^{a}	51.2 ± 4.3^{a}

Superscript lowercase letters signify statistical differences (p-value < 0.05) as determined by Tukey's-test. Tukey's-test was done for each microalga separately to determine the statistical significance among various media.

The removal of nitrate by both microalgae in TAP-N5 medium was 100 % by the third day. On the sixth day, *Chlamydomonas* sp. MACC-216 exhibited nitrate removal efficiencies of ~84% and ~53% in TAP-N10 and TAP-N15, respectively, while *Chlorella* sp. MACC-360 exhibited nitrate removal efficiencies of ~72% in TAP-N10 medium and ~51% in TAP-N15 medium.

Table 4.3. Nitrate removal rate of *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360 at three different time points. Values are represented as mean ± standard deviation.

Medium	Nitrate removal rate (nmol cell^{-1} h^{-1})					
	Chlamydomonas sp. MACC-216			*Chlorella* sp. MACC-360		
	3 h	6 h	9 h	3 h	6 h	9 h
TAP-N5	36.7 ± 2.8^{a}	61 ± 1.7^{a}	47.4 ± 1.3^{b}	6.6 ± 0.9^{b}	11.3 ± 0.0^{a}	10.7 ± 0.1^{c}
TAP-N10	30.1 ± 13.4^{a}	55.9 ± 2.8^{a}	53.9 ± 1.1^{ab}	3.8 ± 0.1^{c}	11.4 ± 0.4^{a}	12.3 ± 0.0^{b}
TAP-N15	25.6 ± 5.2^{a}	55 ± 5.3^{a}	59 ± 3.6^{b}	10 ± 0.3^{a}	12.3 ± 0.01^{a}	13.3 ± 0.2^{a}

Superscript lowercase letters signify statistical differences (p-value < 0.05) as determined by Tukey's-test. Tukey's-test was done for each microalga separately to determine the statistical significance among various media.

Chlamydomonas sp. MACC-216 had a higher nitrate removal rate than *Chlorella* sp. MACC-360 from the tested media, as determined by calculating the nitrate removal rates of both microalgae (Table 4.3). From nitrate removal rate calculations, it was observed that *Chlamydomonas* sp. MACC-216 showed concentration-dependent nitrate removal at a 9 h time point only, whereas *Chlorella* sp. MACC-360 showed a concentration-dependent nitrate removal rate at 6 h and 9 h time points. The only concentration-dependent nitrate removal rate that was found to be significant was of *Chlorella* sp. MACC-360 at 9 h time point.

4.1.3 ROS production in Chlorella sp. MACC-360

ROS production in both microalgae was measured using DCF (2′,7′-dichlorofluorescein) fluorescence. In both microalgae, a time-dependent increase in DCF fluorescence was observed. *Chlamydomonas* sp. MACC-216 exhibited less DCF fluorescence in TAP-N5, TAP-N10, and TAP-N15 media compared to standard TAP medium, indicating that nitrate did not cause significant stress in this particular microalga (Figure 4.3a). However, *Chlorella* sp. MACC-360 exhibited a significant increase in DCF fluorescence when grown in TAP-N15 medium, indicating that 15 mM nitrate caused *Chlorella* sp. MACC-360 to experience significant stress (Figure 4.3b).

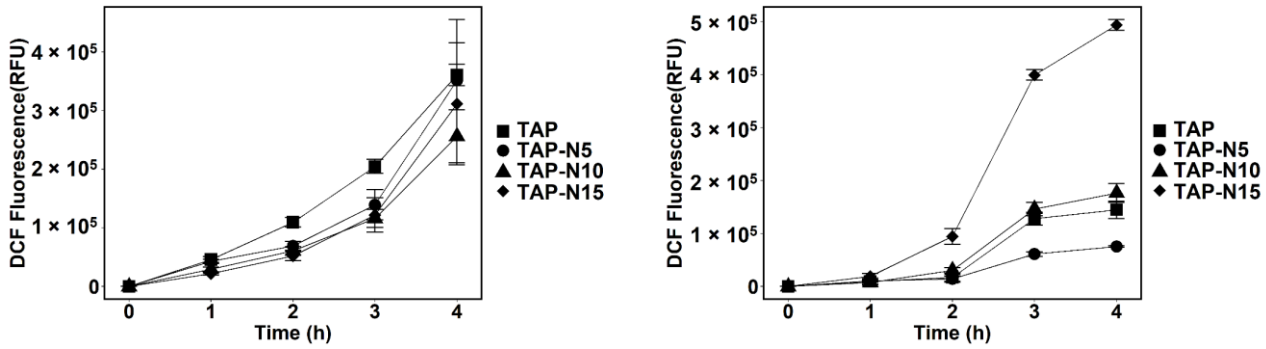


Figure 4.3. ROS production in *Chlamydomonas* sp. MACC-216 (a) and *Chlorella* sp. MACC-360 (b). Error bars represent standard deviations.

4.1.4 Nitrate affects total pigments production

3-day-old cultures of both *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360 were used to extract total pigments. As the nitrate concentration increased, the total pigments content of both *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360 decreased (Figure 4.4a, b). However, in the case of *Chlamydomonas* sp. MACC-216, this decrease in the amount of pigments among different media was not found to be significant, whereas the decrease in chlorophyll-a and carotenoids in *Chlorella* sp. MACC-360 was significant. The amount of total pigments in *Chlamydomonas* sp. MACC-216 was found to be generally greater than that of *Chlorella* sp. MACC-360. Moreover, *Chlorella* sp. MACC-360 contained notably less chlorophyll-b than *Chlamydomonas* sp. MACC-216.

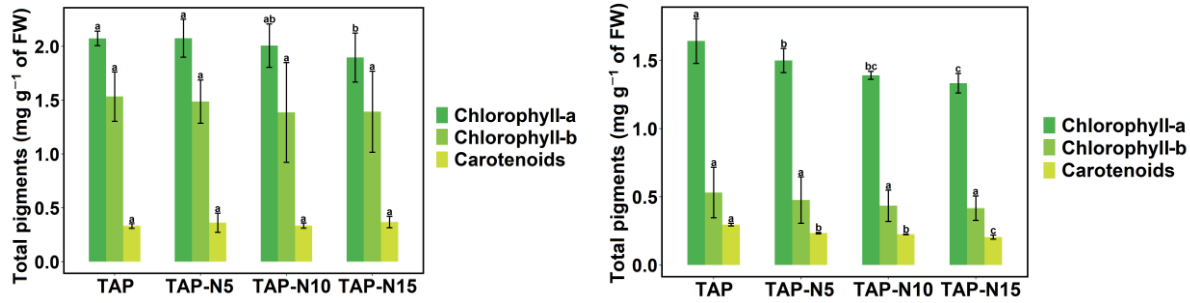
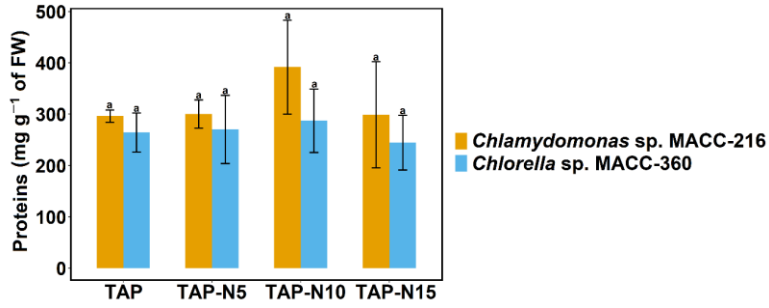


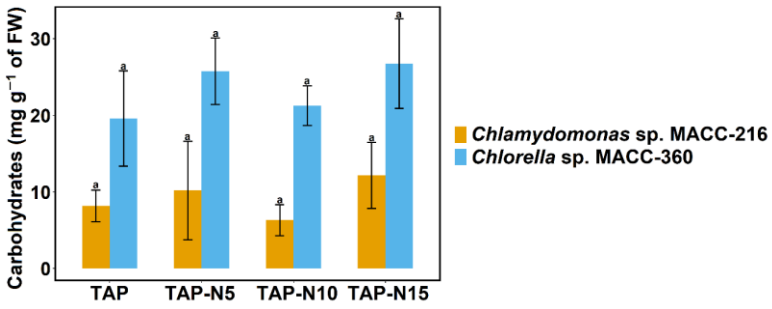
Figure 4.4. Total pigments of *Chlamydomonas* sp. MACC-216 (a) and *Chlorella* sp. MACC-360 (b) in TAP, TAP-N5, TAP-N10, and TAP-N15 media. Error bars represent standard deviations. Tukey's-test was done for each pigment separately. Lowercase letters signify statistical differences (p-value < 0.05) as determined by Tukey's-test. FW: Fresh weight.

4.1.5 Effect of nitrate on total protein and carbohydrate contents

The total protein contents of *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360 increased when the concentration of nitrate was increased from 5 mM to 10 mM and decreased when the concentration was further increased from 10 mM to 15 mM, but these changes were not statistically significant. *Chlamydomonas* sp. MACC-216 produced a greater amount of total proteins than *Chlorella* sp. MACC-360 (Figure 4.5a). The total protein contents of *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360 grown in TAP, TAP-N5, TAP-N10, and TAP-N15 media were not significantly different. Nitrate had no effect on the total carbohydrate content of either *Chlamydomonas* sp. MACC-216 or *Chlorella* sp. MACC-360. Similar to the protein content, no statistically significant difference in total carbohydrate content was observed among TAP, TAP-N5, TAP-N10, and TAP-N15 samples of both microalgae (Figure 4.5b). *Chlorella* sp. MACC-360 did exhibit a higher total carbohydrate content than *Chlamydomonas* sp. MACC-216 (Figure 4.5b).



(a)



(b)

Figure 4.5. Total protein (a) and carbohydrate (b) contents of *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360. Error bars represent standard deviations. Error bars represent standard deviations. Tukey's-test was done for each microalga separately. Lowercase letters signify statistical differences (p-value < 0.05) as determined by Tukey's-test. FW: Fresh weight.

4.1.6 Nitrate increases lipid content in Chlamydomonas sp. MACC-216

To determine whether nitrate affects lipid production in *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360, total lipid content was measured. Two distinct methods were used to determine lipid accumulation in the microalgae of interest. First, BODIPY dye was used for the labelling of neutral lipids inside the microalgae cells, while the second method was the quantification of extracted lipids using the sulpho-phospho-vanillin reagent assay.

The fluorescence of the BODIPY dye increased with increasing nitrate concentration in *Chlamydomonas* sp. MACC-216, indicating the presence of an increased amount of lipids (Figure 4.6a-d). The maximum fluorescence was observed in *Chlamydomonas* sp. MACC-216 grown in TAP-N15 medium (Figure 4.6d). In comparison, nitrate triggered BODIPY fluorescence in a small percentage of *Chlorella* sp. MACC-360 cells (5-10 per 100 cells), but in TAP medium, no cells fluoresced (Figure 4.6e-h).

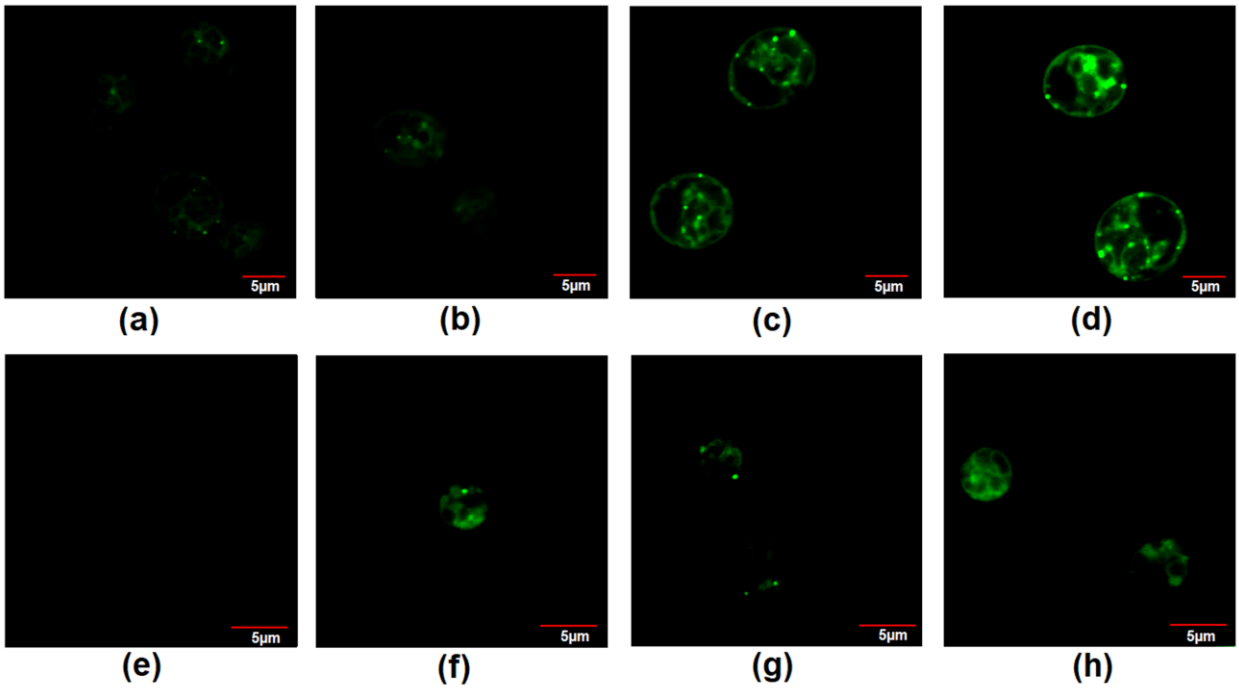


Figure 4.6. Staining of neutral lipids by BODIPY dye in *Chlamydomonas* sp. MACC-216 grown in TAP (a), TAP-N5 (b), TAP-N10 (c), TAP-N15 (d) media and *Chlorella* sp. MACC-360 grown in TAP (e), TAP-N5 (f), TAP-N10 (g), TAP-N15 (h) media.

In *Chlamydomonas* sp. MACC-216, the total lipid content increased from 19.66 mg g^{-1} of FW in TAP-grown microalgae to 37.51 mg g^{-1} of FW in TAP-N15-grown microalgae (Figure 4.7). The total lipid content of *Chlorella* sp. MACC-360 microalgae cultivated in TAP, TAP-N5, TAP-N10, and TAP-N15 media did not differ significantly (Figure 4.7).

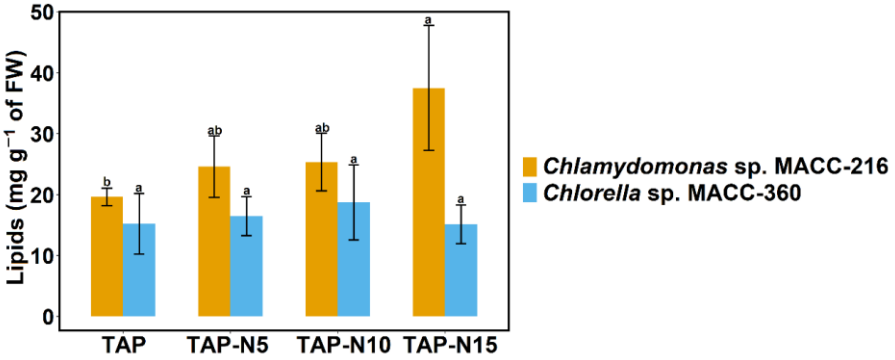


Figure 4.7. Total lipid contents of *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360 grown in TAP, TAP-N5, TAP-N10 and TAP-N15 media. Error bars represent standard deviations. Tukey's-test was done for each microalga separately. Lowercase letters signify statistical differences (p-value < 0.05) as determined by Tukey's-test. FW: Fresh weight.

4.1.7 Growth and nitrate removal in synthetic wastewater

In order to test the efficacy of axenic *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360 at removing nitrate from a wastewater model system, SWW was prepared with varying nitrate concentrations (5 mM, 10 mM, 25 mM, and 50 mM). *Chlamydomonas* sp. MACC-216 grew more efficiently in 5 mM and 10 mM SWW than in 25 mM and 50 mM and exhibited the least amount of growth in 50 mM SWW (Figure 4.8a). *Chlorella* sp. MACC-360 grew more efficiently in SWW with 25 mM and 50 mM nitrate than in SWW with 5 mM and 10 mM nitrate (Figure 4.8b).

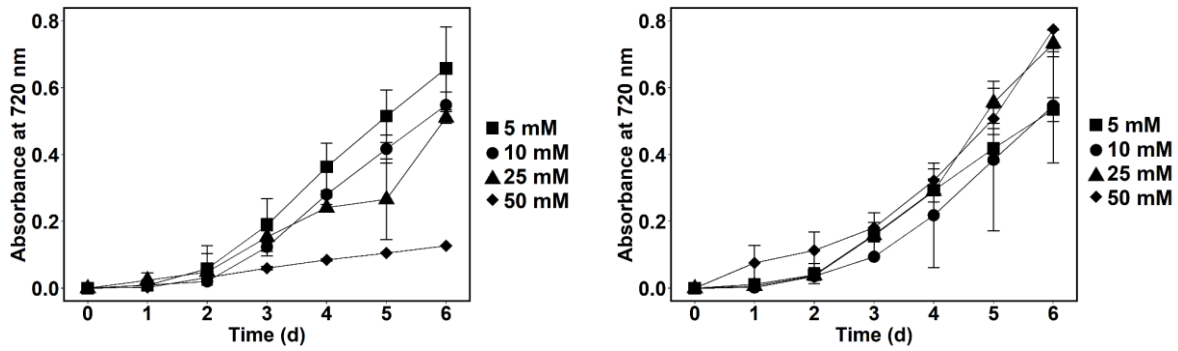


Figure 4.8. Growth of *Chlamydomonas* sp. MACC-216 (a) and *Chlorella* sp. MACC-360 (b) in SWW supplemented with 5 mM, 10 mM, 25 mM, and 50 mM nitrate. Error bars represent standard deviations.

Nonetheless, *Chlamydomonas* sp. MACC-216 outperformed *Chlorella* sp. MACC-360 in nitrate removal. On the sixth day, *Chlamydomonas* sp. MACC-216 demonstrated a nitrate

removal efficiency of ~35% in 50 mM SWW, whereas *Chlorella* sp. MACC-360 only demonstrated a nitrate removal efficiency of ~28%. The removal of total nitrate by both microalgae increased with increasing nitrate concentration in the SWW (Table 4.4). Though increased nitrate removal was observed with increasing nitrate concentration in both microalgae, no significant difference was observed between the nitrate removal efficiency of both microalgae, except at 50 mM nitrate concentration. At 50 mM nitrate concentration, *Chlamydomonas* sp. MACC-216 showed significantly higher nitrate removal efficiency than *Chlorella* sp. MACC-360.

Table 4.4. Total nitrate removal by *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360 in SWW supplemented with 5 mM, 10 mM, 25 mM and 50 mM nitrate after six days. Values are represented as mean ± SD.

Concentration of nitrate in SWW	*Chlamydomonas* sp. MACC-216		*Chlorella* sp. MACC-360	
	Total nitrate removal (mM)	Nitrate removal efficiency (%)	Total nitrate removal (mM)	Nitrate removal efficiency (%)
5 mM	1.8 ± 0.2^{a}	35.8 ± 4.8^{a}	1.6 ± 0.1^{a}	31.9 ± 2.8^{a}
10 mM	2.8 ± 0.2^{a}	27.9 ± 1.7^{a}	3.7 ± 0.3^{a}	37.1 ± 3.1^{a}
25 mM	9.5 ± 0.9^{a}	38 ± 4^{a}	8.1 ± 0.8^{a}	32.6 ± 3.1^{a}
50 mM	17.3 ± 0.8^{a}	34.6 ± 1.6^{a}	13.8 ± 0.8^{b}	27.6 ± 1.5^{b}

Superscript lowercase letters signify statistical differences (p-value < 0.05) as determined by Tukey's-test. Tukey's-test was performed for the comparison between the two microalgae at each concentration.

4.2 Objective 2

4.2.1 Growth of Chlamydomonas sp. MACC-216 under various light conditions

The growth of *Chlamydomonas* sp. MACC-216 was mainly influenced by the light intensity in TAP-N5 and TAP-N10 media (Figure 4.9). *Chlamydomonas* sp. MACC-216 grew more slowly at 50 µmol m^{-2} s^{-1} light intensity compared to 100 µmol m^{-2} s^{-1} and 250 µmol m^{-2} s^{-1} light intensities. It was also observed that up to 250 µmol m^{-2} s^{-1}, the growth of *Chlamydomonas* sp. MACC-216 is directly proportional to light intensity, as the microalgal growth increased when light intensity was increased from 50 µmol m^{-2} s^{-1} to 250 µmol m^{-2} s^{-1}. The growth curves of *Chlamydomonas* sp. MACC-216 grown in TAP-N5 and TAP-N10 media under any light condition did not differ significantly.

4.2.2 Nitrate removal efficiency under various light conditions

Chlamydomonas sp. MACC-216 grown under TAP-N5 medium exhibited the highest nitrate removal efficiency under Blue 25 + Red 25 and Red 50 light conditions (Figure 4.10). Least nitrate removal efficiency was observed in the case of the White 50 light condition. Furthermore, increasing the light intensity from 50 µmol m^{-2} s^{-1} to 100 µmol m^{-2} s^{-1} enhanced the nitrate removal efficiency. However, when light intensity was increased to either 100 µmol

m^{-2} s^{-1} or 250 µmol m^{-2} s^{-1}, no significant difference in nitrate removal efficiency was observed among various light conditions.

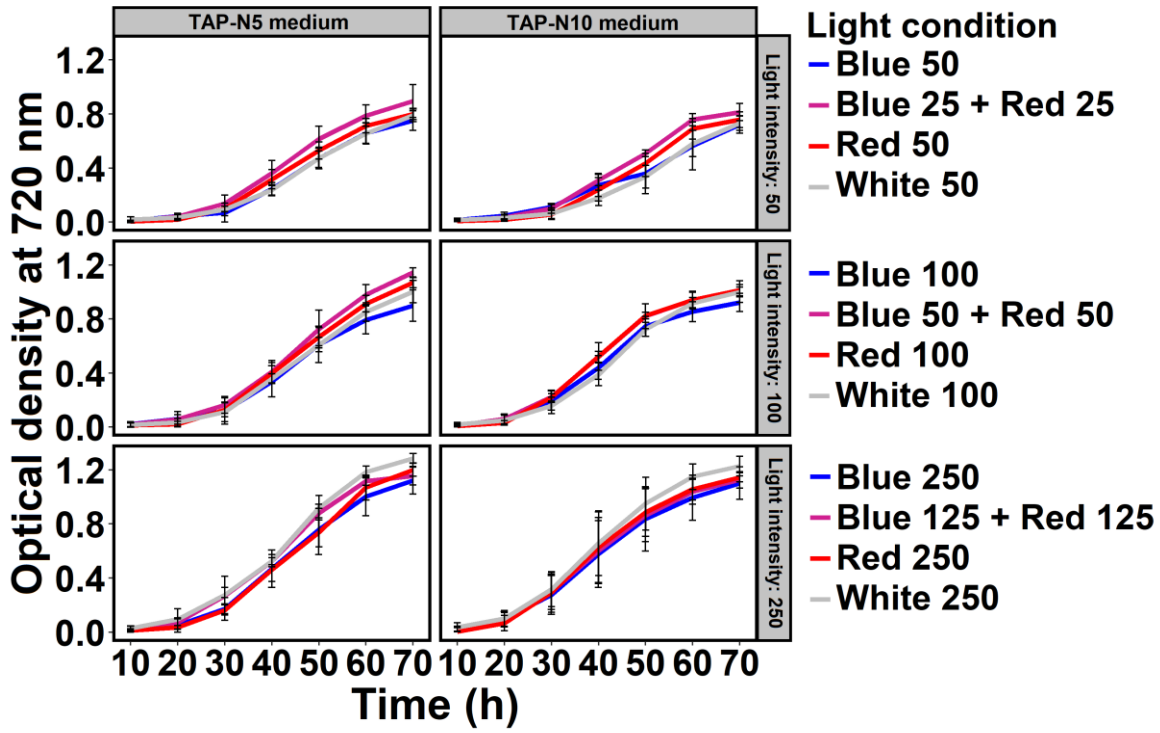


Figure 4.9. Growth of *Chlamydomonas* sp. MACC-216 under various light conditions in TAP-N5 and TAP-N10 media. Numbers 25, 50, 100, 125, and 250 mentioned in the figure represent light intensity. The unit of light intensity is µmol m^{-2} s^{-1}.

When *Chlamydomonas* sp. MACC-216 was cultivated in TAP-N10 medium at 50 µmol m^{-2} s^{-1} light intensity, no significant difference in nitrate removal efficiency was observed among the various light conditions (Blue 50, Blue 25 + Red 25, Red 50, and White 50). An increase in the light intensity from 50 µmol m^{-2} s^{-1} to 250 µmol m^{-2} s^{-1} led to significantly higher nitrate removal efficiency. Thus, *Chlamydomonas* sp. MACC-216 grown under Blue 250 removed more nitrate than *Chlamydomonas* sp. MACC-216 grown under Blue 50 and so on. A clear role of light intensity was observed in total nitrate removal as higher nitrate removal efficiency was observed at higher light intensity. Moreover, compared to all other light conditions, *Chlamydomonas* sp. MACC-216 grown under Blue 125 + Red 125 exhibited significantly higher nitrate removal efficiency in TAP-N10 medium. *Chlamydomonas* sp. MACC-216 removed 9.64 mM nitrate when grown under Blue 125 + Red 125 light condition (Table S1). At 250 µmol m^{-2} s^{-1} light intensity, the least nitrate removal efficiency was observed under Blue 250 light condition.

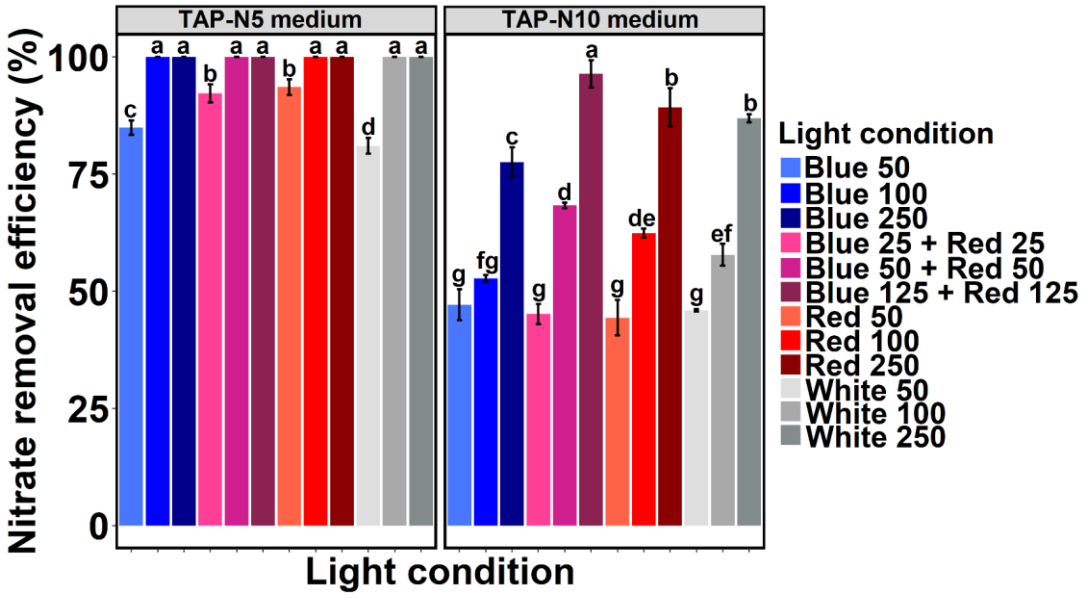


Figure 4.10. Nitrate removal efficiency of *Chlamydomonas* sp. MACC-216 under various light conditions in TAP-N5 and TAP-N10 media. Numbers 25, 50, 100, 125, and 250 mentioned in the figure represent light intensity. The unit of light intensity is µmol m^{-2} s^{-1}. Error bars represent standard deviations. Tukey's-test was done for each nitrate concentration separately. Lowercase letters signify statistical differences (p-value < 0.05) as determined by Tukey's-test.

4.2.3 Growth, nitrate removal efficiency and nitrate reductase activity in SWW

The light intensity 250 µmol m^{-2} s^{-1} was chosen for SWW experiments because *Chlamydomonas* sp. MACC-216 exhibited the highest growth and nitrate removal efficiency in TAP-N5 and TAP-N10 media under this light intensity. The growth curves of *Chlamydomonas* sp. MACC-216 grown under Blue 250, Blue 125 + Red 125, Red 250, and White 250 light conditions did not differ significantly (Figure 4.11a). However, at 250 µmol m^{-2} s^{-1} light intensity, *Chlamydomonas* sp. MACC-216 grew slower in SWW (Figure 4.11a) than in TAP-N5 or TAP-N10 medium (Figure 4.9). Even in SWW, *Chlamydomonas* sp. MACC-216 showed the highest nitrate removal efficiency under Blue 125 + Red 125 light condition (Figure 4.11b). In contrast to *Chlamydomonas* sp. MACC-216 grown under Red 250 and White 250 light conditions, which removed 3.67 mM and 3.60 mM of nitrate, respectively, *Chlamydomonas* sp. MACC-216 grown under Blue 125 + Red 125 light condition removed 4.22 mM (Table S2). The least amount of nitrate was removed by *Chlamydomonas* sp. MACC-216 grown under Blue 250 light condition (3.51 mM).

To examine the impact of various lighting conditions on the nitrate reductase activity of *Chlamydomonas* sp. MACC-216, a quantitative analysis was performed. The highest nitrate

reductase activity was found in *Chlamydomonas* sp. MACC-216 grown under Blue 125 + Red 125 light, compared to any other lighting conditions used in the experiment (Figure 4.11c). Nitrate reductase of *Chlamydomonas* sp. MACC-216 grown under Blue + Red 125 light condition produced 31.09 µmol g^{-1} of FW min^{-1} NO_2^-, whereas *Chlamydomonas* sp. MACC-216 grown under Blue 250, Red 250, and White 250 light conditions produced 18.47 µmol g^{-1} of FW min^{-1} NO_2^-, 28.41 µmol g^{-1} of FW min^{-1} NO_2^-, and 25.4 µmol g^{-1} of FW min^{-1} NO_2^-, respectively (Table S2).

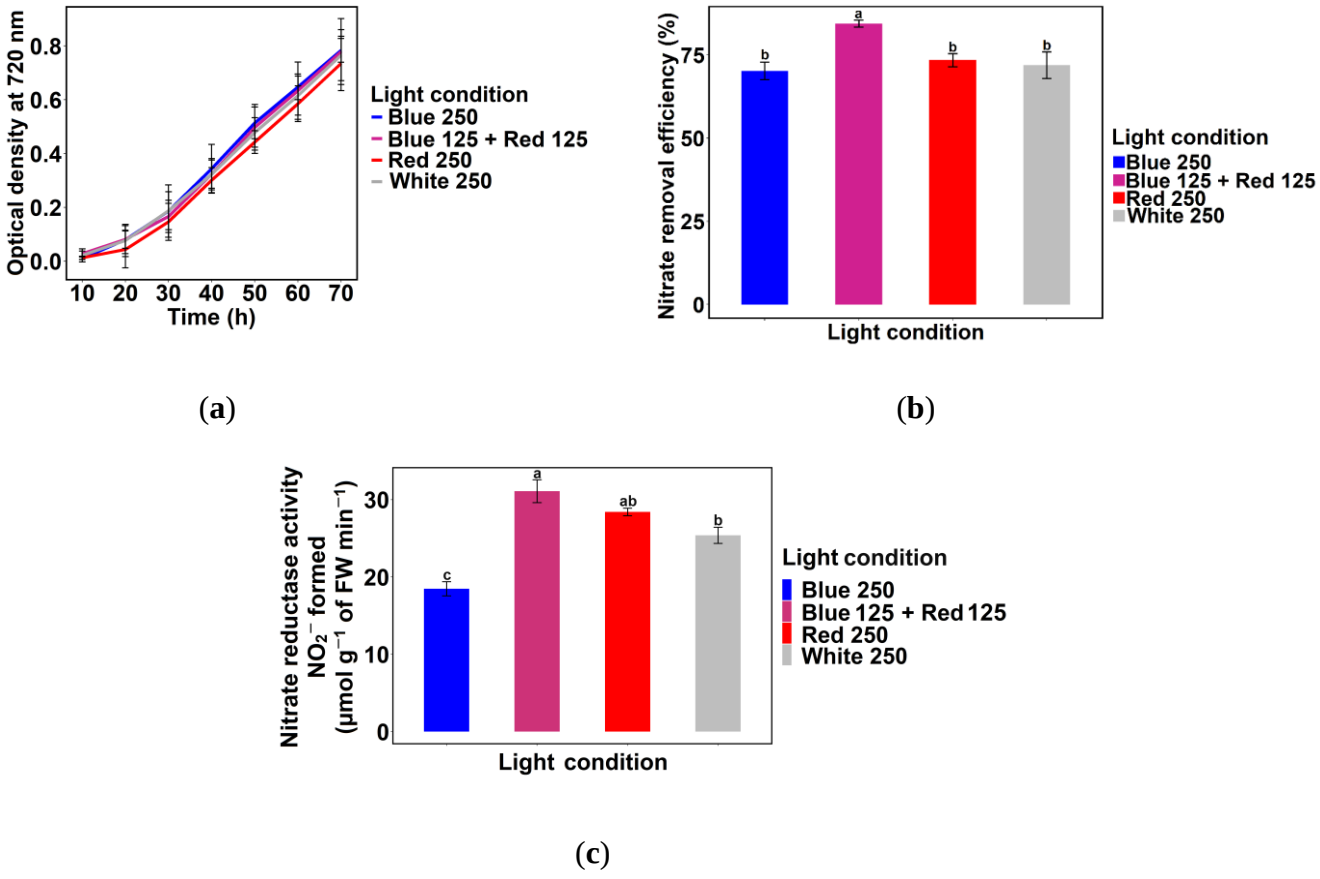


Figure 4.11. Growth (a), nitrate removal efficiency (b), and nitrate reductase activity (c) of *Chlamydomonas* sp. MACC-216 grown under various light conditions in SWW. Error bars represent standard deviations. Lowercase letters signify statistical differences (p-value < 0.05) as determined by Tukey's-test. FW: Fresh weight.

4.2.4 Expression of genes involved in nitrate transport and reduction

The expression of genes involved in nitrate transport and reduction in *Chlamydomonas* sp. MACC-216 was investigated. Five genes were selected namely, *NRT1*, *NRT2*.1, *NRT2*.2, *NIA1*, and *MCP1* where *NRT1*, *NRT2*.1, *NRT2*.2 code for nitrate and/or nitrite transporters, *NIA1* codes for nitrate reductase, and *MCP1* codes for moco carrier protein. Expression of

NRT1, *NRT2*.1, *NRT2.2*, *NIA1*, and *MCP1* was significantly higher in *Chlamydomonas* MACC-216 grown under the Blue 125 + Red 125 light condition (Figure 4.12 and Table S3). After the Blue 125 + Red 125 light condition, *NRT1*, *NRT2*.1, *NRT2.2*, *NIA1*, and *MCP1* genes showed high expression under the Red 250 light condition.

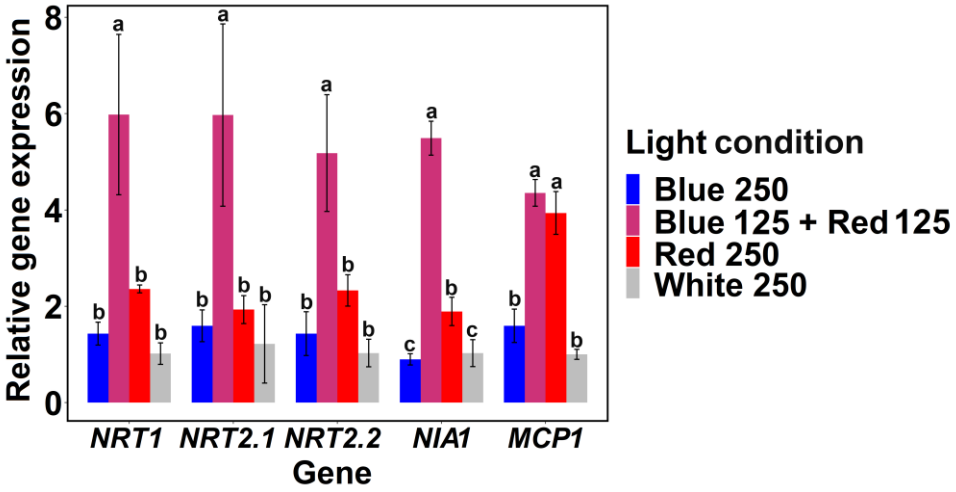


Figure 4.12. Relative gene expression of *NRT1*, *NRT2.1*, *NRT2.2*, *NIA1*, and *MCP1* genes in *Chlamydomonas* sp. MACC-216 grown under various light conditions in SWW. Error bars represent standard deviations. Tukey's-test was done for each gene separately. Lowercase letters signify statistical differences (p-value < 0.05) as determined by Tukey's-test.

4.2.5 Growth and nitrate removal efficiency of Chlorella spp. in SWW

Chlorella sp. MACC-38 and *Chlorella* sp. MACC-360 were cultivated in SWW under Blue 250, Blue 125 + Red 125, Red 250, and White 250 light conditions to determine if they would perform similarly to *Chlamydomonas* sp. MACC-216. No major difference was observed among the growth curves of *Chlorella* sp. MACC-38 grown under various light conditions (Figure 4.13a), whereas *Chlorella* sp. MACC-360 grew better under the Blue 125 + Red 125 light condition in comparison to other light conditions (Figure 4.13b). Under Blue 125 + Red 125 light conditions, only *Chlorella* sp. MACC-360 exhibited an exceptionally high nitrate removal efficiency (Figure 4.13c, d). Even under Blue 125 + Red 125 light condition, both microalgae removed less nitrate in comparison to *Chlamydomonas* sp. MACC-216. *Chlorella* sp. MACC-38 and *Chlorella* sp. MACC-360 removed 1.67 mM and 1.37 mM nitrate, respectively, whereas *Chlamydomonas* sp. MACC-216 removed 4.22 mM nitrate under the same light conditions (Tables S2 and S4). Both microalgae removed the least nitrate under Red 250 and White 250 light conditions.

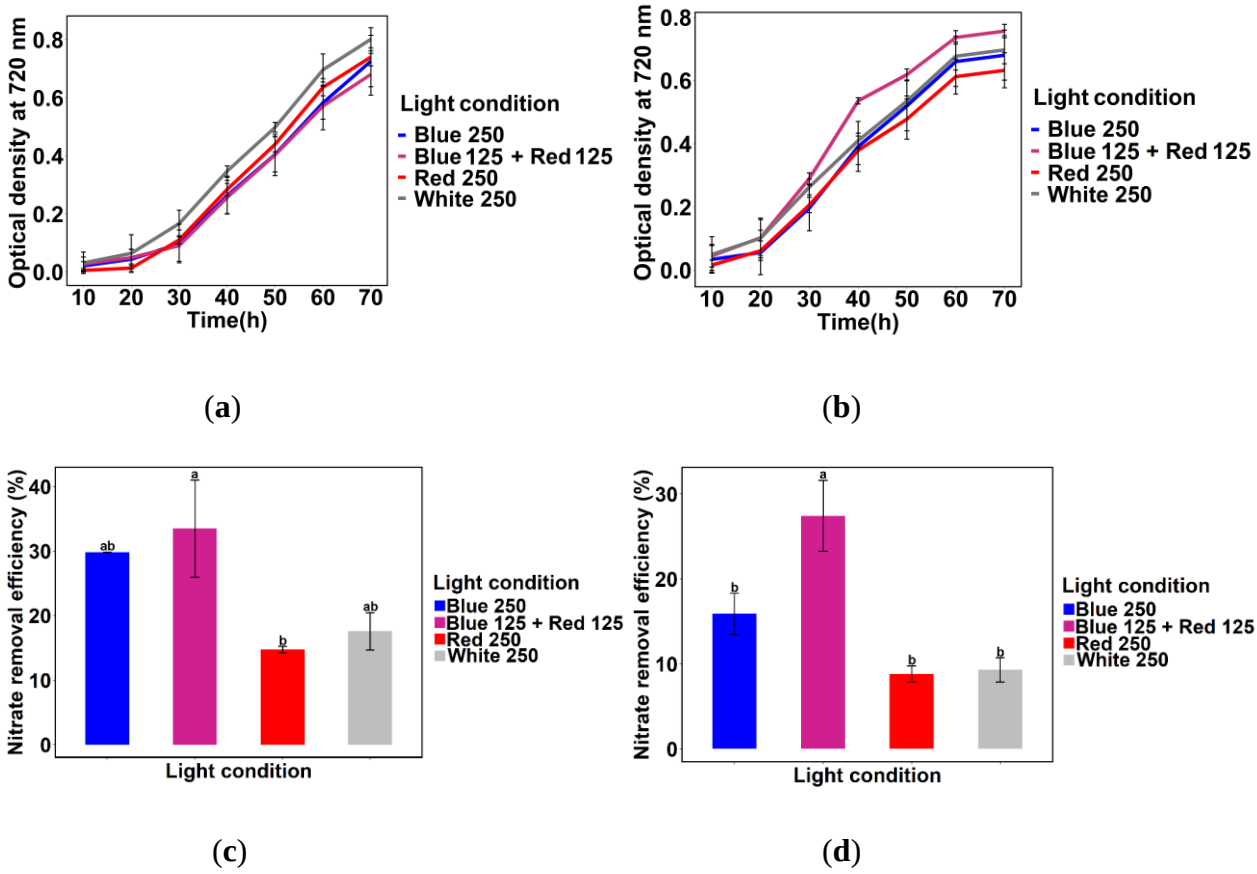


Figure 4.13. Growth of *Chlorella* sp. MACC-38 (a) and *Chlorella* sp. MACC-360 (b) under various light conditions in SWW. Nitrate removal efficiency of *Chlorella* sp. MACC-38 (c) and *Chlorella* sp. MACC-360 (d) under various light conditions in SWW. Error bars represent standard deviations. Lowercase letters in (c, d) signify statistical differences (p-value < 0.05) as determined by Tukey's-test.

4.3 Objective 3

4.3.1 Growth and nitrate removal efficiency

The short-term changes in gene expression in response to the absence and presence of nitrate were studied by cultivating *Chlamydomonas* sp. MACC-216 and *C. reinhardtii* cc124 for 6 hours in TAP-N0 (nitrate-deplete) and TAP-N15(nitrate-replete) growth media or conditions. The initial cell density of both microalgae in both conditions was 6 x 10^6 cells mL^{-1}. No significant change in cell number for *Chlamydomonas* sp. MACC-216 was observed in either of the conditions after 6 hours (Table 4.5). However, in the case of *C. reinhardtii* cc124, there was a significant decrease in cell number in TAP-N15 condition after 6 hours, whereas there was no significant change in the TAP-N0 condition. Furthermore, *Chlamydomonas* sp.

MACC-216 showed ~22% nitrate removal efficiency from the TAP-N15 medium, whereas *C. reinhardtii* cc124 showed only ~4% nitrate removal efficiency.

Table 4.5. Cell density and nitrate removal efficiency under TAP-N0 and TAP-N15 growth conditions. Values are represented as mean ± SD.

Microalgae	Growth condition	Cell density after 6 hours ($\times 10^6$ cells mL^{-1})	Nitrate removal efficiency (%)
***C. reinhardtii* cc124**	TAP-N0	6.39 ± 0.07	–
	TAP-N15	4.61 ± 0.63	4.21 ± 1.87
***Chlamydomonas* sp. MACC-216**	TAP-N0	6.24 ± 0.7	–
	TAP-N15	5.98 ± 0.02	21.52 ± 2.09

4.3.2 Reads trimming and mapping

RNA sequencing generated paired reads of 150 bp size. Raw paired reads were error corrected to obtain a total of 39 million read pairs from TAP-N0 and 35 million read pairs from TAP-N15 samples in *C. reinhardtii* cc124. A total of 33 million read pairs from TAP-N0 and around 35 million read pairs from TAP-N15 were obtained from *Chlamydomonas* sp. MACC-216 samples. After trimming using Trimmomatic v0.39, around 30 million read pairs from TAP-N0 and 27 million read pairs from TAP-N15 were obtained in *C. reinhardtii* cc124, whereas approximately 26 million read pairs from TAP-N0 and 27 million read pairs from TAP-N15 were obtained in *Chlamydomonas* sp. MACC-216. Finally, trimmed read pairs were mapped against *Chlamydomonas reinhardtii* v5.6 transcripts.

4.3.3 Cross-species comparison through PCA plot

Principal component analysis (PCA) of transcript abundances for all samples was done to visualize similarity in gene expression pattern. All replicates from TAP-N0 and TAP-N15 conditions used for *C. reinhardtii* cc124 clustered together which indicates no major transcriptional reorganization. However, in *Chlamydomonas* sp. MACC-216, replicates from each TAP-N0 and TAP-N15 condition clustered together but were separated by condition, indicating significant transcriptional changes between the two conditions (Figure 4.14). Based on these results, the differential expression pattern between the two species was analyzed separately.

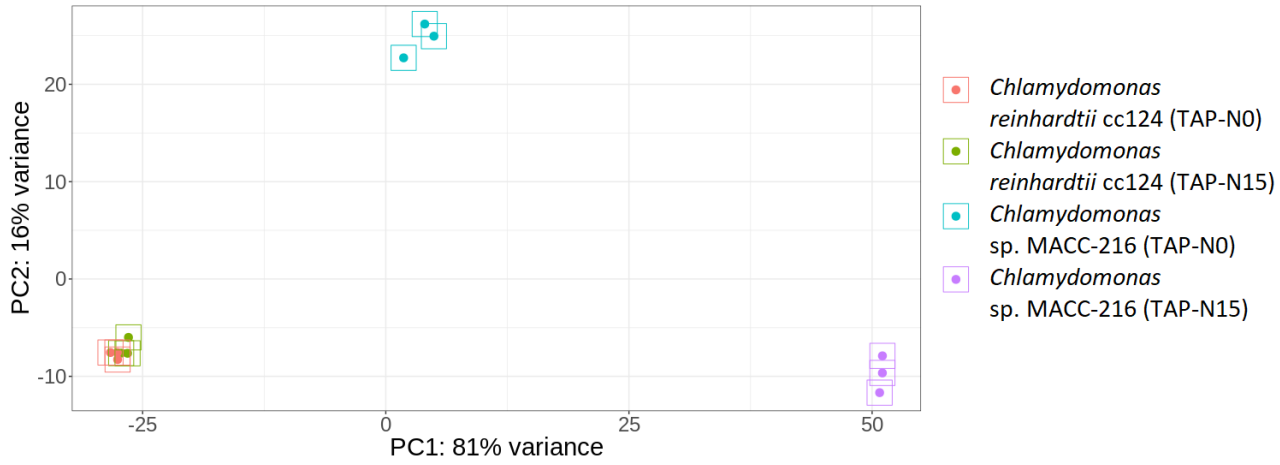


Figure 4.14. PCA plot showing the variation between samples from TAP-N0 and TAP-N15 conditions in *C. reinhardtii* cc124 and *Chlamydomonas* sp. MACC-216.

4.3.4 Differential gene expression and gene ontology enrichment analysis

Differential gene expression analysis was conducted by using TAP-N0 condition as the control and TAP-N15 condition as the treatment condition. The PCA plot revealed low variability between the two conditions in *C. reinhardtii* cc124 and only 45 genes were identified to be differentially regulated with a log2 fold change of 1 and an adjusted p-value < 0.05. Of these 45 genes, 23 were found to be upregulated under the TAP-N15 condition, and 22 were found to be downregulated (Figure 4.15). In *C. reinhardtii* cc124, upregulation of genes involved in nitrate transport and metabolism was not observed. Instead, genes involved in urea transport were found to be upregulated which was interesting as urea was not provided in the growth medium to the cultivated microalgae. As nitrogen limitation or starvation is linked with increased accumulation of lipids, differential regulation of genes involved in this process was also checked, but no differential expression of such genes was observed.

Gene ontology (GO) enrichment studies were performed on the upregulated and downregulated genes to identify important groups differentially expressed under the TAP-N15 condition. Figures 4.16a and b show enrichment terms in upregulated and downregulated gene lists. In the upregulated gene list, GO terms such as ammonium transmembrane transporter, thiamine biosynthetic process, protein processing, proline biosynthetic process, glycerol 3-phosphate catabolic process, glycerol-3-phosphate dehydrogenase (NAD+) activity, and branched-chain amino acid biosynthetic process were identified. In the downregulated GO terms list, voltage-gated potassium channel activity, ionotropic glutamate receptor activity, and potassium ion transport were identified.

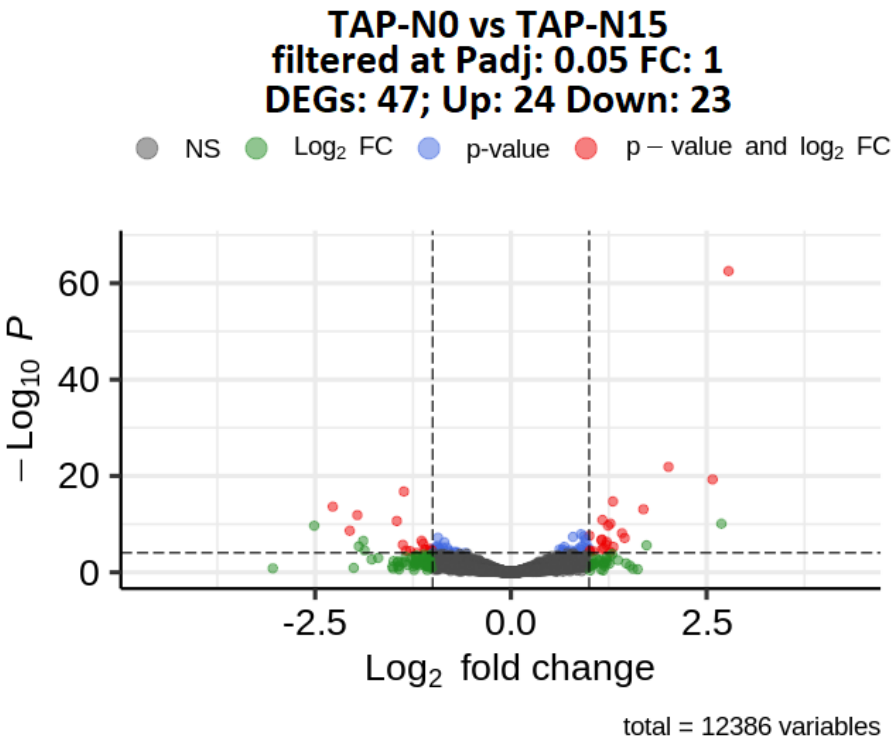
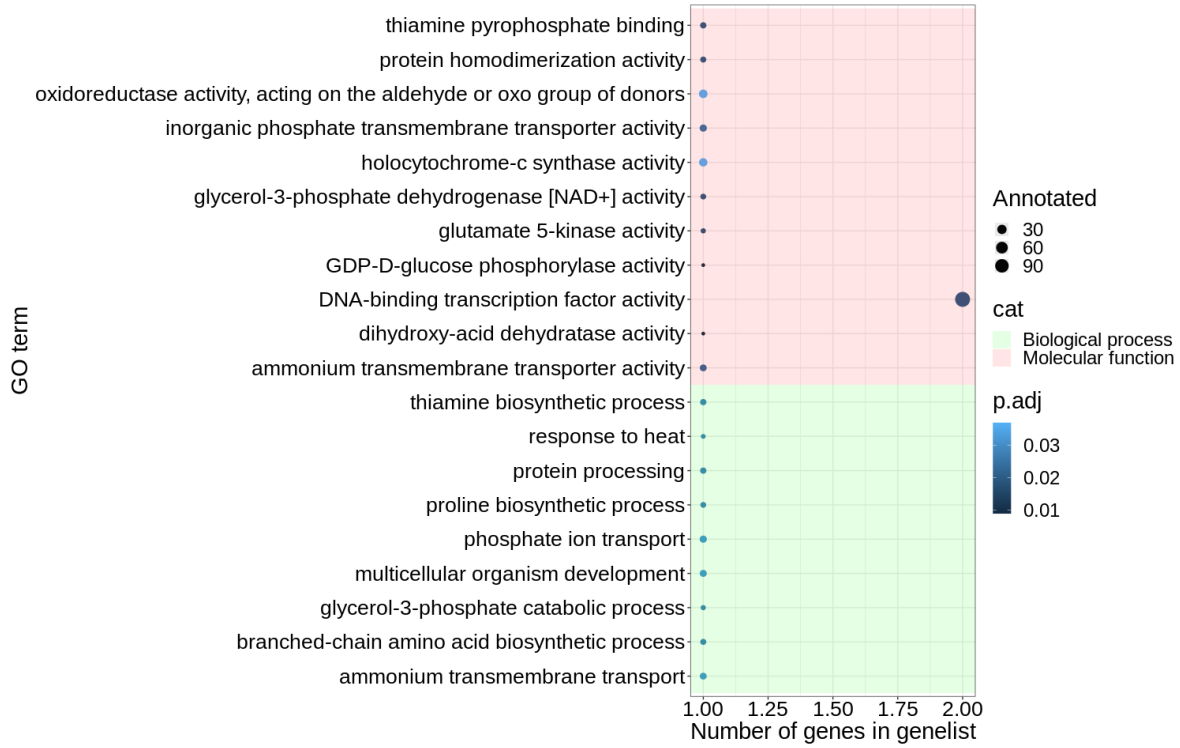
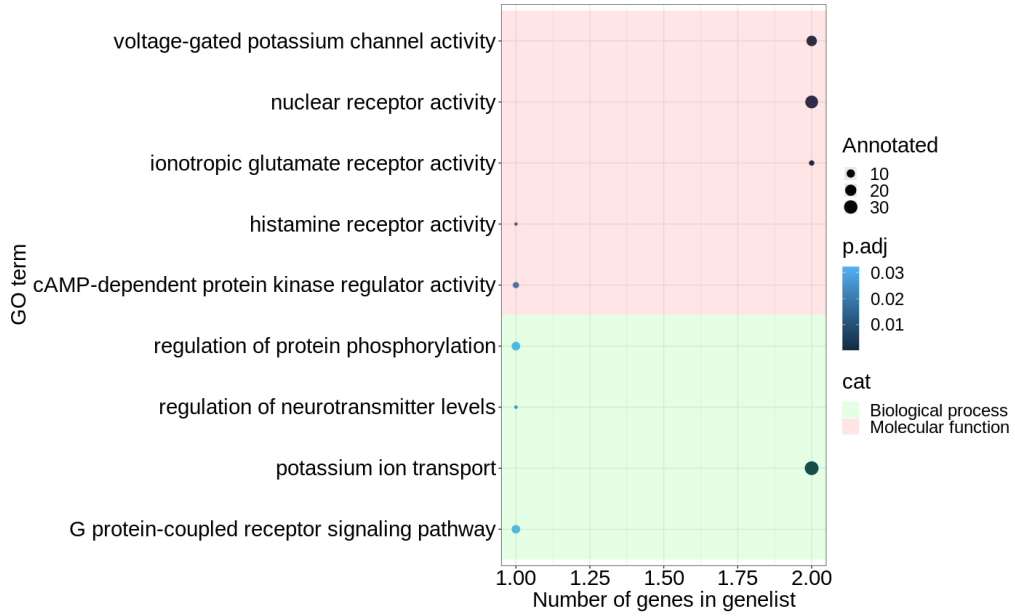


Figure 4.15. Volcano plot showing differentially expressed genes in *C. reinhardtii* cc124.



(a)



(b)

Figure 4.16. Enriched GO terms in upregulated (a) and downregulated (b) gene lists for *C. reinhardtii* cc124.

In *Chlamydomonas* sp. MACC-216 also, differential expression analysis was performed by using TAP-N0 condition as control and TAP-N15 condition as treatment condition. A total of 3143 genes were differentially regulated at a log2 fold change of 1 and adjusted p-value < 0.05. 1604 genes were upregulated, while 1539 genes were downregulated under the TAP-N15 condition (Figure 4.17). Genes coding for nitrate transporters, accessory proteins, nitrate metabolism, and nitrite metabolism were observed to be upregulated under nitrate-replete conditions. Nitrate transporters NRT2.1, NRT2.2, and NRT2.4 encoded by gene ids Cre09.g410850, Cre09.g410800, and Cre03.g150101, respectively were upregulated, whereas nitrate transporters NRT2.3, NRT2.5, NRT2.6 encoded by gene ids Cre09.g396000, Cre03.g150151, and Cre02.g110800, respectively were downregulated. Furthermore, gene ids coding for nitrate reductase (Cre09.g410950) and nitrite reductase (Cre09.g410750) were also upregulated. Gene ids coding for nitrite transporters were downregulated except gene ids Cre12.g541250 and Cre01.g012050 which coded for NAR1.5 and NAR1.6, respectively.

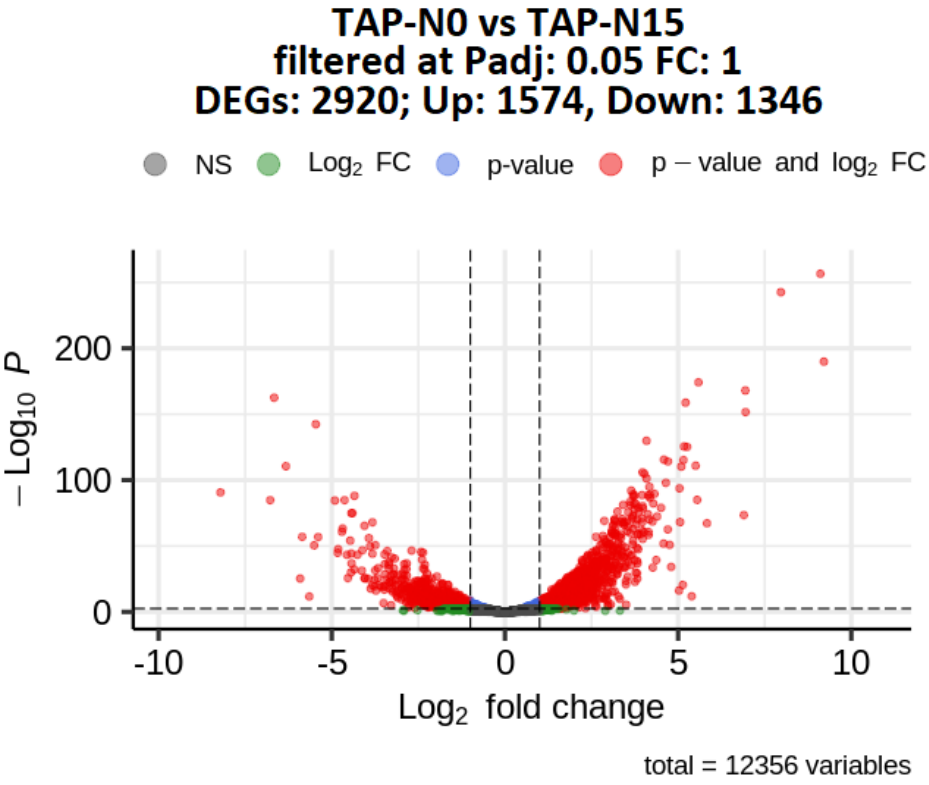
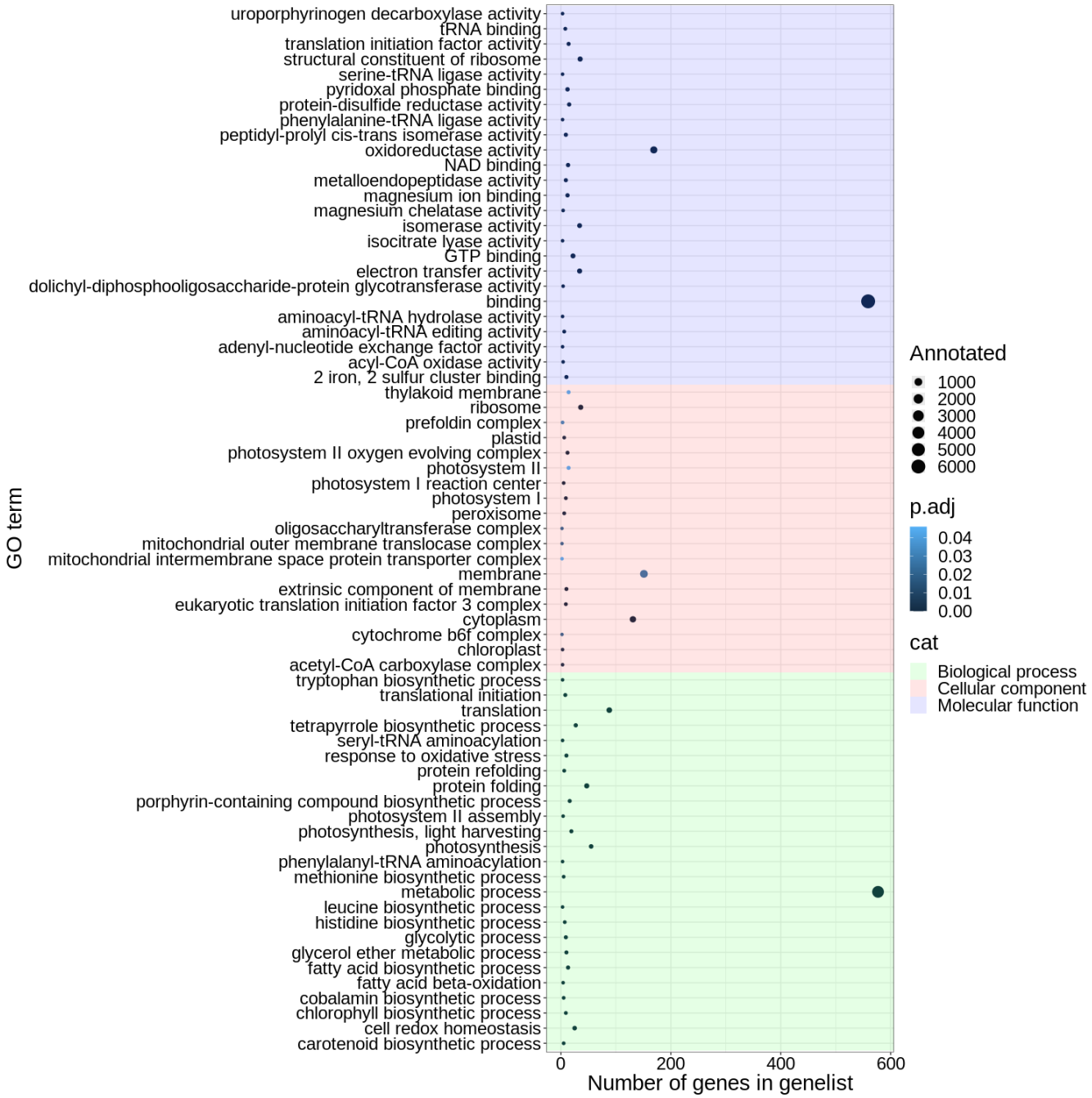


Figure 4.17. Volcano plot showing differentially expressed genes in *Chlamydomonas* MACC-216.

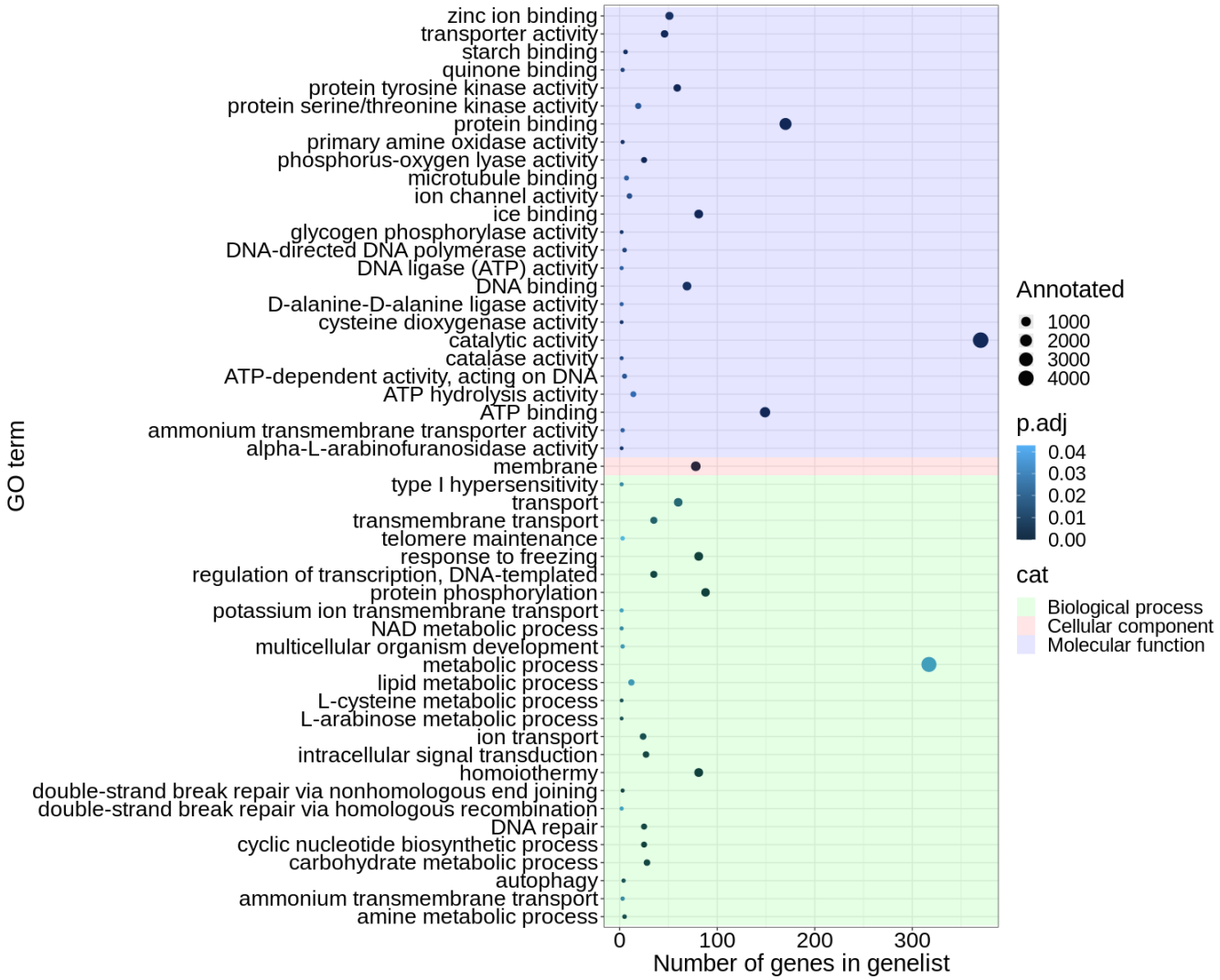
GO enrichment analysis produced a comprehensive list of upregulated and downregulated GO terms from *Chlamydomonas* sp. MACC-216 in comparison to *C. reinhardtii* cc124. GO enrichment analysis indicated GO terms related to photosynthesis and mitochondrial respiration to be enriched terms in the upregulated list under nitrate-replete condition (Figure 4.18a). Furthermore, GO terms related to the biosynthesis of certain amino acids (methionine, leucine, histidine), biosynthesis of tetrapyrrole, biosynthesis of fatty acids, and acetyl-CoA carboxylase complex were enriched in nitrate-replete samples. Acetyl-CoA carboxylase (ACCase) complex is known to play a role in the synthesis and elongation of fatty acids. All mRNAs encoding the different ACCase subunits were upregulated under nitrate-replete condition. One gene id (Cre17.g699100) related to the hydrolysis of triacylglycerols was observed to be upregulated. Finally, two gene ids coding for RHP1 and RHP2 were also found to be upregulated. These genes are hypothesized to function as channels for carbon dioxide gas transport.

In downregulated list (Figure 4.18b), GO terms such as ammonium transmembrane transporter activity, and ion transport were enriched terms. Gene ids Cre14.g62992, Cre13.g569850, Cre09.g400750, Cre02.g111050, and Cre12.g531000 coding for AMT2,

AMT4, AMT5, AMT7, and AMT8, respectively were downregulated except gene id Cre07.g355650 which codes for AMT6 which was found to be upregulated. Furthermore, GO term related to zinc ion binding was found to be enriched for. This is a novel finding that has not been previously reported. Moreover, stress-related response i.e., response to freezing was also noticed to be downregulated.



(a)



(b)

Figure 4.18. Enriched GO terms in upregulated (a) and downregulated (b) gene lists for *Chlamydomonas* MACC-216.

4.3.5 Cross-species analysis of differentially regulated genes

A heat map was generated to illustrate the differential expression of 28 genes under TAP-N0 and TAP-N15 conditions in *Chlamydomonas* sp. MACC-216 and *C. reinhardtii* cc124 (Figure 4.19 and Table 4.6). An upregulation of *NIA1*, *NII1*, *NRT2.1*, *NRT2.2*, *NRT2.4*, *NAR1.5*, *NAR1.6*, and *NAR2* genes was observed under TAP-N15 condition in *Chlamydomonas* sp. MACC-216, whereas downregulation of these genes was observed in *C. reinhardtii* cc124 under the same condition (Figure 4.19). These genes are known to play a major role in nitrate metabolism in *Chlamydomonas*.

Table 4.6. List of important genes used for heat map generation.

Gene name	Gene id	Gene symbol	Function
Nitrate transporter NRT1 family	Cre04.g224700	*NRT1.1*	Nitrate uptake
Nitrate transporter	Cre09.g410850	*NRT2.1*	Nitrate uptake
High affinity nitrate transporter	Cre09.g410800	*NRT2.2*	Nitrate uptake
Nitrate/nitrite transporter	Cre09.g396000	*NRT2.3*	Nitrate uptake
Nitrate/nitrite transporter	Cre03.g150101	*NRT2.4*	Nitrate uptake
Nitrate/nitrite transporter	Cre03.g150151	*NRT2.5*	Nitrate uptake
Nitrate/nitrite transporter	Cre02.g110800	*NRT2.6*	Nitrate uptake
Nitrite transporter accessory protein	Cre09.g410900	*NAR2*	Accessory protein
Nitrate reductase	Cre09.g410950	*NIA1/ NIT1*	Nitrate reductase
Inorganic carbon channel localized at chloroplast membrane	Cre06.g309000	*LCIA, NAR1.2*	Nitrite transport
Formate/nitrite transporter	Cre04.g217915	*NAR1.3*	Nitrite transport
Formate/nitrite transporter	Cre07.g335600	*NAR1.4*	Nitrite transport
Nitrite transporter	Cre12.g541250	*NAR1.5*	Nitrite transport
Formate/nitrite transporter	Cre01.g012050	*NAR1.6*	Nitrite transport
Nitrite reductase	Cre09.g410750	*NII1*	Nitrite reductase
Glutamine synthetase	Cre02.g113200	*GLN1*	Glutamine synthetase
Glutamine synthetase	Cre12.g530650	*GLN2*	Glutamine synthetase
Glutamine synthetase	Cre12.g530600	*GLN3*	Glutamine synthetase
Glutamine synthetase	Cre03.g207250	*GLN4*	Glutamine synthetase
Glutamate synthase, NADH-dependent	Cre13.g592200	*GSN1*	Glutamate synthase
Ferredoxin-dependent glutamate synthase	Cre12.g514050	*GSF1*	Glutamate synthase
Transcription factor regulating nitrogen metabolism	Cre03.g177700	*NIT2*	Transcription factor
Nitrogen regulatory protein PII	Cre07.g357350	*GLB1*	Regulatory protein
Urea carboxylase/allophanate hydrolase	Cre08.g360050	*DUR1*	Urea enzyme
Allophanate hydrolase	Cre08.g360100	*DUR2*	Urea enzyme
Urea active transporter	Cre08.g360200	*DUR3A*	Urea active transporter
Urea active transporter	Cre08.g360250	*DUR3B*	Urea active transporter
Urea active transporter	Cre17.g703800	*DUR3C*	Urea active transporter

Furthermore, it was observed that genes related to urea transport, *DUR3A*, *DUR3B*, and *DUR3C*, were upregulated under TAP-N15 condition in *C. reinhardtii* cc124. However, downregulation of *DUR3A, DUR3B*, and *DUR3C* was observed under TAP-N15 condition in *Chlamydomonas* sp. MACC-216. However, in TAP-N0 condition, genes related to urea metabolism (*DUR1* and *DUR2*) were found to be upregulated in *Chlamydomonas* sp. MACC-

216, but there was no change in the expression of these two genes in *C. reinhardtii* cc124 between the two conditions. From the heatmap, it was evident that gene ids related to nitrogen assimilation and metabolism were not highly expressed in *C. reinhardtii* cc124, and the microalgae behaved more or less in a similar manner whether it was cultivated under TAP-N0 or TAP-N15 medium. This was in stark contrast to the expression of nitrate assimilatory genes in *Chlamydomonas* sp. MACC-216.

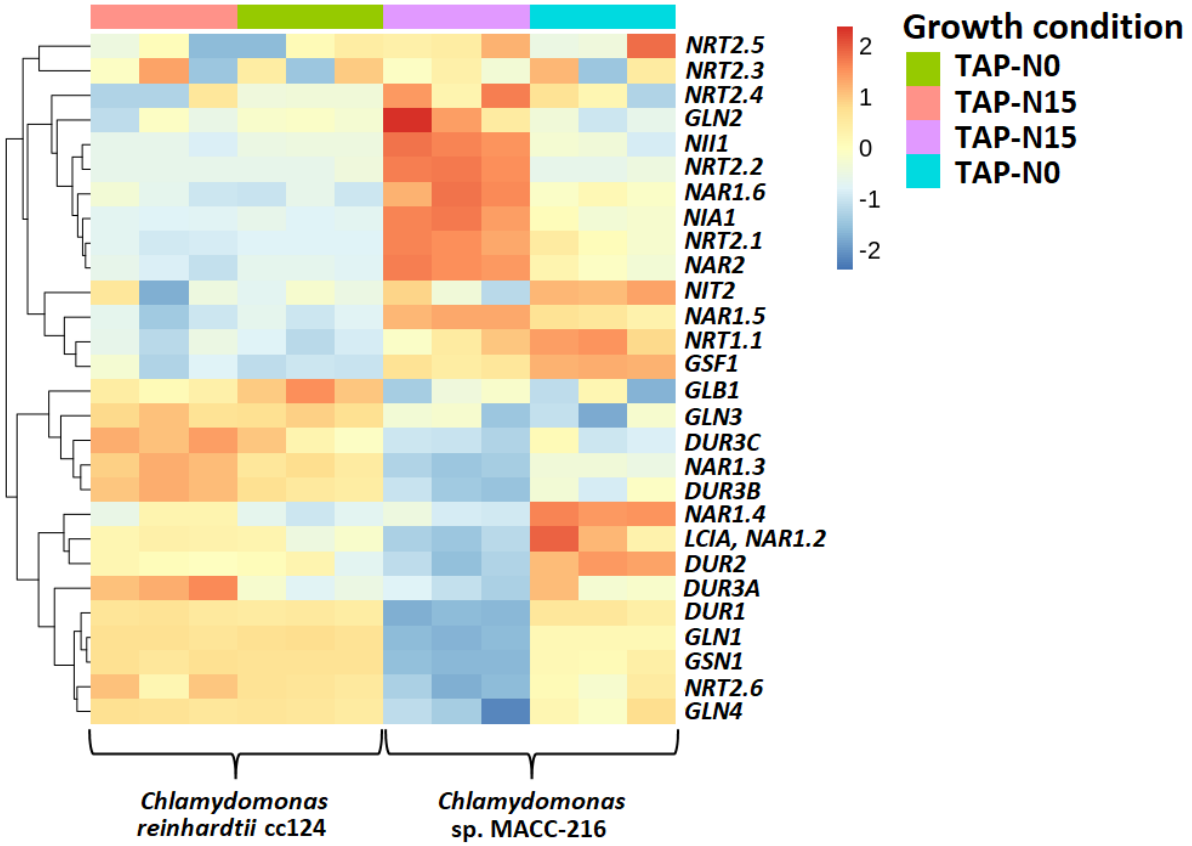


Figure 4.19. Heatmap of important differentially expressed genes in *C. reinhardtii* cc124 and *Chlamydomonas* MACC-216.

5 DISCUSSION

5.1 Effect of nitrate on two green microalgae

Initial screening was performed to select three nitrate concentrations for further experimentation, for which both *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360 were grown under various concentrations of nitrate ranging between 1 and 100 mM (Figure S1). At 100 mM nitrate, the growth was affected, but both microalgae still managed to grow. Similarly, *Chlorella vulgaris* has also been shown to grow at a high nitrate concentration of 97 mM (Jeanfils et al., 1993). The specific growth rate of *Chlamydomonas* sp. MACC-216 remained unaffected at 5 mM and 10 mM nitrate concentration, but decreased when the nitrate concentration was increased to 15 mM. The specific growth rate of *Chlorella* sp. MACC-360 decreased as the nitrate concentration increased, correlating with the findings of Jeanfils et al. (1993), who observed a decrease in the growth of *Chlorella vulgaris* when the nitrate concentration exceeded 12 mM. In contrast, other studies have demonstrated an increase in growth with an increase in nitrate concentration (Xin et al., 2010; Sayadi et al., 2016). In addition, the presence of nitrate appeared to affect the size of the cells. The cell size of both microalgae grown in TAP medium was smaller than in TAP-N5, TAP-N10, and TAP-N15 media (Table 4.1). The results indicate that as the concentration of nitrate increases, the cell size of microalgae also increases. In the case of *Chlamydomonas* sp. MACC-216, this increase in cell size may have been caused by the accumulation of lipids within the cells. In *Chlorella* sp. MACC-360, this increase in cell size could be due to an increase in the size of the vacuole to maintain the osmotic balance inside the microalgae (Dickson and Kirst, 1987; Kosmic-Buchmann et al., 2014).

Chlamydomonas sp. MACC-216 and *Chlorella* sp. MACC-360 showed 100% nitrate removal efficiency in TAP-N5 medium by the third day. This nitrate removal can be explained by high nitrate transport and high nitrate reduction activity. Su et al. (2012) demonstrated a 99% nitrate removal efficiency by *Chlamydomonas reinhardtii* and *Chlorella vulgaris* on the fourth and sixth day, respectively. The high uptake of nitrate by microalgae is significant because nitrate is an essential nitrogen source for microalgal survival; in the absence of nitrogen sources, electron acceptors, i.e., NADP+, which play a fundamental role in photosynthesis, are depleted (Kiran et al., 2016). Studies have shown that nitrogen deprivation leads to the loss of photosystem II activity in *Isochrysis zhangjiangensis* and *Tetraselmis subcordiformi*s (Feng et al., 2011; Yao et al., 2012;

Wang et al., 2014). Photosystem II plays a role in the transfer of electrons to photosystem I for the reduction of $NADP^+$ during photosynthesis. In *Chlamydomonas* sp., a decrease of 65% in certain Photosystem II -associated proteins was observed under nitrogen depletion (Plumley et al., 1989). In *Chlorella sorokiniana* C3, an increase in damaging nonphotochemical quenching, an indicator of the extent of damage to Photosystem II was observed, while an increasing cyclic electron flow around Photosystem I was observed which supplies ATP to triacylglycerol synthesis pathway (Zhang et al., 2013).

The nitrate removal rate of *Chlorella* sp. MACC-360 was found to be dependent on the nitrate concentration in the medium, whereas this was not the case for *Chlamydomonas* sp. MACC-216 (Tables 4.2 and 4.3). Similarly, Jeanfils et al. (1993) also noted that increasing the nitrate concentration from 2 mM to 29 mM increased the rate of nitrate uptake by *Chlorella vulgaris*. This could be due to the dependence of nitrate reductase activity on the actual concentration of nitrate (Tischner and Lorenzen, 1979).

ROS, including superoxide anion (O_2^-), hydroxyl radical (OH^-), singlet oxygen ($^1O_2^*$) and hydrogen peroxide (H_2O_2), are the final products of metabolic pathways occurring in the mitochondria, chloroplasts, peroxisomes, endoplasmic reticulum, chloroplast, cell wall, and plasma membrane of freshwater microalgae (Ugya et al., 2020). ROS are produced in response to a variety of environmental stresses, including high salt concentration, heavy metals, drought, ultraviolet irradiation, pathogens, extreme temperatures, etc. (Mallick and Mohn, 2000). ROS in high concentrations are toxic to cells and cause oxidative damage, which ultimately results in cell death. By measuring DCF fluorescence, the production of total ROS in microalgal cells exposed to varying concentrations of nitrate was assessed. The cell-permeable indicator DCFH-DA dye is hydrolyzed by cellular esterases to form the non-fluorescent DCFH, which is then transformed to the highly fluorescent DCF in the presence of ROS which emits green fluorescence at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. Among the microalgae tested, only *Chlorella* sp. MACC-360 grown in TAP-N15 media showed the highest ROS production, suggesting that the high concentration of nitrate caused significant stress to *Chlorella* sp. MACC-360. Perhaps, an increase in accumulation of NO as a result of increasing concentration of nitrate could be responsible for the presence of a high amount of ROS in *Chlorella* sp. MACC-360, as it has been shown that nitrate reductase is the source of NO in algae. Nitrate reductase is an essential partner protein of the NOFNiR (nitric oxide-forming nitrite reductase), which catalyzes nitrite

conversion to nitric oxide (Calatrava et al., 2017). Tischner et al. (2004) reported a reduction of nitrite into NO in *Chlorella sorokiniana*.

The concentration of nitrate decreased the abundance of photosynthetic pigments (chlorophyll-a, chlorophyll-b, and carotenoids) in both *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360. Similarly, the amount of chlorophyll-a in *Scenedesmus obliquus* decreased when the nitrate concentration was increased from 12 mM to 20 mM (Çelekli and Balcı, 2009). Zarrinmehr et al. (2020) demonstrated that *Isochrysis galbana* produces significantly more photosynthetic pigments in the presence of nitrate than in the absence of nitrate. When the concentration of nitrate was increased from 72 mg L^{-1} to 288 mg L^{-1}, they also observed a sharp decline in the pigment content. On the other hand, *Neochloris oleoabundans*, exhibited normal chlorophyll concentrations at 15 mM and 20 mM, in contrast to lower concentrations of nitrate (3 mM, 5 mM, and 10 mM), where a sharp decrease in chlorophyll concentration was observed (Li et al., 2008). It appears that different types of algae respond to nitrate stress by altering their pigment levels in different ways. Overall nitrate concentration 10 mM and 15 mM did have an effect on the total pigments of both microalgae.

The effect of different nitrate concentrations on protein content was also studied. When the concentration of nitrate was increased from 5 mM to 10 mM, it appeared that the total protein contents of both microalgae increased, but it decreased when the concentration was increased from 10 mM to 15 mM. Similarly, Xie et al. (2017) observed maximum protein production at 14.7 mM nitrate, while protein content decreased above and below this concentration. Rückert and Giani (2004) demonstrated through their research that the amount of protein was higher in the presence of nitrate than when ammonium was used as the nitrogen source. Comparing microalgae grown in different nitrate concentrations did not reveal a continuous increase or decrease in total carbohydrates. This could be likely due to the fact that carbohydrate accumulation is known to occur under sulfur and nitrogen deprivation or nitrogen limitation, as demonstrated in previous studies (Kiran et al., 2016; Bránykiová et al., 2011; Morsy, 2011).

Under environmental stress, microalgae are known to accumulate neutral lipids, primarily in the form of triacylglycerols. The accumulation of lipids allows microalgae to store carbon and energy in order to endure harsh environmental conditions. *Chlorella* sp. MACC-360 did not accumulate lipids significantly in response to an increasing nitrate concentration, whereas

Chlamydomonas sp. MACC-216 accumulated a substantial amount of lipids in response to high nitrate concentrations. Lipid accumulation in *Chlorella* sp. MACC-360 could not be considered significant because BODIPY staining results did not match with lipid content results obtained using the sulpho-phospho-vanillin reagent method. The possible explanation behind lipid accumulation in *Chlamydomonas* sp. MACC-216 at high nitrate concentration could be the presence of unutilized nitrate in the media and probably, the accumulation of other nitrogenous compounds, such as nitrite and ammonia, which act as stress factors after nitrate assimilation within the microalgae. Similarly, in *Isochrysis galbana*, lipid content increased when nitrate concentration was increased from 0 mg L^{-1} to 144 mg L^{-1} (Zarrinmehr et al., 2020). Contrary to our results, previous studies have shown that it is nitrogen limitation that results in lipid production in various microalgae like *Nannochloropsis oceanica*, *Nannochloropsis oculata*, and *Chlorella vulgaris* (Converti et al., 2009; Rodolfi et al., 2009; Juneja et al., 2013; Wan et al., 2013).

Chlorella sp. MACC-360 showed maximum growth in 50 mM SWW in comparison to *Chlamydomonas* sp. MACC-216 which showed maximum growth in 5 mM SWW. SWW consists of peptone and meat extract and probably *Chlorella* sp. MACC-360 utilizes these two components better than *Chlamydomonas* sp. MACC-216. Amino acids found in peptone are a source of organic nitrogen, whereas meat extract is a great source of vitamins, carbohydrates, and salts as well as other organic nitrogen compounds. Despite exhibiting slower growth, *Chlamydomonas* sp. MACC-216 performed notably better than *Chlorella* sp. MACC-360 at removing nitrate from 50 mM SWW. From Table 4.3, it can be seen that *Chlorella* sp. MACC-360 showed low nitrate removal in comparison to *Chlamydomonas* sp. MACC-216. Therefore, the possible reason behind less nitrate removal by *Chlorella* sp. MACC-360 in comparison to *Chlamydomonas* sp. MACC-216 could be the presence of low nitrate transporter and nitrate reductase activity in *Chlorella* sp. MACC-360. As a consequence, even after showing high growth at 50 mM SWW, *Chlorella* sp. MACC-360 still could not overtake the nitrate removal ability of *Chlamydomonas* sp. MACC-216. Previous research has demonstrated that microalgae are capable of removing nitrogen from wastewater or its sources (Sydney et al., 2011; Kim et al., 2013; Zhu et al., 2013; McGaughy et al., 2019; Gupta et al., 2020). *Chlorella* sp., as measured on the fifth day of cultivation, removed 56% of nitrate from wastewater containing 9.3 mg L^{-1} nitrate (McGaughy et al., 2019). Three different algae species, LEM-IM 11, *Chlorella vulgaris*, and *Botryococcus braunii*, removed 235 mg L^{-1}, 284 mg L^{-1}, and 311 mg L^{-1} of nitrate from an initial nitrate concentration of 390 mg L^{-1}

after 14 days of cultivation (Sydney et al., 2011). Through this objective, it was determined that both *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360 can grow and reproduce in SWW containing a high concentration of nitrate, and both microalgae are effective at removing a substantial proportion (28–35%) of the initial nitrate concentration after just 6 days of cultivation.

5.2 Light condition affects nitrate removal capacity

The second objective was to determine the effects of combinations of various light colours (blue, red, and white) and intensities (50 µmol m^{-2} s^{-1}, 100 µmol m^{-2} s^{-1}, and 250 µmol m^{-2} s^{-1}) on the nitrate removal capacity of *Chlamydomonas* sp. MACC-216. Light intensity and light spectrum have been known to affect growth, lipid accumulation, carotenoids, and the composition of fatty acids in microalgae (Maltsev et al., 2021). Therefore, the combination of these two factors was utilized to estimate the nitrate removal capacity of *Chlamydomonas* sp. MACC-216 as not so many studies are done on the effect of this combination on the nitrate removal capacity of a microalga. As the absorption bands of photosynthetic pigments lie in the blue and red ranges of visible light, we were also interested in determining whether the combination of blue and red light increases the growth and nitrate removal efficiency of *Chlamydomonas* sp. MACC-216. Numerous studies (Das et al., 2011; Atta et al., 2013; Kim et al., 2013; Zhang et al., 2015; Gunawan et al., 2018) have demonstrated the effects of different light colours or wavelengths and intensities on the growth of various microalgae; however, only a few studies (Kim et al., 2013; Yan and Zheng, 2014) have employed a blue + red light colour combination. The only factor that appeared to affect the growth of *Chlamydomonas* sp. MACC-216 in TAP-N5 and TAP-N10 media was light intensity, with the maximum light intensity used being 250 µmol m^{-2} s^{-1} (Figure 4.9). Similarly, a continuous increase in the growth of *Chlorella* sp. 800 has been reported with increasing light intensity until 500 µmol m^{-2} s^{-1} (Hempel et al., 2012). Light intensity has been shown to play a crucial role in the lifecycle of microalgae by affecting their photosynthesis and, consequently, their growth. In general, the light intensity can increase the growth rate of microalgae up to a certain point, which depends on specific microalgae species. A light intensity that is too high can cause photoinhibition, while a light intensity that is too low can reduce the growth of microalgae (Van Wagenen et al., 2012; Sforza et al., 2014; Lehmuskero et al., 2018; Nzayisenga et al., 2020; Maltsev et al., 2021). Through this objective, it was observed that nitrate removal efficiency was affected by light colour, light intensity, and nitrate concentration. At a nitrate concentration of 5 mM, no significant difference was observed in nitrate removal efficiency under different light

conditions (Figure 4.10). However, at 10 mM nitrate concentration, *Chlamydomonas* sp. MACC-216 removed the highest amount of nitrate under the Blue 125 + Red 125 light condition. Our findings of high nitrate removal under blue + red light combination are consistent with the findings of Kim et al. (2013), where *Scenedesmus* sp. was shown to remove nitrate at a higher rate when grown under a combination of blue + red light as opposed to blue, red, and white light alone. Moreover, the nitrate removal efficiency of *Chlamydomonas* sp. MACC-216 in TAP-N5 and TAP-N10 media increased with increasing light intensity (Figure 4.10). Consistent with our observations, the *Chlamydomonas reinhardtii* strain 21 gr also exhibited high nitrate removal when the light intensity was increased from 400 µmol m^{-2} s^{-1} to 1000 µmol m^{-2} s^{-1} (Garbayo et al., 2000). Perhaps, the high nitrate removal at high light intensity could be due to increased demand for nitrogen in microalgae grown under high light intensity to repair light-induced damage caused to their photosystem II (Pirastru et al., 2012; Lehmuskero et al., 2018; Maltsev et al., 2021; Esmaeili et al., 2022). Additionally, continuous light for cultivation was chosen for this study because earlier research has shown that continuous light promotes better growth and nitrate removal (Lee and Lee, 2001; Su et al., 2012). In one study, the nitrate removal efficiency of *Chlorella kessleri* under continuous illumination and 12h:12h (Light:Dark) lighting was observed to be 19% and 9%, respectively (Lee and Lee, 2001). In the same study, continuous illumination resulted in higher cell concentrations than 12h:12h (Light:Dark) lighting. In a separate study, the microalgal biomass of a mixed microalgae culture (*Chlamydomonas reinhardtii*, *Scenedesmus rubescens*, and *Chlorella vulgaris*) increased under continuous illumination versus 12h:12h (Light:Dark) lighting (Su et al., 2012). The decrease in cell biomass observed under the 12h:12h (Light:Dark) condition may be the result of respiration-induced biomass loss (Lee and Lee, 2001).

Chlamydomonas sp. MACC-216 grown in SWW under Blue 250, Blue 125 + Red 125, Red 250, and White 250 light conditions exhibited slower growth and lower nitrate removal efficiency than when grown in TAP-N5 and TAP-N10 media. *Chlamydomonas* sp. MACC-216 grown in SWW under Blue 125 + Red 125 exhibited the highest nitrate removal efficiency in comparison to all other light conditions (Figure 4.11b). Similar findings were observed for *Chlorella* sp. MACC-38 and *Chlorella* sp. MACC-360 (Figure 4.13c, d). High nitrate reductase activity of *Chlamydomonas* sp. MACC-216 grown under Blue 125 + Red 125 light condition correlated with its high nitrate removal efficiency in SWW (Figure 4.11b, c). Similarly, the low nitrate reductase activity of *Chlamydomonas* sp. MACC-216 grown under Blue 250 light condition corresponded

to its low nitrate removal efficiency under Blue 250 light condition. It has been demonstrated that red light, through the phytochrome system, stimulates the de novo synthesis of nitrate reductase in some higher plants, such as etiolated pea and tissues of maize (Hillman, 1967). Red light is known to photoconvert the pigment phytochrome from physiologically inactive Pr (red light absorbing phytochrome) to photoactive Pfr (far-red light absorbing phytochrome) (Hillman, 1967; Sharrock, 2008). Figueroa (1993) suggested that the red algae *Corallina elongate* contains two types of photoreceptors, phytochrome and B-light photoreceptor for red and blue light, respectively. By studying *Monoraphidium braunii*, Aparicio and Quiñiones (1991) demonstrated the significance of blue + red light. When *Monoraphidium braunii* cells were grown in red light alone, nitrate uptake was slower compared to when they were grown in red + blue light continuously and red + intermittent blue light. Furthermore, blue light is required for the in vivo activation of inactive nitrate reductase in *Chlamydomonas reinhardtii* (Azuara and Aparicio, 1983). In conclusion, red light may be required for the biosynthesis of nitrate reductase, whereas blue light is likely required for nitrate uptake and enzyme activation. This may account for the high nitrate removal efficiency and nitrate reductase activity observed in our study under Blue 125 + Red 125 light condition.

The expression of five genes, namely *NRT1*, *NRT2.1*, *NRT2.2*, *NIA1*, and *MCP1*, was analyzed by quantitative RT-qPCR to determine whether different light conditions modulate the transcription of genes involved in nitrate uptake and reduction. *NRT1*, *NRT2.1*, and *NRT2.2* represent nitrate and/or nitrite transporters, *NIA1* represents nitrate reductase, and *MCP1* represents the Moco (molybdenum cofactor) carrier protein. *Chlamydomonas* nitrate and/or nitrite transport has been attributed to three families of transporters, namely NRT1, NRT2, and NAR1 (Fernandez and Galvan, 2007; Sanz-Luque et al., 2015a). The NRT1, NRT2, and NAR1 transporters are composed of one, six, and six transporters, respectively (Fernandez and Galvan, 2007). The *Chlamydomonas* NRT1 transporter, which is encoded by a single *NRT1* gene, is a member of the nitrate peptide transporter family (NPF) (Sanz-Luque et al., 2015a; Calatrava et al., 2017). *Arabidopsis* NRT1 transporter is well studied as a dual affinity nitrate transporter, but *Chlamydomonas* NRT1 transporter is poorly understood (Azuara and Aparicio, 1983; Aparicio and Quiñiones, 1991). NRT2 transporters belong to the NNP family, which is a member of the major facilitator superfamily (Sanz-Luque et al., 2015a). Six genes (*NRT2.1*-2.6) in *Chlamydomonas* code for the NRT2 family of transporters. NRT2.1 and NRT2.2 transporters require NAR2 protein for full functionality; therefore, NRT2.1 and NAR2 make transporter system

I bi-specific for nitrate and nitrite, whereas NRT2.2 and NAR2 make nitrate specific transporter system II (Fernandez and Galvan, 2007; Sanz-Luque et al., 2015a). Other members of the NRT2 family (NRT2.3-6) are nitrate transporters with low affinity (Fernandez and Galvan, 2007; Higuera et al., 2016). NAR1 transporters only play a role in nitrite and bicarbonate transportation; therefore, *NRT1*, *NRT2.1*, and *NRT2.2* genes were selected from the nitrate/nitrite transporter families for expression study, as these three appear to be the primary nitrate transporters. In comparison to other light conditions, the highest expression of these three genes was observed under the Blue 125 + Red 125 light condition (Figure 4.12). Based on the aforementioned findings, this high expression correlates with high nitrate reductase activity and high nitrate removal. Therefore, it is evident from our study that the colour of light influences nitrate transport and, consequently, the expression of genes encoding nitrate transporters in *Chlamydomonas* sp. MACC-216.

Nitrate reductase encoded by the *NIA1* gene is involved in the cytoplasmic conversion of nitrate to nitrite. The genome of *Chlamydomonas* algae contains a single nitrate reductase-encoding gene (Fernandez and Galvan, 2007; Sanz-Luque et al., 2015a). Moco carrier protein, which is encoded by the *MCP1* gene, is known to play a role in the biosynthesis and transfer of the molybdenum cofactor to nitrate reductase, which is required for the enzyme function (Fischer et al., 2006; Fernandez and Galvan, 2007). Similar to the nitrate transporters, the expression of *NIA1* and *MCP1* was found to be light colour dependent, as both genes were highly expressed in the Blue 125 + Red 125 light condition relative to other light conditions (Figure 4.12). This increased expression of *NIA1* and *MCP1* is probably to assimilate large amount of nitrate uptake by nitrate transporters. Although there was no significant difference between the growth curves of *Chlamydomonas* sp. MACC-216 grown under various light conditions, *Chlamydomonas* sp. MACC-216 grown under the Blue 125 + Red 125 light condition exhibited the highest nitrate removal efficiency and gene expression associated with nitrate transport and reduction. This non-correlation between the growth and expression of different genes under various light conditions could be due to the regulation of gene expression at the translational or post-translational level in *Chlamydomonas* MACC-216.

The second objective of this thesis confirmed the greater role of the Blue 125 + Red 125 light condition in nitrate removal at the gene expression level. Although *Chlamydomonas* sp. MACC-216 was cultivated in SWW under the above-mentioned light conditions, the next step could be to use these light conditions to conduct total nitrogen and phosphorus removal studies in

actual wastewater collected from various industrial plants. Additionally, transcriptome analysis could be conducted on *Chlamydomonas* sp. MACC-216 grown under Blue 125 + Red 125 light. This analysis would be useful for observing the detailed transcriptional reorganization occurring within *Chlamydomonas* sp. MACC-216 under the Blue 125 + Red 125 light condition, which could lead to a better understanding of this light condition.

5.3 Transcriptome analysis under nitrate-replete and nitrate-deplete conditions

The third objective aimed to compare the transcriptome of *C. reinhardtii* cc124 and *Chlamydomonas* MACC-216 in nitrate-replete and nitrate-deplete conditions. Preliminary results indicated that *C. reinhardtii* cc124 struggles to grow in TAP medium when nitrate is the sole nitrogen source (Figure S2). Therefore, this objective sought to examine the initial changes occurring in the transcriptome of both microalgae after short-term exposure to nitrate. Nitrate measurement revealed that *C. reinhardtii* cc124 did not remove a significant amount of nitrate from the TAP-N15 medium compared to *Chlamydomonas* sp. MACC-216, which removed 3.22 mM nitrate after 6 hours. This inability to remove nitrate within the first six hours further suggests that *C. reinhardtii* cc124 has difficulty in the uptake and metabolism of nitrate.

PCA plot showed that there was extremely low variability between the samples of *C. reiinhardtii* cc124 grown under TAP-N0 and TAP-N15 conditions. This suggests that there is no significant reorganization in the transcriptome of *C. reinhardtii* cc124 between the two conditions, and this may be due to the inability of *C. reinhardtii* cc124 to sense nitrate properly in the TAP-N15 medium. Additionally, no enriched GO terms related to nitrate assimilation were observed in *C. reinhardtii* cc124, even when it was cultivated in TAP-N15 medium. Carbonic anhydrase 1 (CAH1; Cre04.g223100) was upregulated in nitrate-replete samples of *C. reinhardtii* cc124. Previous studies have identified the upregulation of mitochondrial CAH5 as an early-stage response to nitrogen starvation (López García de Lomana et al., 2015). Glycerol-3-phosphate dehydrogenase (GPDH) activity and glycerol-3-phosphate catabolic process were enriched GO terms in upregulated GO terms list; these two terms are associated with the production of glycerol. NAD^+-dependent Glycerol-3-phosphate dehydrogenase (GPDH) is known for catalyzing the reduction of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P) by using NADH as an electron donor (Casais-Molina et al., 2015). G3P is subsequently converted to glycerol by the action of a G3P phosphatase (GPP) or perhaps via the reversible reaction of a glycerol kinase

(Driver et al., 2017). G3P plays a crucial role in osmotic stress response and is a major metabolite in triacylglycerol production (Cai et al., 2013; Casais-Molina et al., 2015; Driver et al., 2017). However, in our results, upregulation of GO term related to lipid accumulation in nitrate-replete samples was not observed, so it was assumed that G3P is playing a role in the stress response as *C. reinhardtii* cc124 does not perform well in TAP-N15 medium. The accumulation of intracellular glycerol has been shown to provide osmotic adjustment to salt stress in various organisms such as *Saccharomyces cerevisiae*, *Dunaliella* spp., *C. reinhardtii* (Ben-Amotz et al., 1982; Husic and Tolbert, 1986; André et al., 1991; León and Galván, 1994; Cai et al., 2013; Driver et al., 2017). Glutamate 5-kinase activity GO term was in the upregulated gene list and it catalyzes the ATP-dependent phosphorylation of L-glutamate to L-glutamate 5-phosphate during the biosynthesis of proline from glutamate (Baich, 1969). Upregulation of this enzyme could explain the identification of proline biosynthesis as an enriched term in the upregulated gene list for *C. reinhardtii* cc124 under nitrate-replete condition. Proline accumulation in stressed plants is linked with reduced damage to membranes and proteins (Alia et al., 1997; Shah and Dubey, 1998; Verma, 1999). Currently, it is uncertain if proline plays a similar role in improving *C. reinhardtii* cc124 survival under nitrate-deplete condition, but future experiments involving exogenous proline supplementation are planned to test this theory. Upregulation of the branched-chain amino acid (BCAA) biosynthetic process was also observed. Leucine, Isoleucine, and Valine possess a common branched aliphatic chain and that is why they are known as branched-chain amino acids. Degradation products of these BCAAs include an acetyl-CoA which is a potential substrate for the de novo synthesis of fatty acids (Binder, 2010). BCAAs are also known to play a role as structural and signalling compounds/molecules besides acting as a respiratory substrate (Kimball and Jefferson, 2006; Binder, 2010). Additionally, ammonium transmembrane transporter was one of the enriched GO terms to be upregulated under nitrate-replete condition; this GO term is probably associated with the detection of any trace of ammonia in the surroundings. GO terms such as ionotropic glutamate receptor activity, voltage-gated potassium channel activity, and potassium ion transport were downregulated for *C. reinhardtii* cc124 cultivated under nitrate-replete condition. Glutamate-like receptors (GLRs) of plants are homologs of mammalian ionotropic glutamate receptors (iGluRs), which were found more than a decade ago, and are speculated to function as possible amino acid sensors in plants (Price et al., 2012). Several studies have shown the involvement of GLRs in many biological processes such as abscisic acid (ABA) biosynthesis

and signalling (Kang et al., 2004), innate immune responses (Kang et al., 2006; Manzoor et al., 2013), carbon and nitrogen balance or metabolism (Kang and Turano 2003). Hence, it can be assumed that GLRs may play a role in conveying nitrogen status and triggering the appropriate response. Schmollinger et al. (2014) observed a high upregulation of GLR1 (Cre16.g685650) under nitrogen deplete condition in three strains of *C. reinhardtii*, whereas through our study we observed the downregulation of two other GLRs (Cre12.g532850 and Cre12.g532950) under nitrate (nitrogen) replete condition which explains a certain role of GLRs during nitrogen starvation. Upregulation of KCN11 potassium channel activity during nitrogen starvation has been shown to be essential in maintaining cell cycle activity and osmoregulation (Xu and Pan, 2020). The lack of this particular protein is connected to loss in growth rate, inhibition of chlorophyll, and triacylglycerol accumulation. Perhaps, that is why a significant decrease in growth under nitrate-replete condition after 6 hours was observed in *C. reinhardtii* cc124 (Table 4.5).

PCA plot of *Chlamydomonas* sp. MACC-216 showed a huge difference between the samples from TAP-N0 and TAP-N15 conditions, indicating a major transcriptional reorganization between the two conditions. In *Chlamydomonas* sp. MACC-216, more upregulated and downregulated enriched GO terms were obtained in comparison to *C. reinhardtii* cc124. GO terms related to photosynthesis and mitochondrial respiration were enriched in the upregulated list of genes under nitrate-replete condition in *Chlamydomonas* sp. MACC-216. Furthermore, enriched GO terms related to ACCase complex and acetyl-CoA oxidase activity were observed in the upregulated gene list. ACCase is an important enzyme that plays a role in the lipid synthesis pathway by catalyzing the formation of malonyl-CoA from acetyl-CoA in the plastid (chloroplast) and the cytosol of plants and algae (Huerlimann and Heimann, 2013). Fatty acid synthase (FAS) then uses plastidial-derived malonyl-CoA for the de novo synthesis of fatty acids, while cytosol-derived malonyl-CoA is used for elongation of fatty acids in the endoplasmic reticulum (Huerlimann and Heimann, 2013). However, no differential transcriptional regulation of ACCase complex was observed in *Poryphyridium cruentum* between nitrogen replete and deplete conditions which indicates species-specific transcriptional regulation of this complex (Wei et al., 2021). Furthermore, Huerlimann et al. (2014), showed that changes in transcripts relating to acetyl-CoA were species-specific and depended on growth phase, and nutrient conditions and did not necessarily lead to fatty acid accumulation. GO terms related to amino acids biosynthesis, tetrapyrrole biosynthesis, chlorophyll biosynthesis, and carotenoid biosynthesis were enriched

terms in upregulated GO terms list. With increasing photosynthesis and respiration, there is an increasing demand for proteins, chlorophyll, and carotenoids and that is why corresponding terms were observed in the upregulated gene list. Furthermore, as tetrapyrrole compounds such as chlorophyll-a, chlorophyll-b, and cobalamin consist of nitrogen in their structure; these compounds may be helpful in accumulating nitrogen. Enzymes involved in the tetrapyrrole pathway were observed to be reduced under nitrogen starvation (Schmollinger et al., 2014). In the downregulated gene list, GO terms such as ion transport, and ammonium transmembrane transporter activity were enriched. These were all previously associated with nitrogen starvation responses. Furthermore, GO term zinc ion binding was observed in the downregulated gene list. Zinc has been known as an important component of various enzymes, low concentration of zinc has been shown to help in the growth of certain algae, whereas high concentration of zinc concentrations has been shown to cause slow growth and reduced cell division (El-Agawany and Kaamoush, 2022). As for ammonium transmembrane transporter activity, downregulation of *AMT2, AMT4, AMT5, AMT7,* and *AMT8*, respectively was observed except *AMT6* which was found to be upregulated under nitrate-replete condition. AMT6 is the largest ammonium transporter made up of 778 amino acids and may assist in reducing nitrogen quota. This transporter was the only downregulated transporter among ammonium transporters in *C. reinhardtii* cultivated under nitrogen deplete condition probably to save nitrogen consumption (Schmollinger et al., 2014). Furthermore, González-Ballester et al. (2004) showed high expression of *AMT6* in microalgae grown under nitrate-replete condition than nitrate-deplete condition. A stress-related response, such as the response to freezing was observed to be downregulated. This suggests that microalgae grown under TAPN-15 conditions were not stressed, and that is why the downregulation of stress-related genes was identified. Moreover, downregulation of ion transport, transmembrane transport, and transporter activity GO terms was observed. Through our data, it was noticed that gene ids associated with the transport of ions were all downregulated for *Chlamydomonas* sp. MACC-216 under nitrate-replete condition. Schmollinger et al. (2014) showed high induction of ion transport-related gene ids under nitrogen deplete condition.

Previous studies have demonstrated that expression of *CrGLB1* gene is upregulated in the presence of nitrate (Zalutskaya et al., 2018). However, no such upregulation was observed through this study either in *C. reinhardtii* cc124 or *Chlamydomonas* sp. MACC-216. In fact, differential expression of this gene was not observed between TAP-N0 and TAP-N15 growth conditions.

CrGLB1 encoded PII protein is responsible for coordinating the central C/N anabolic metabolism (Zalutskaya et al., 2015). Furthermore, PII proteins have been shown to play a role in the regulation of triacylglycerol accumulation (Zalutskaya et al., 2015).

The *NRT1.1* gene was already expressed under nitrate-replete conditions, but its expression increased further under nitrate-deplete conditions in *Chlamydomonas* sp. MACC-216. The NRT1 transporter is not widely studied in microalgae, although its functions are well-explored in plants. One of the best-studied NRT1 transporters from *Arabidopsis* is CHL1 or AtNRT1.1 which is dual affinity nitrate transporter that changes its affinity according to the nitrate conditions (Ho et al., 2009). AtNRT1.1 is phosphorylated at low nitrate conditions and thereby functions as high-affinity transporter, whereas, at high nitrate conditions, this transporter is dephosphorylated and becomes a low-affinity transporter. In fact, this explains why a higher expression of this transporter was observed under nitrate-deplete condition as it became a high-affinity nitrate transporter. Furthermore, an upregulation in *NRT2.1*, *NRT2.2* and *NRT2.4* was observed under nitrate-replete condition in *Chlamydomonas* sp. MACC-216. The NRT2 transporters are capable of transporting nitrate or nitrite, and some of them are two-component systems that necessitate the presence of the NAR2 protein in order for them to function properly. Hence, NRT2.1 together with NAR2 constitutes a bispecific high-affinity nitrate/nitrite transporter and NRT2.2 together with NAR2 is a high-affinity nitrate transporter. NRT2.4 transporter has been shown to be associated with high-affinity transport of nitrite. Furthermore, upregulation of nitrate reductase (*NIA1*) and nitrite reductase (*NII1*) was observed. Nitrate reductase reduces nitrate to nitrite after that nitrite reductase catalyzes the reduction of nitrite to ammonium. Interestingly all the upregulated genes (*NRT2.1*, *NRT2.2*, *NRT2.4*, *NAR2*, *NIA1*, and *NII1*) observed in *Chlamydomonas* sp. MACC-216 under nitrate-replete conditions were downregulated in *C. reinhardtii* cc124 under the same nitrate condition. *C. reinhardtii* cc124 displayed similar expression of these genes under nitrate-deplete and nitrate-replete conditions. Furthermore, upregulation of *NAR1.5*, and *NAR1.6* and downregulation of *NAR1.2*, and *NAR1.4* was observed under nitrate-replete condition in *Chlamydomonas* sp. MACC-216. NAR1 transporters are known to play a role in the transport of nitrite and bicarbonate. Apart from genes directly related to nitrogen assimilation and metabolism, downregulation of *GLN1* and *GLN4* genes was also observed. These two genes code for cytosolic GS1, whereas no significant difference was observed in the expression of *GLN2* and *GLN3* genes coding for chloroplastic GS2 under nitrate-replete condition in *Chlamydomonas* sp. MACC-216.

Additionally, NIT2, a regulatory protein for the nitrate assimilation pathway showed no differential expression under nitrate-deplete and nitrate-replete conditions in *C. reinhardtii* cc124. However, it was highly expressed under nitrate-deplete condition in *Chlamydomonas* sp. MACC-216. Finally, genes responsible for urea assimilation (*DUR1* and *DUR2*) were expressed in a similar manner under both nitrate-replete and nitrate-deplete conditions in *C. reinhardtii* cc124, indicating that these proteins might perform important functions for cells going through nitrogen starvation. However, genes responsible for urea transport (*DUR3A*, *DUR3B*, and *DUR3C*) were highly expressed under nitrate-replete condition in *C. reinhardtii* cc124. Additionally, *DUR1* and *DUR2* were upregulated in *Chlamydomonas* sp. MACC-216 cultivated under nitrate-deplete condition, further supporting the idea that these genes play a role in nitrogen scavenging. Despite no urea being provided to either species of the *Chlamydomonas* during cultivation, the expression of these genes related to urea metabolism lends evidence to the idea that these genes might have a role in nitrogen scavenging during nitrogen-starved condition. Previously, Park et al. (2015) have also shown the upregulation of *DUR1*, *DUR2*, *DUR3B*, and *DUR3C* under nitrogen starvation. Furthermore, they also detected the presence of intracellular urea in *C. reinhardtii* even when the growth medium did not have any urea (Park et al., 2015). Overall, the results from the heat map suggest that the expression of genes in *C. reinhardtii* cc124 grown under nitrate-deplete and nitrate-replete condition showed similar gene expression. No major difference was observed between the two conditions in this microalga which points towards the incapability of *C. reinhardtii* cc124 to sense nitrate as a nitrogen source. On the other hand, in *Chlamydomonas* sp. MACC-216, a clear difference in the expression of genes between the two conditions could be observed.

6 CONCLUSIONS

Through the work carried out for the present thesis, following conclusions can be drawn:

- *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360 are able to grow in growth medium supplemented with nitrate.
- *Chlamydomonas* sp. MACC-216 performs better in nitrate removal than *Chlorella* sp. MACC-360.
- Increasing concentration of nitrate leads to lipid accumulation in *Chlamydomonas* sp. MACC-216.
- *Chlamydomonas* sp. MACC-216 shows high nitrate removal efficiency under the blue + red light combination in comparison to solely Blue, Red, or White light colour.
- Nitrate removal efficiency of *Chlamydomonas* sp. MACC-216 increases as the light intensity is increased from 50 µmol m^{-2} s^{-1} to 250 µmol m^{-2} s^{-1}.
- Blue + red light combination affects nitrate removal at the gene expression level.
- Blue + red light combination with high light intensity represents an optimal light condition for efficient nitrate removal from wastewater.
- In the presence of nitrate, transcriptome of *Chlamydomonas* sp. MACC-216 goes through a big transcriptional reorganization, where upregulation of genes related to nitrate transport and reduction can be observed.
- In *Chlamydomonas reinhardtii* cc124, no major transcriptional reorganization takes place in the presence of nitrate, where upregulation of genes related to nitrate transport and reduction cannot be observed. However, genes related to urea transport are upregulated.
- *Chlamydomonas reinhardtii* cc124 cannot sense the presence of nitrate as *Chlamydomonas* sp. MACC-216 does.
- Nitrate metabolism is a species-specific complex pathway.

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SUMMARY

Nitrogen pollution, often in the form of nitrate, has emerged as one of the major causes behind the eutrophication of water bodies all over the world (Mohensi-Bandpi et al., 2013). The major factors behind eutrophication are the extensive use of fertilizers in agricultural fields, improper disposal of industrial wastewater, and the release of human sewage into the water bodies. Algal blooms have resulted from the eutrophication of surface waters such as lakes, streams, and drinking water reservoirs. One of the most obvious effects of these blooms is the depletion of oxygen levels, which leads to massive fish deaths and the subsequent degradation of lakes and rivers (Moss et al., 2011; Maberly et al., 2020). Microalgae are the primary photosynthesizers, and nitrogen is one of the most important nutrients for their growth and can be acquired from both organic (urea and amino acids) and inorganic (nitrate, nitrite, and ammonia) nitrogen sources. Microalgae have traditionally been utilized for the treatment of wastewater because of their capacity to consume nitrogen and phosphorus. This eco-friendly treatment consumes less energy, significantly reduces carbon emissions, and can lead to the production of biofuels (Prasad et al., 2017). Furthermore, recovered nitrogen- and phosphorus-rich algal biomass can be exploited as low-cost plant biostimulants or as animal feed (Wilkie and Mulbry, 2002; Su et al., 2012). The aim of this thesis was to investigate the capacity of microalgae for their nitrate removal efficiency and understand nitrate assimilation, as nitrate is one of the major pollutants causing eutrophication.

Through the first objective, the ability of two eukaryotic green microalgae, *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360, to grow and remove nitrate at varied nitrate concentrations was determined. Three concentrations of nitrate were selected for this purpose, i.e., 5 mM, 10 mM, and 15 mM. Both microalgae were capable of removing 100 % nitrate when grown in TAP medium supplied with 5 mM nitrate. However, as nitrate concentration increased, nitrate removal efficiency decreased. The nitrate removal rate was determined for both microalgae over a span of 9 hours, where *Chlamydomonas* sp. MACC-216 outperformed *Chlorella* sp. MACC-360. Furthermore, to observe whether the presence of nitrate is causing any stress on both microalgae, reactive oxygen species (ROS) production was determined. No major ROS production was observed in *Chlamydomonas* sp. MACC-216, but *Chlorella* sp. MACC-360 showed high ROS production at 15 mM nitrate concentration, signifying the stress caused by this particular nitrate concentration. Additionally, *Chlamydomonas* sp. MACC-216 showed enhanced lipid accumulation with increasing nitrate concentration. Furthermore, after observing the nitrate removal capacity of both microalgae in

TAP-N (Tris-acetate-phosphate medium supplied with nitrate as the sole nitrogen source) medium, the nitrate removal capacity of both microalgae was determined in synthetic wastewater (SWW). Both microalgae could grow well in SWW supplied with different nitrate concentrations; *Chlamydomonas* sp. MACC-216 showed better growth in SWW supplied with either 5 mM or 10 mM nitrate, and *Chlorella* sp. MACC-360 showed better growth in SWW supplied with higher nitrate, i.e., 25 mM and 50 mM nitrate. However, even in SWW, *Chlamydomonas* sp. MACC-216 showed better nitrate removal than *Chlorella* sp. MACC-360.

The effect of combinations of different light intensities and colours (or wavelengths) on the nitrate removal efficiency of *Chlamydomonas* sp. MACC-216 was determined through the second objective. In total, 12 different light conditions made up of permuted combinations of three light colours (referred to as blue, red, and white light) and three light intensities (50 µmol m^{-2} s^{-1}, 100 µmol m^{-2} s^{-1}, and 250 µmol m^{-2} s^{-1}) were used to cultivate *Chlamydomonas* sp. MACC-216 in TAP-N5 (TAP-N medium supplied with 5 mM nitrate) and TAP-N10 (TAP-N medium supplied with 10 mM nitrate) media. No major effect of different light conditions was observed on the growth of the microalgae. However, in comparison to monochromatic light colours (blue, red, and white), *Chlamydomonas* sp. MACC-216 showed better nitrate removal efficiency under the combination of blue + red light colour combination. An increase in the light intensity from 50 µmol m^{-2} s^{-1} to 250 µmol m^{-2} s^{-1} led to significantly higher nitrate removal efficiency. Therefore, the nitrate removal efficiency was found to be light intensity dependent. Furthermore, *Chlamydomonas* sp. MACC-216 was cultivated under Blue 250, Red 250, Blue 125 + Red 125, and White 250 light conditions in SWW, where numbers 125 and 250 define light intensity with unit µmol m^{-2} s^{-1}. The nitrate removal efficiency of *Chlamydomonas* sp. MACC-216 was observed to be highest under Blue 125 + Red 125 light condition in SWW; similar results were observed for nitrate reductase activity too. Expression of five genes (*NRT1*, *NRT2.1*, *NRT2.2*, *NIA1*, and *MCP1*) participating in nitrate transport and reduction was thoroughly analyzed, and all these genes showed the highest expression under the Blue 125 + Red 125 light condition.

As *Chlamydomonas* sp. MACC-216 showed efficient nitrate removal in the first two objectives, it was decided to compare the transcriptome of microalgae (*Chlamydomonas* sp. MACC-216) which can remove nitrate efficiently, to the transcriptome of microalgae (*C. reinhardtii* cc124) which cannot even grow well in the presence of nitrate. Therefore, for the third objective, transcriptome analysis was performed to compare the transcriptional changes occurring in the presence and absence of nitrate in *Chlamydomonas* sp. MACC-216 and

Chlamydomonas reinhardtii cc124, which revealed interesting results. Differential expression analysis of *C. reinhardtii* cc124 grown in the presence of nitrate revealed that only 45 genes were differentially regulated, where 23 genes were upregulated, and 22 genes were downregulated. In *Chlamydomonas* sp. MACC-216, differential expression analysis revealed that 3143 genes were differentially regulated, where 1604 genes were upregulated, and 1539 genes were downregulated in the presence of nitrate. From the results, it was observed that there is a great transcriptional reorganization of the genes related to nitrate transport and reduction in *Chlamydomonas* sp. MACC-216 when it is grown in the presence of nitrate, but no similar results were observed for the *C. reinhardtii* cc124. In *Chlamydomonas* sp. MACC-216, major upregulation of *NRT2.1*, *NRT2.2*, *NAR2*, *NIA1*, *NAR1.5*, *NAR1.6*, and *NII1* genes was observed in the presence of nitrate. However, in *C. reinhardtii* cc124, upregulation of genes related to the urea transport (*DUR3A*, *DUR3B*, and *DUR3C*) was observed, which is interesting because no such nitrogen source was provided to this microalga in the growth medium. This objective demonstrated a clear picture of species-specific regulation of nitrate metabolism in *Chlamydomonas*.

ÖSSZEFOGLALÓ

A gyakran nitrát formájában megjelenő nitrogénszennyezés, világszerte a víztestek eutrofizációjának egyik fő oka (Mohensi-Bandpi et al., 2013). Az eutrofizáció hátterében álló fő tényezők legfőképpen a műtrágyák kiterjedt használata a mezőgazdasági területeken, az ipari szennyvizek nem megfelelő elhelyezése és az emberi szennyvizek víztestekbe történő kibocsátása. Az algavirágzások a felszíni vizek, például tavak, patakok és ivóvíztározók eutrofizálódása miatt alakulnak ki. E virágzások egyik legnyilvánvalóbb hatása az oxigénszint csökkenése, ami tömeges halpusztuláshoz, majd a tavak és folyók állapotának romlásához vezet (Moss et al., 2011; Maberly et al., 2020). A mikroalgák elsődleges fotoszintetizálók, a növekedésükhöz egyik legfontosabb tápanyag pedig a nitrogén, amelyet szerves (karbamid és aminosavak) és szervetlen (nitrát, nitrit és ammónia) nitrogénforrásokból egyaránt fedezhetnek. A mikroalgákat hagyományosan a nitrogén- és foszforfelhasználási képességük miatt alkalmazzák a szennyvíz tisztítására. Ez a környezetbarát kezelés kevesebb energiát fogyaszt, jelentősen csökkenti a szén-dioxid-kibocsátást, illetve bioüzemanyagok előállításához is hozzájárulhat (Prasad et al., 2017). A visszanyert nitrogén- és foszforban gazdag algabiomassza továbbá hasznosítható alacsony költségű növényi biostimulánsként vagy állati takarmányként is (Wilkie and Mulbry, 2002; Su et al., 2012). Ezen dolgozat célja a mikroalgák nitráteltávolító képességének vizsgálata és a nitrátasszimiláció megértése volt, mivel a nitrát az egyik legfontosabb eutrofizációt okozó szennyezőanyag.

Az első célkitűzés révén két eukarióta zöld mikroalga, a *Chlamydomonas* sp. MACC-216 és a *Chlorella* sp. MACC-360 növekedési és nitráteltávolítási képességét határoztuk meg különböző nitrátkoncentrációk mellett. Három nitrátkoncentrációt; 5 mM, 10 mM és 15 mM választottunk ki erre a célra. 5 mM nitrátot tartalmazó TAP tápoldatban növesztve mindkét mikroalga képes volt a nitrát 100 %-os eltávolítására. A nitrátkoncentráció növekedésével azonban a nitrát eltávolításának hatékonysága csökkent. A nitráteltávolítási sebességet mindkét mikroalga esetében 9 óra alatt határoztuk meg, ahol a *Chlamydomonas* sp. MACC-216 felülmúlta a *Chlorella* sp. MACC-360 alga nitráteltávolító képességét. Annak eldöntésére, hogy a nitrát jelenléte okoz-e stresszt a mikroalgák számára, meghatároztuk a reaktív oxigén származékok (ROS) képződését. A *Chlamydomonas* sp. MACC-216 alga esetében nem volt megfigyelhető jelentős ROS-termelődés, de a *Chlorella* sp. MACC-360 15 mM nitrátkoncentrációnál magas ROS-termelődést mutatott, ami az adott nitrátkoncentráció okozta stresszt jelzi. Emellett a *Chlamydomonas* sp. MACC-216 fokozott lipidfelhalmozódást is mutatott a nitrátkoncentráció növekedésével. Továbbá, miután megfigyeltük mindkét

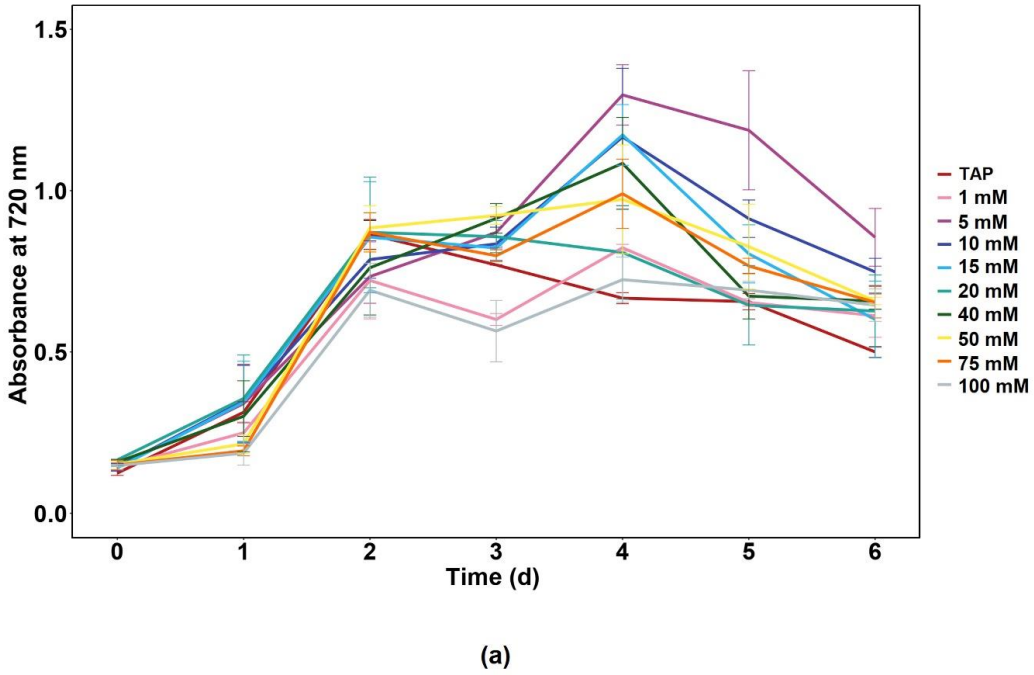
mikroalga nitráteltávolító képességét TAP-N (tris-acetát-foszfát tápoldat, amelyben nitrát szerepel egyedüli nitrogénforrásként) tápoldatban, meghatároztuk mindkét mikroalga nitráteltávolító képességét szintetikus szennyvízben (SWW) is. Mindkét mikroalga jól növekedett a különböző nitrátkoncentrációjú szintetikus szennyvízben; a *Chlamydomonas* sp. MACC-216 az 5 mM vagy 10 mM nitrátot tartalmazó szintetikus szennyvízben, míg a *Chlorella* sp. MACC-360 a magasabb nitrátkoncentrációjú, azaz 25 mM és 50 mM nitrátot tartalmazó szintetikus szennyvízben mutatott erőteljesebb növekedést. Tehát a *Chlamydomonas* sp. MACC-216 szintetikus szennyvízben is jobb nitráteltávolítást mutatott, mint a *Chlorella* sp. MACC-360.

A második célkitűzéssel a különböző fényintenzitások és színek (vagy hullámhosszok) kombinációinak a *Chlamydomonas* sp. MACC-216 alga nitráteltávolítási hatékonyságára gyakorolt hatását kívántuk meghatározni. Összesen 12 különböző fénybeállítást alkalmaztunk, amelyek három szín (kék, vörös és fehér fény) és három fényintenzitás (50 µmol m^{-2} s-^{1}, 100 µmol m^{-2} s^{-1} és 250 µmol m^{-2} s^{-1}) permutált kombinációiból álltak. A *Chlamydomonas* sp. MACC-216 növesztéséhez TAP-N5 (5 mM nitrátot tartalmazó TAP-N tápoldat) és TAP-N10 (10 mM nitrátot tartalmazó TAP-N tápoldat) tápoldatot használtunk. Elmondható, hogy a különböző fényviszonyoknak nem volt jelentős hatása a mikroalgák növekedésére. Azonban monokromatikus fényszínekkel (kék, vörös és fehér) összehasonlítva a *Chlamydomonas* sp. MACC-216 jobb nitráteltávolítási hatékonyságot mutatott a kék + vörös fényszínkombináció alkalmazása mellett. A fényintenzitás 50 µmol m^{-2} s^{-1}-ről 250 µmol m^{-2} s^{-1}-re történő növelése is szignifikánsan magasabb nitráteltávolítási hatékonyságot eredményezett. Tehát a nitráteltávolítás hatékonysága fényintenzitásfüggőnek bizonyult. Továbbá a *Chlamydomonas* sp. MACC-216 algát kék 250, piros 250, kék 125 + piros 125 és fehér 250 fényviszonyok között növesztettük SWW-ben, ahol a 125 és 250-es számok egységnyi µmol m^{-2} s^{-1} fényintenzitást jelölnek. A *Chlamydomonas* sp. MACC-216 nitráteltávolítási hatékonysága szintetikus szennyvízben kék 125 + vörös 125 fényviszonyok mellett bizonyult a legmagasabbnak; hasonló eredményeket figyeltünk meg a nitrátreduktáz-aktivitás tekintetében is. A nitrát szállításában és redukciójában részt vevő öt gén (*NRT1*, *NRT2.1*, *NRT2.2*, *NIA1* és *MCP1*) expresszióját alaposan megvizsgáltuk, és ezen gének mindegyike a kék 125 + piros 125 fényviszonyok mellett mutatta a legmagasabb expressziót. Összességében a kék + vörös fény kombinációja nagy fényintenzitással optimális fényviszonyokat jelentett a szintetikus szennyvízből történő hatékony nitráteltávolításhoz.

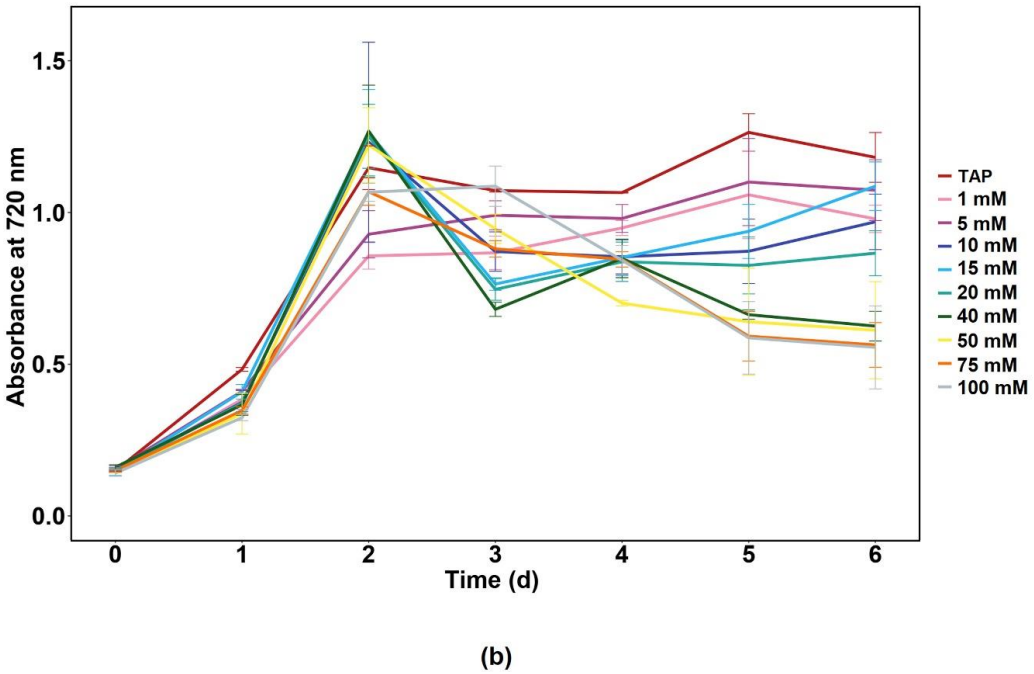
Mivel a *Chlamydomonas* sp. MACC-216 az első két célkitűzésben hatékony nitráteltávolítást mutatott, úgy döntöttünk, hogy összehasonlítjuk a nitrát hatékony eltávolítására képes mikroalga (*Chlamydomonas* sp. MACC-216) transzkriptomját a nitrát jelenlétében gyengén növekvő (*C. reinhardtii* cc124) mikroalga transzkriptomjával. Ennek érdekében a harmadik célkitűzésünk vizsgálatára transzkriptom-elemzést végeztünk, hogy összehasonlítsuk a nitrát jelenlétében és hiányában bekövetkező transzkripciós változásokat *Chlamydomonas* sp. MACC-216 és *Chlamydomonas reinhardtii* cc124 algákban, ami érdekes eredményeket hozott. A nitrát jelenlétében növesztett *C. reinhardtii* cc124 differenciális expressziós elemzése kimutatta, hogy csak 45 gén volt differenciálisan szabályozott, ebből 23 gén felülszabályozott, illetve 22 gén alulszabályozott volt. A *Chlamydomonas* sp. MACC-216 -ban a differenciális expressziós elemzés kimutatta, hogy 3143 gén volt differenciálisan szabályozott, 1604 gén felülszabályozott, továbbá 1539 gén alulszabályozott nitrát jelenlétében. Az eredményekből megfigyelhettük, hogy a *Chlamydomonas* sp. MACC-216 algát nitrát jelenlétében növesztve, a nitrát transzportjával és redukciójával kapcsolatos gének nagymértékű transzkripciós átrendeződést mutattak, míg a *C. reinhardtii* cc124 esetében nem tapasztaltunk hasonló eredményeket. A *Chlamydomonas* sp. MACC-216 algában az *NRT2.1*, *NRT2.2*, *NAR2*, *NIA1*, *NAR1.5*, *NAR1.6* és *NII1* gének jelentős felülszabályozódását figyeltük meg nitrát jelenlétében. A *C. reinhardtii* cc124 algában azonban a karbamid transzporttal kapcsolatos gének (*DUR3A*, *DUR3B*, és *DUR3C*) felülszabályozódását figyeltük meg hasonló körülmények között, ami azért érdekes, mert ennek a mikroalgának nem biztosítottunk ilyen nitrogénforrást a tápoldatban. A harmadik célkitűzéssel ezek a vizsgálatok világos képet adtak a nitrát-anyagcsere fajspecifikus szabályozásáról *Chlamydomonas* algákban.

APPENDIX

Supplementary Figures:

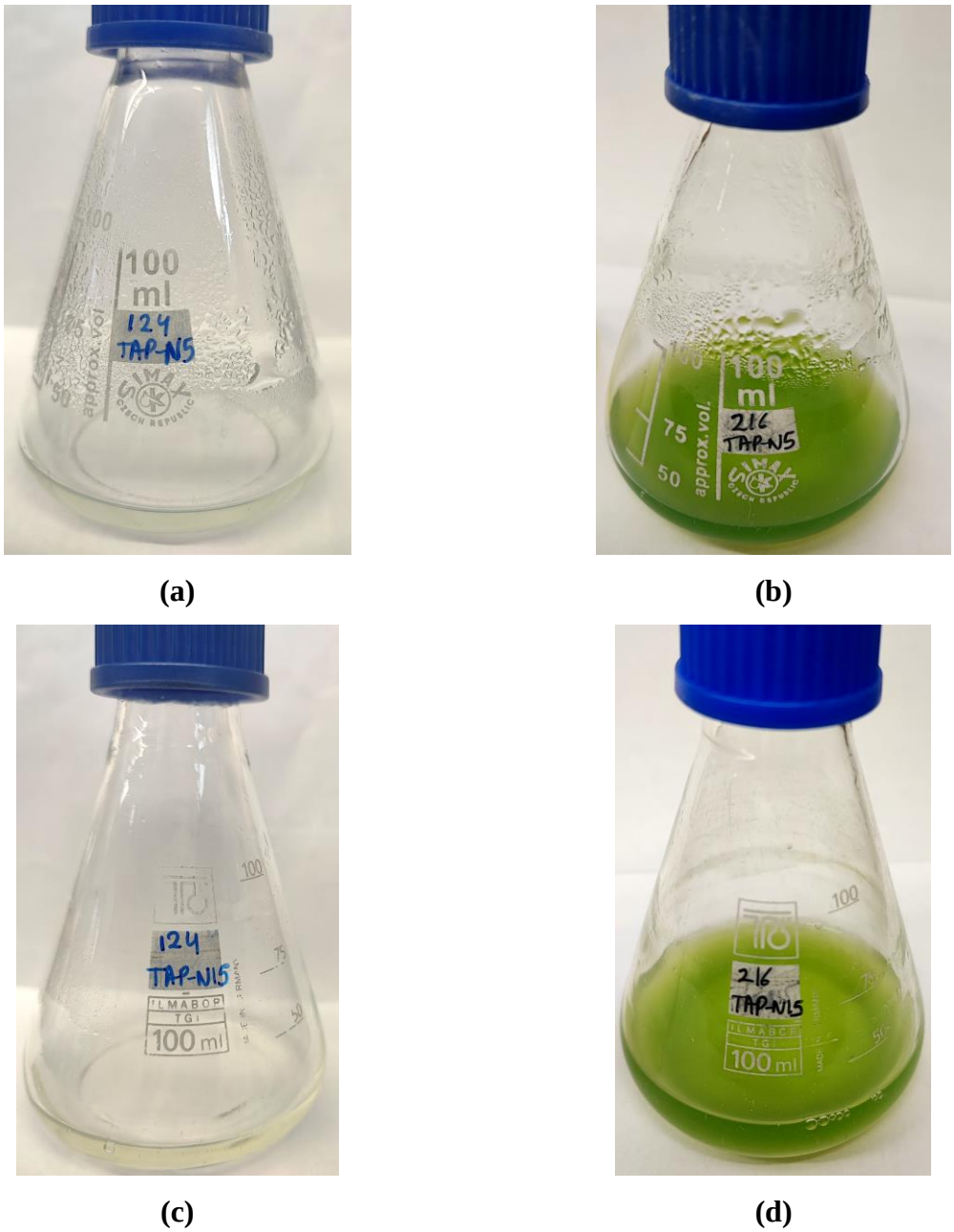


(a)



(b)

Figure S1. Growth of *Chlamydomonas* sp. MACC-216 (**a**) and *Chlorella* sp. MACC-360 (**b**) under standard TAP medium and TAP medium supplemented with different concentrations of nitrate. Error bars represent standard deviations.



(a) **(b)**

(c) **(d)**

Figure S2. Growth of *Chlamydomonas reinhardtii* cc124 (a, c) and *Chlamydomonas* sp. MACC-216 (b, d) in TAP-N5 and TAP-N15 media after 5 days.

Supplementary Tables:

Table S1. Total nitrate removal by *Chlamydomonas* sp. MACC-216 under various light conditions in TAP-N5 and TAP-N10 media. Values are represented as mean ± standard deviation.

Light condition	Total nitrate removal in TAP-N5 medium (mM)	Total nitrate removal in TAP-N10 medium (mM)
Blue 50	4.25 ± 0.07^{c}	4.71 ± 0.33^{g}
Blue 100	5 ± 0.0^{a}	5.27 ± 0.07^{fg}
Blue 250	5 ± 0.0^{a}	7.76 ± 0.31^{c}
Blue 25 + Red 25	4.61 ± 0.09^{b}	4.52 ± 0.21^{g}
Blue 50 + Red 50	5 ± 0.0^{a}	6.83 ± 0.05^{d}
Blue 125 + Red 125	5 ± 0.0^{a}	9.64 ± 0.3^{a}
Red 50	4.68 ± 0.08^{b}	4.44 ± 0.37^{g}
Red 100	5 ± 0.0^{a}	6.24 ± 0.1^{de}
Red 250	5 ± 0.0^{a}	8.92 ± 0.41^{b}
White 50	4.05 ± 0.08^{d}	4.60 ± 0.02^{g}
White 100	5 ± 0.0^{a}	5.78 ± 0.23^{ef}
White 250	5 ± 0.0^{a}	8.69 ± 0.08^{b}

Numbers 50, 100 and 250 mentioned in the table represent light intensity. The unit of light intensity is µmol m^{-2} s^{-1}.
Superscript lowercase letters signify statistical differences (p-value <0.05) as determined by Tukey's-test. Tukey's-test was done for each nitrate concentration separately.

Table S2. Values of total nitrate removal and nitrate reductase activity under various light conditions in SWW. Values are represented as mean ± standard deviation.

Light condition	Total nitrate removal (mM)	Nitrate reductase activity NO_2^- formed (µmol g^{-1} of FW min^{-1})
Blue 250	3.51 ± 0.13^{b}	18.47 ± 0.91^{c}
Blue 125 + Red 125	4.22 ± 0.05^{a}	31.09 ± 1.48^{a}
Red 250	3.67 ± 0.09^{b}	28.41 ± 0.48^{ab}
White 250	3.60 ± 0.19^{b}	25.4 ± 1.04^{b}

FW: Fresh weight
Superscript lowercase letters signify statistical differences (p-value <0.05) as determined by Tukey's-test.

Table S3. Values of the relative gene expression of *NRT1, NRT2.1, NRT2.2, NIA1,* and *MCP1* in *Chlamydomonas* sp. MACC-216 grown under various light conditions in SWW. Values are represented as mean ± standard deviation.

Gene	Relative gene expression			
	Blue 250	**Blue 125 + Red 125**	**Red 250**	**White 250**
NRT1	1.43 ± 0.23^{b}	6.0 ± 1.7^{a}	2.36 ± 0.08^{b}	1.01 ± 0.22^{b}
NRT2.1	1.59 ± 0.32^{b}	6.0 ± 1.9^{a}	1.93 ± 0.29^{b}	1.21 ± 0.80^{b}
NRT2.2	1.43 ± 0.46^{b}	5.18 ± 1.21^{a}	2.32 ± 0.32^{b}	1.02 ± 0.29^{b}
NIA1	0.90 ± 0.11^{c}	5.49 ± 0.35^{a}	1.89 ± 0.29^{b}	1.02 ± 0.28^{c}
MCP1	1.6 ± 0.34^{b}	4.35 ± 0.28^{a}	3.93 ± 0.44^{a}	1.0 ± 0.10^{b}

Superscript lowercase letters signify statistical differences (p-value <0.05) as determined by Tukey's-test.

Table S4. Total nitrate removal by *Chlorella* sp. MACC-38 and *Chlorella* sp. MACC-360 under various light conditions in SWW. Values are represented as mean ± standard deviation.

Light condition	Total nitrate removal (mM)	
	Chlorella sp. MACC-38	*Chlorella* sp. MACC-360
Blue 250	1.50 ± 0.01^{ab}	0.79 ± 0.12^{b}
Blue 125 + Red 125	1.67 ± 0.38^{a}	1.37 ± 0.20^{a}
Red 250	0.73 ± 0.02^{b}	0.44 ± 0.05^{b}
White 250	0.88 ± 0.14^{ab}	0.46 ± 0.07^{b}

Superscript lowercase letters signify statistical differences (p-value <0.05) as determined by Tukey's-test.

LIST OF PUBLICATIONS

Peer-reviewed publications for the completion of PhD:

1. **Rani**, V., Maróti, G. 2021. Assessment of Nitrate Removal Capacity of Two Selected Eukaryotic Green Microalgae. *Cells*. 10:2490. (IF_{2021}: 7.666)
2. **Rani**, V., Maróti, G. 2023. Light-Dependent Nitrate Removal Capacity of Green Microalgae. *International Journal of Molecular Sciences*. 24:77. (IF_{2023}: 6.208)

MTMT: 10084705