Chapter 1

Introduction

Microalgae is a photosynthetic organism and capable of converting light energy, nutrients, and carbon dioxide into biomass (Mata et al., 2010). For more than 40 years, some microalgae strains are known as sources of carbohydrates, proteins, lipids, and vitamins. For that reason these are mostly used as food and feed additives. Microalgae with valuable properties were used only in aquaculture and the composition of the algal biomass considering protein, lipid and carbohydrate contents and their quality determines its overall economic potential (Williams & Laurens, 2010).

Addition of larger proportions of plant-based materials may be beneficial for the sustainable healthy diets for humans and animals (Sabaté& Soret, 2014) (Westhoek et al., 2014). Many species of microalgae are able to produce additional metabolites that are not available from natural terrestrial plant sources that's why phototrophic microalgae are promising source of alternative food and feed ingredients (Wells et al., 2017) (Mühlroth et al., 2013).

World aquaculture is fully dependent on those feed ingredients which are derived from capture fisheries, so there is an urgency to find substitute materials that reduce environmental costs (Chauton et al., 2015) (Norambuena et al., 2015). Replacing feed ingredients with a source that is single cell oil and protein producer and have faster growth rate, can minimize the risks related to the traditional sources and ensure the nutritional quality issues and pollutants impacts from the marine food chains (Bellou et al., 2016).The Proximate composition is the body forming chemical factors like protein, carbohydrates, lipid, etc. that form the entire body of any organism. Previous studies demonstrated that microalgae contain on average 30-40% protein, 10-20% lipids, and 5-15% carbohydrates (Brown et al., 1997) (Reitan et al., 1997). Microalgal lipids are extensively favored for the production of biofuel for alternative energy(Li et al., 2008)

Microalgae growth is nutrient specific and environmental factors can alter the natural behavior of microalgae like *Nannochloropsis sp.*, *Tetraselmis* sp. and so on. Culture

condition influence the biomass and pigments productions of microalgae(Rao et al., 2007).

Depletion of inorganic phosphorus and nitrogen in the growth medium can influence the accumulation of lipids in oleaginous algae but also triggers cell remodeling processes whereby membrane lipids are broken down and their forming fatty acids are rebuilt into TAG. When microalgae oils are incorporated into food and feed, this redistribution of fatty acids amongst the different types of lipids could influence the fatty acid chains bioavailability (Kagan et al., 2013).

In this study, *Nannochloropsis* sp. was used which is a non-motile unicellular microalga. They are mostly found in the marine environments but also found in fresh and brackish water environments. The incorporation of *Nannochloropsis* sp. can only be economically feasible if their biochemical compositions and growth performances are satisfactory. Growth performance can be determined by cell counting, optical density, biomass determination, Specific Growth Rate (SGR) etc.

Nannochloropsis sp. is a good source of biodiesel because of its high growth rate and high lipid content, ranging from 31% to 68%(Chisti, 2007). It has rapid growth performance with various nutritional components like N,P,Vitamins etc.

The use of *Nannochloropsis* has been recognized for human diets and there are several studies about the enrichment of foods such as noodles with this biomass, to improve their nutritional profile. Feeding experiments have demonstrated the beneficial effect of reducing blood pressure in hypertensive rats (Seto, et al; 1992). The bioavailability of EPA from algal sources was evaluated in rats fed with diets supplemented with this biomass.

As rotifers feed and fish hatcheries feed ingredients, *Nannochloropsis sp.* has been as great source due to its nutritional value. The microalgae *Nannochloropsis sp* – is a promising alternative ingredient for aquafeeds as well as various industrial applications due to its favorable protein and lipid content.

The nitrogen sources in the culture media are key factors for the growth of microalgae. The objectives of the thesis work is given below-

a) To evaluate the growth rate of *Nannochloropsis* sp. cultured in different nitrogen concentration.
b) To determine the proximate composition of *Nannochloropsis* sp. cultured in different nitrogen concentration.

And the thesis paper will answer the following specific questions –

a) What is the effect of nitrogen concentration fluctuation on the growth of *Nannochloropsis* sp.?
b) Can nutritional value of *Nannochloropsis* sp. be improved with the changes of nitrogen concentrations?

If we can answer the above questions from the study, it might be a successful study outcome.

Chapter 2

Review of Literature

2.1: *Nannochloropsis* sp.:

Nannochloropsis sp. is a genus of microalgae comprising approximately 6 species. The species typically known from the marine environment, but also occur in fresh and brackish water. *Nannochloropsis* are small sizes species, nonmotile and cannot be recognized by either light or electron microscope. They have a diameter of about 2 µm. *Nannochloropsis* sp. are widely used in marine hatcheries as food sources as well as to maintain water quality (Riquelme and Avendano-Herrera, 2003; Khatoon *et al*., 2007 in Banerjee *et al*., 2011). It is considered as potential algae for industrial applications as a consequence of its capability to build up high levels of polyunsaturated fatty acids. It is primarily used as an energy-rich food supply for fish larvae and rotifers. *Nannochloropsis* sp. just has been explored for biofuel production. *Nannochloropsis* are commercially cultured for extensive use in the aquaculture industry for growing small zooplankton such as rotifers, copepod, daphnia and artemia (Banerjee *et al.,* 2011), for feeding SPS corals and other filter-feeders that require extremely small phytoplankton to bloom as well as to establish the initial step of an artificial food chain.

2.2 Importance of *Nannochloropsis* sp.

There are many importance of microalgae, *Nannochloropsis* sp. such as for the nutrition in aquaculture, as a highly potential species for production of biodiesel, able to inhibit the opportunist pathogen *vibrio* in larvae and rotifer culture, and also as a great alternative to fresh microalgae. *Nannochloropsis* sp are considered among the high nutritional microalgae which have been widely used as a food supply in the aquaculture industry for hatchery grown herbivores, such as larval and juvenile bivalves (Okauchi, 2004) and it was recognized as the source for different valuable pigments, such as chlorophyll a, zeaxanthin, canthazanthin, and astaxanthin which was produced at high level (Lubi'an*et al.* 2000) and also as a great potential source of essential ω-3 LC-PUFA eicosapentaenoic acid (EPA; 20:5 ω 3) (Renaund*et.al.* 1991; Renaund*et al.* 1994; Sukenik*et al*. 1993). These nutrients, especially EPA was important for the growth and development of marine

fish larvae, either by directly added to the culture system or by incorporating into rotifer as the live food for the culture system. The nutritional value of rotifers fed to fish larvae depends on the rotifer dry weight, caloric content and biochemical composition, and is highly correlated with the type of food they are fed (Lubenz*et al.* 1995).

Furthermore, as the primary producer in the aquatic food-chain, it provides many phytonutrients including in particular, PUFAs – EPA as mentioned before, arachidonic acid (AA) and DHA, which was important for growth and metamorphosis of many larvae (Becker *et.al.* 2004). The marine Eustigmatophyte, *Nannochloropsis* sp. has been recommended as a preferable food for the rotifer *Branchionus plicatilis* which was proven to be efficiently transfers the algal PUFA (mostly eicosapentaenoic acid (EPA) - 20: 5 n-3) to marine fish larvae (James *et al.* 1988; Seto *et al.* 1992). Moreover, it was able to support high rate of survival and growth and also the rotifer reproduction (egg carrying rotifer).

Besides that, another importance of *Nannochloropsis* sp. is its ability as a bioremediation as it was able to absorb dissoluble nitrogen and phosphate as nutrient (Okauchi*et al.* 1995). This is beneficial in controlling water quality which can deteriorate because of dead larvae and also after feeding with artificial diet. Not only that, *Nannochloropsis* sp. has been proven to be able to inhibits the opportunist pathogen vibrio (Eguchi*et al.* 2011), which infects grouper larvae (Liao *et al.* 2001) and retards the growth of rotifers (Tinh*et al.* 2007).

Cultivation of photosynthetic microalgae have received bigger interest as a potential sources of high-value biochemical components, such as natural colorant, polyunsaturated fatty acids, proteins and polysaccharides (Chen *et al.* 2010) as well as being a potential biofuel source of food material. *Nannochloropsis* sp. also has a great potential as feedback for biodiesel production on long term perspectives as it was able to accumulate large amount of lipids which can reach concentration up to 65%-70% of total dry weight (Hodgson *etal.* 1991; Rodolfi *et al.* 2009).

2.3: Growth phases of microalgae:

Growth phase of microalgae consist of five growth phases which were, the lag phase, exponential phase, phase of declining relative growth, stationary phase and death phase (Lavens *et al*. 1996).

The initial phase was lag phase and during this phase the increase in the cell density was low because at this phase, the microalgae cell undergoes physiological adaptation of the cell metabolism involved in cell division and carbon fixation.

The second phase was the log phase or also known as the exponential phase. During this phase, the cell density increases as a function of time t logarithmic function:

$$Ct = C_0.emt$$

Where, Ct and C_0 = the cell concentrations at time t and 0, and m = specific growth rate. The specific growth rate is mainly dependent on algal species, light intensity & temperature.

The third phase is the phase of declining growth and at this time, cell division slows down and nutrients, light, pH, carbon dioxide or other physical and chemical factors begin to limit growth.

The fourth phase is the stationary phase and the cell density was constant for some times at this phase because the limiting factor and the growth rate is balance. This is the phase with the highest microalgae density.

The last phase was the death phase. At this phase, the water quality deteriorates and nutrient depleted to the level incapable of sustaining growth and the cell density decreased rapidly. The culture collapsed.

2.4. Nutritional value of *Nannochloropsis* sp.

The biochemical composition of microalgae may change when cultured in different environmental condition, such as salinity, pH and temperature. Protein, carbohydrate and lipid are among the major composition in microalgae.

Proteins are long chain of amino acids that is important for growth and development of marine animal and larvae. It makes up about 65-75% of dietary dry weight of many animals including fish and shrimps and it also makes up a large portion of microalgae's biomass and cyanobacteria (Lopez *et al.* 2010). Protein composition of microalgae may change when in different culture condition such as salinity, light intensity, temperature and other factors. Under light-limited conditions, protein percentage increased with increasing irradiance at the expense of lipid percentage and carbohydrate to a lesser extent (Fabregas et al. 2004). The protein content per cell, which is considered as one of the most important factors that determines the nutritional

value of micro-algae as feed in aquaculture, was found to be more susceptible to medium-induced variation than the other cellular constituents. *Nannochloropsis*sp. in exponential phase have the highest crude protein content (33.99%) and the lowest (28.33%) in stationary phase (Huang *et al*. 2004).

Lipids are organic molecules containing many carbon atoms (C) in a variety of chain and are founds in the tissues of plants and animal.Lipid is one of major portion of phytoplankton biomass and it plays an important role in controlling growth and reproduction of many marine animals (Mansour *et al.* 2005). Lipid are consist of five main classes; fatty acids, triglycerides, phospholipid, glycolipids, waxs and sterols.

Microalgae biosynthesize lipid compounds as phospholipids and triacyglycerols in cell membrane and intracellular energy storage compound (Brown *et.al.* 1996). The total lipid content of algae in phase of declining relative growth was significantly higher than those in exponential phase and stationary phase (Huang *et al.* 2004). *Nannochloropsis* sp. contains 29.81 % of lipid when cultured in salinity of 33ppt and less than 12 hour light and 12 hour dark cycle (Alsull *et al.* 2012).

Carbohydrate in microalgae biomass is mainly in the cell wall and starch in the plastids without lignin and can be readily converted into fermentable sugar (Chen *et al.* 2013). Composition and metabolism of carbohydrate may differ significantly from species to species (Rismani-Yazdi *et al.* 2011). The amount of carbohydrate in *Nannochloropsis* sp. was 0.48 pg.cell^{-1}(Lavens *et al.* 1996).

2.5. Factors Affecting Microalgae growth

2.5.1: Temperature:

Microalgae growth can be influenced by temperature fluctuations. Besides light and nutrients, temperature also influences the growth of microalgae. Due to the greenhouse effect, the wide range of temperature fluctuation from 10 to 45°C has been experienced in photo-bioreactor culture of microalgae in temperate regions. (Béchet et al., 2010), thereby including temperatures above-tolerated thresholds of most commercialized algae species(Mata et al., 2010) (Ma et al., 2015) stated that the optimal temperature conditions for *Chlorella vulgaris* are 25° - 28°C; on the other hand, (Leyva et al., 2014)state that it is 27°C. The cultivation temperature of *Chlorella* sp. is much higher than the stated temperature.

Temperature also affect the nitrogen availability, growth, fatty acid profile and cell density of the microalgae culture (Navarro-Peraza et al., 2017). Different study illustrated that the temperature of 25-30°C and salinity of 30‰ induced optimum cell proliferation in *Skeletonema costatum*

2.5.2: Salinity:

Biochemical and physiological mechanisms like growth and lipid production are also influenced by the variations in salinity (Fava and Martini, 1988). It is recommended that cultivate the ideal species which have wide range of salinity tolerance in high salinity condition and thus will provide higher biomass.

When the salinity in the open waterbody or pond increase, it could be possible to produce continuous production of higher biomass by cultivating halotolerant microalgae organisms (Ishika et al., 2018). Between salinities of 22 and 34 PSU,*N. salina*exhibited highest biomass accumulation and growth rates (Bartley et al., 2013). Salt stresses also induce the accumulation of lipids in most algal cells but it is species specific.

Lipids were stored in salt-stressed cells and were degraded when stressed cells are crossed the optimal conditions (Shetty et al. 2019). A significant proportional increase in the lipid content of biomass occurred with an increase in salinity (up to 35 ppt), with

a concomitant decrease in EPA percentage, in *N. oculata* (Renaud and Parry1994). Complete growth retardation also observed with the higher salinity levels in *Nannochloropsis sp.* (H. Hu & Gao, 2003); but higher EPA percentage has been noticed with lower brackish water salinities. (Hu & Gao, 2003)

2.5.3: Light:

When growing autotrophically, microalgae and cyanobacteria are strongly depended on light supply and temperature (Laura Barsanti, 2006). Biomass productivities and Specific growth rates are strongly dependent on these factors. Similarly Solovechenko et al. have shown that high light intensities, especially under nitrogen starvation cause slow biomass and TFA accumulation by the microalga *Parietochlorisincisa* and cause cell damage (Solovchenko et al., 2008).

With the increasing light intensity, *S. obliquus* and *Desmodesmus* sp. showed higher fatty acid contents, declined protein content and unchanged carbohydrates content. The same results also observed for the microalgae *Dunaliella tertiolecta* (Cuhel et al., 1984).

2.5.4: pH:

Many factors influence the microalgae growth, this includes the pH of the culture medium to grow them. Depending on the microalgae species, some of them can adapt in acidic conditions but most of them preferred neutral and alkaline conditions for optimum growth (Lam et al., 2017). An increase in the pH of the culture medium resulted in a reduction of the total amount of the structural lipids (Spilling et al., 2013). Microalgae biomass attachment was much preferable at alkaline conditions than acidic conditions. While pH9 the highest dry weight of attached microalgae biomass, the lipid content was lower. Therefore pH6 was selected to be the best culture condition for *Chlorella vulgaris* for both suspended growth and attachment, accompanied by high lipid content compared to other pH values (Rosli et al., 2018).

2.6: Effect of different nutrients on the growth of microalgae:

2.6.1: Phosphorus:

Phosphorus is a vital component required for normal development and growth of algal cells. It is the primary limiting factor for microalgal growth (Larned, 1998). Algae constitute 1% phosphorous of their dry weight, (Vymazal, 1988) whatever it still not enough for microalgae that minute amount as all of the added phosphorus are not bioavailable because of their binding nature with metal ions. Calvin-Benson cycle is a vital carbon fixation cycle, are substrate dependent. These substrate could not be produced due to any shortage of available and required phosphorus (Laura Barsanti, 2006).

Phosphorus limitation in the microalgae culture also results in lipid accumulation. With a reduction in the total phosphorus (as phosphate) concentration of 0.1 to 0.2 mg per liter,total lipid content in *Scenedesmus* sp. was noticed to increase from 23% to 53% (Xin et al., 2010). Phosphatidylglycerol (PG) was observed to decrease with phosphorus limitation in *Chlamydomonas reinhartdtii* (Sato et al., 2000). It is essential for normal structure-function of the photosynthesis-2 complexcell growth, and the maintenance ofchlorophyll-protein complex levels. N-3 PUFA synthesis also reduces due to the limitation of phosphorus (Reitan et al., 1994).

2.6.2: Trace metals:

There are so many trace metals exist in algal cells in minute amount (less than 4 ppm). These are manganese (Mn), Iron (Fe), cobalt (Co), copper (Cu), nickel (Ni) andzinc (Zn). The six trace metals are most important and required for their metabolic processes. (Bruland et al., 1991)

Algal growth is also dependent on the trace metals availability as these are limiting factors. Whereas these are excess or lower in concentration, may inhibit growth, impair photosynthesis,damage the cell membrane and deplete anti-oxidants.

For photosynthesis and respriration in alagae, iron is a vital trace element. Oxidation reduction reaction process is necessary for nitrogen assimilation; there it acts like a catalyst. As a catalyst it also participates in many processes, such as- photosynthesis,

electron transport reactions etc. (Abadia, 1986). Cellular chlorophyll concentration also reduces due to iron limitation (Greene et al., 1992) (Liu et al., 2008) observed that, to increase the lipid content, the higher concentration of iron in cultivation of *Chlorella vulgaris* were key factor. Decrease in iron content also reduces carotenoid composition also observed to be reduced because of iron content deficiency (Kobayashi et al., 1993) (van Leeuwe & Stefels, 1998).

2.6.4: Nitrogen:

Nitrogen is an important nutritional element for the microalgal growth because the formation of amino acids, proteins, coenzymes, enzymes, chloroplast, and so on is related with the assimilation of nitrogen (Turpin, 1991) A variety of nitrogen sources (such as ammonia, nitrite, nitrate,and urea) can be used by microalgae, while various nitrogen sources may induce their biochemical composition. More importantly, under nitrogen-depletion conditions various microalgal species could convert proteins or peptides to carbohydrates or lipids as energy reserve elements (Huo et al., 2011). (Illman et al., 2000) showed that a 55% carbohydrate content was found when culturing*C. vulgaris* on a depleted nitrogen medium.(D'Souza & Kelly, 2000) also explained that the culture of *T. suecica* under nitrogen depletion with carbon-di-oxide feeding could suddenly enhance the carbohydrate content of cell from 10% to 57%. Literature showed various examples like that, the limitation of nitrogen availability seems to be the effective way of inducing the carbohydrates accumulation in microalgae (Ho et al., 2011) However, some studies also showed that there was a negative relationship between lipid and carbohydrate yield under the different stresses of environment (e.g., nitrogen depletion) because the pathways of metabolism are related with production and depletion of energy-rich compounds (e.g., lipids and carbohydrates) are closely linked (Ho et al., 2012) (Rismani-Yazdi et al., 2011) (Siaut et al., 2011).

Nitrogen and light limitation are the most specific and influential factors that affect both the biochemical composition and cellular productivity(Angles et al., 2017). Among the other nutrients, the concentration and source of nitrogen can induce and make important alteration the biochemical compositions of microaolgae cells. *Nannochloropsis* sp. is a great candidate as a feedstock for the biofuels production and

food. Under the nitrogen limitation, growth, cell density, and fatty acid profiles are highly induced. (Navarro-Peraza et al., 2017) In that condition, the chemical composition of microalgal cells is also known to differ during their growth phase, particularly concerning their lipid component (Sukenik & Carmeli, 1990).

Chapter3

Materials and Methods

3.1: Culture and Maintenance of *Nannochloropsis* sp.:

The marine microalgae *Nannocloropsis* sp. was obtained from the culture collection of the Aquaculture Department of Chattogram Veterinary and Animal Sciences University (CVASU). To amplify the volume of the culture, it was cultured again as a stock culture. The species was cultured in Conway media. The surrounding environment was controlled by placing the stock in a Laminar Airflow (Esco laminar airflow). The Temperature was 25 ± 2°C

This thesis work was accomplished in two different phases. The first phase was the growth curve and parameters determination experiment followed by the experiment to determine the proximate composition of the *Nannochloropsis* sp. Both of the phases were maintained at different nitrogen concentrations of Conway Media. The experiments were carried out in the Live Feed Corner of the Department of aquaculture in CVASU.

3.2: Purpose of the Growth Curve Experiment:

The growth curve experiment was carried out to look at the growth of *Nannocholoropsis*sp. at different nitrogen concentrations. The growth curve helped in the decision making of harvesting of the species for the second phase of the experiment. As the day before the stationary phase always shows the highest growth condition of any species, the growth curve certainly helps to make the decision to harvest.

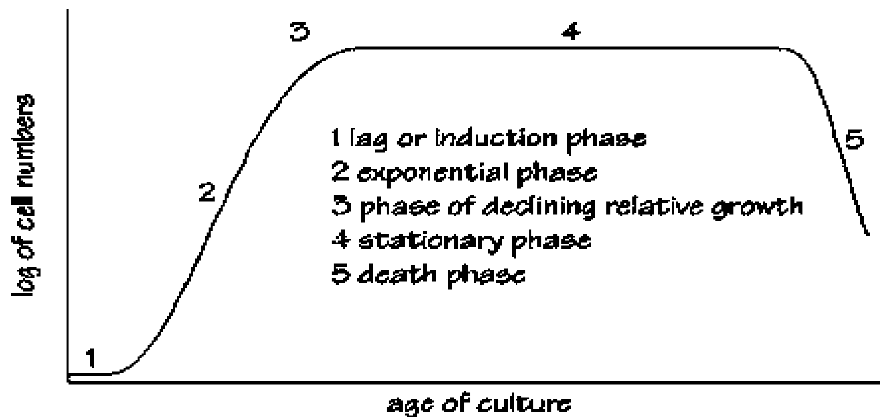


Fig 1: Growth Curve of Microalgae

3.3: Preparation of Culture Media:

3.3.1: Processing of Natural Sea Water:

Natural seawater was used as a culture media of the *Nannochloropsis* sp. The Seawater was collected from the Halishahar beach, Chattogram and the salinity was 26ppt measured by using a hand refractometer. The seawater was kept in a jar for several days for siltation. After that seawater was separated by siphoning. Then the seawater was filtered with the vacuum pump filtration unit (Rocker300) using microfiber filter papers (Whatman Glass Microfibre filter paper, GF/C 47mmǾ Circles). Then the seawater was autoclaved at 121°C for 15 minutes at 15lb/inch^2 using an autoclave (Human Lab, DAC-60). Finally, the seawater was kept in a dry and cool place for use.

3.3.2: Preparation of Conway media:

The direct use of natural seawater alone is not feasible in culturing algae because the yield is normally quite low for culture maintenance and laboratory experiments (Harrison, 2014). Laboratory experiments need complete control of culture media to grow the microalgae. Therefore culture media should be filled with adequate nutrients. Conway media is a combination of main minerals stock, trace minerals, and vitamins. It also provides adequate nutrients for marine microalgae. It was used as a form of solution and added with the prepared natural seawater. Each of the solutions was prepared by following the media preparation chart. 1ml of main minerals stock, 0.5ml of trace metals, and 0.1 ml of vitamins were added per 1000 ml filtered seawater. The pH of the culture media was maintained through the use of 1:3 HCl and Water solution.

Fig 2: Conway Media (solution a, b & c)

3.3.3: Main minerals stock solution preparation:

The minerals solution in Conway media is called Solution A. It is a combination if different compounds with definite quantity. By using the electrical weigh machine, these need to be measured accurately. The chemicals weighed were, potassium nitrate (100g), sodium orthophosphate (20g), sodium EDTA (25g), boric acid (33.4g), ferric chloride (1.3g), manganese chloride (0.36g). The method used was from James, 1996. Then, half of a 1000 ml volumetric flask was filled with fresh milli-equivalent water. The first weighed chemical, potassium nitrate, was added to the milli-equivalent water and was dissolved while stirring continuously. After it had dissolved completely, the second chemical, sodium orthophosphate, was added to the solution and was also dissolved while stirring continuously. This step was repeated for all weighed chemicals in particular order until all of them were added to the solution. Next, the solution was diluted to final volume of 1000 ml with fresh milli-equivalent water. Finally, the main minerals stock solution was stored in a Schott- Duran® bottle and kept in a refrigerator until later use.

3.3.4: Trace minerals preparation:

All of the required chemicals were weighed precisely with the electrical weigh machine (RADWAG, AS 220.R2). The required chemicals weighed were zinc chloride (4.2 g), cobalt chloride (4.0 g), copper sulphate (4.0 g), and ammonium molybdate (1.8 g). The

method originated from James, 1996. Half of the 1000 ml volumetric flask used was filled with fresh milli-equivalent water. Next, the first weighed chemical, zinc chloride, was added to the milli-equivalent water and was dissolved while stirring continuously. When it was completely dissolved, the second chemical, cobalt chloride, was added to the solution and was dissolved while stirring continuously too. This procedure was repeated for the rest of the chemicals in particular order until all of them were added to the solution. After that, the solution was diluted to final volume of 1000 ml with fresh milli-equivalent water. Lastly, the trace metals solution was stored in a Schott- Duran® bottle and kept in a refrigerator (Walton, WNJ-5A-0102) until later use.

3.3.5: Vitamin solution preparation:

Half of the 50 ml volumetric flask was filled with fresh milli-equivalent water. 100 mg of vitamin B (thiamine) was weighed with the analytical balance. Then, the vitamin B (thiamine) was added to the water. After the vitamin had dissolved, the solution was diluted to final volume of 50 ml with fresh milli-equivalent water. Finally, the vitamin B (thiamine) solution was kept in a tight Schott- Duran® bottle and stored in the refrigerator until later use.

3.3.6: Modified potassium nitrate in Conway media solution preparation:

Conway media was prepared firstly with the standard prescribed formula. Then only solution A which composed potassium nitrate was remade by following different concentration of potassium nitrate. But other minerals were remaining same in quantity as the experiment's target was to find out the impact of potassium nitrate concentration at different concentration on *Nannochloropsis* sp. The method used was adapted from James, 1996. Hence, main minerals stock solutions with 50 g/L KNO3, 75 g/L KNO3, 125 g/L KNO3, and 150 g/L KNO3 were prepared separately. Each solution were kept in centrifuge tube (Biolab centrifuge tube eppendrof) and stored in the refrigerator. Here the grams per liter of nitrate concentration resembled the concentrations in solution A. Only 1ml of solution A was added so the actual concentration in the media was milligrams per liter in terms of unit. But in this experiment we used the nitrate concentration from the view of solution A composition.

3.4: Experimental Design:

3.4.1: Growth Curve Experiment:

Nannochloropsis sp. was inoculated in 300ml culture media for growth curve experiment. 5% *Nannochloropsis sp.* was stocked in the 300ml media. It was stocked in a 500ml volumetric flask. The stock of microalgae was designed by following three duplicates for each of the treatments. The number of treatments was five (50g KNO_3, 75gKNO_3, 100gKNO_3, 125gKNO_3, 150gKNO_3 Conway media). The 100g KNO_3 was the control treatment for the other four treatments. The growth curve experiment was conducted until the stationary phase was determined for each of the treatments. The stationary phase was required for the phase of the experiment. The culture environment was maintained with 25±2°C temperature, 2000lux light intensity, 24:0 photoperiods and 7.8 pH. Plate 3.2 Main minerals stock solution with 50 g/L, 75 g/L, 100 g/L (Control), 125 g/L and 150 g/L concentration of potassium nitrate (KNO3) The cultures were aerated continuously with natural sterile air using an air pump (SHANDA &SeBO).

3.4.2. Analysis of growth parameters

Microalgae growth was measured using cell count and optical density.

3.4.2.1 Determination of cell density:

Cell density was carried out every day using a Neubaer haemacytometer. Haemacytometer was cleaned with distilled water to make sure that it is free of dust, lint and grease. Then small drop of properly mixed sample was transferred into the counting chamber. Meanwhile, the pipette was held at an angle until a small drop has arisen at the tip of it. Then the drop was put between the cover glass and the counting chamber base as a result of the capillary effect. No bubbles and the cells were allowed to settle 3-5 minutes for better counting. The cells were count in five small squares in the centre block. The total number of cells was calculated using the formula:

$$\text{Cell density (cells/ml) for 25 squares} = \frac{\text{total number of cells counted} \times 10^6}{10 \times 4}$$

Where, 10= the squares of 2 chamber

$4x10^{-6}$= the volume of samples over the small squares area which is equivalent to 0.004mm^3 (0.2x0.2x0.1) expressed in cm^3 (ml)

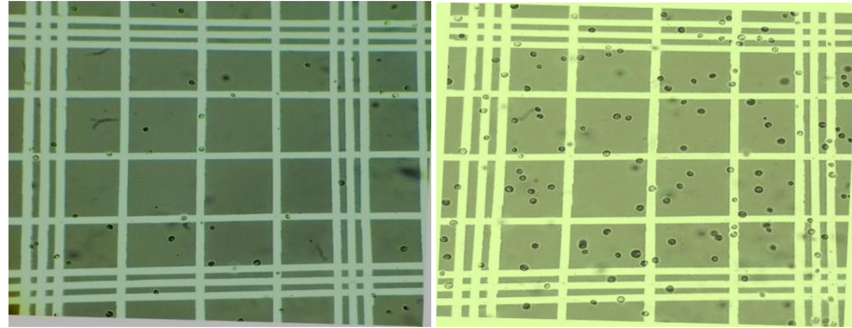


Fig 3: Cell counting for growth curve using hemacytometer under light microscope

3.4.2.2: Specific Growth Rate (SGR):

The obtained cell count data with different days were used to determine the SGR. Specific growth rate (μ) was calculated using the following formula (Fogg&Thake, 1987):

$$\mu \text{ (Cells / day)} = \frac{\ln x2 - \ln x1}{t2 - t1}$$

Note:

μ = growth rate of per unit cell concentration, x_1 = cell concentration at time 1, x_2 = cell concentration at time 2, t_1 = time 1, t_2 = time 2

3.4.2.3: Cell Doubling Time (td):

It was calculated from the obtained μ (SGR). Doubling time can be calculated using the formula (Agarwal et al., 2012):

$$\text{Td (Day)} = \frac{\ln 2}{\mu}$$

Here, μ = SGR

3.4.3: Culture of *Nannochloropsis* sp. using different nitrogen concentration:

3.4.3.1: Phase 1:

This phase was the determination of stationary phase from the growth curve of the *Nannochloropsis* sp. that was cultured at culture media of altered KNO_3 nutrient. It was initiated with cleaning of 15 conical flasks by following the laboratory glassware cleaning protocols. These conical flasks (Glacier UK) were autoclaved at 121°C for 15 minutes and 15 lb/inch^2 pressure. Then the flasks were poured 200ml and 5% of 300ml *Nannochloropsis sp.* cultured stock equivalent 15ml was mixed with the 200ml media and poured more media upto 300ml volume. The cultured *Nannochloropsis* sp. density that was inoculated was 6.65×10^6 cells/ml.

The phase-1 experiment was monitored by determining some factors like cell density, optical density etc. Cell density was measured every day. The procedure to determine the cell density procedure is described in the 3.4.2.1.

3.4.3.2: Phase 2:

After the completion of the phase 1, there was a decision making position to make decision when the cultures of different nitrogen concentration were reached the stationary phase. According to that information, another 15 conical flasks with same treatments were cultured again for the biomass collection. But this time the cultures volume was 5 times to the phase 1. At the early stationary phase of the treatments, these were centrifuged. A centrifugal machine comprising six eppendrof tubes were used for the centrifugation process. Centrifugation of the cultures was carried out in a centrifuge tube(HERMLE Z 206A). There were four different stresses and a control treatment to be centrifuged.

Table.1: Different Treatments

Nitrogen Concentration	Title of the treatment
50g/L KNO_3	Stress 1
75g/L KNO_3	Stress 2
100g/L KNO_3	Control

125g/L KNO_3	Stress 3
150g/L KNO_3	Stress 4

Each of them was 1.5L×3= 4.5L and centrifuged to make algae pellets. The control treatment was used in order take the normal algae growth and proximate compositions as a standard for the*Nannochloropsis* sp. against the different concentrations outcome.

There were four types of treatments in this study to determine the effects of different nitrogen concentrations (KNO_3) on growth and proximate composition of*Nannochloropsis*sp. Treatments were determined by decreasing and increasing the concentration of nitrogen (KNO_3) present in Conway medium relative to the concentration used in the control. Hence, the treatments were composed of 50 g/L, 75 g/L, 125 g/L, and 150 g/L of nitrogen (KNO_3) in Conway medium respectively. There were three replicates for control and the stress treatments. The experiment was terminated when the *Nannochloropsis* sp. culture reached the stationary phase.

3.5: Proximate composition analysis

Proximate analysis was carried out after the culture was harvested at stationary phase.

3.5.1 Protein analysis

For every sample, 5-6 mg of freeze dried microalgae was taken and made into 25 ml solution by mixing with distilled water. From the 25 ml of sample prepared, 0.5ml was taken from each sample for protein analysis. Prior to that, Reactive 1 (1% NP tartrate) and Reactive 2 (2g of $NaCO_3$ in 100ml of 0.1 NaOH) were prepared. For the analysis (Lowry *et al.*, 1951), 50 ml of Reactive 2 and 1 ml of Reactive 1 were mixed. After which 0.5ml of sample was added with 0.5 ml of 1N NaOH and it was kept in 100°C water bath for 5 minutes. It was then cooled in a water bath and 2.5 ml of the prepared mixed reagent was added 10 minutes after cooling. The mixed solution added with 0.5ml of Falin and was kept in dark places for 30 minutes. The reading of the mixed solution was taken with spectrophotometer (UV-1601, Shidmadzu) at the wavelength of 750nm.

2000mg/L of standard stock solution was prepared and a series of standards were also be prepared (20mg/L, 40mg/L, 80mg/L, 100mg/L and 200mg/L) from the stock

solution. The same procedures as described for protein analysis were done on the standard series and a calibration line was plotted according to the results and the protein composition for every sample was determined.

3.5.2 Lipid analysis

1ml of culture was centrifuged and washed with 0.9% saline solution and freeze dried. Then lipid analysis was conducted using carbonization method (Marsh and Weinstein, 1966). The samples were extracted from 4.5 ml of chloroform: methanol (1:2 concentrations) and it was then centrifuged at 10,000rpm for 10 minutes. After separating the supernatant from the biomass, 1.5ml of chloroform and 1.5ml of distilled water was added and the sample was centrifuged again to facilitate the separation of two phases. After centrifugation, the polar phase was removed with a pipette and evaporated under vacuum with a water-bath at 35°C. The dry-residue was resolubilized in 1ml chloroform. Then, 3 aliquots of 200µL each was taken from this solution and transferred into test tubes and the solvent was evaporated again. When completely dried, 2ml of concentrated sulphuric acid was added. The carbonization process was carried out at 200°C for 15 minutes, then the tubes were cooled and 3ml of water was added into each tubes. The optical density was measured at 375nm.

Then, solution of tripalmitin in chloroform at 3 g/L and a series of standard solution of tripalmitinwas prepared (0µg, 30µg, 90µg, 120µg, 150µg, 180µg, 210µg, 270µg). The same lipid analysis was done on the standard series and a calibration line was plotted according to results and the lipid composition for every sample was determined.

3.5.3 Carbohydrate analysis

For each sample, 5-6 mg was taken and made into 25 ml solution by mixing with distilled water. Prior to analysis, 5% phenol solution and concentrated sulfuric acid was prepared. For analysis, 1 ml was taken from the prepared 25 ml solution and 5% phenol solution and 5 ml of sulfuric acid was added (Dubois *et al.* 1956). Then, the sample was kept in the cold water bath and reading was taken with spectrophotometer with wavelength at 488nm. 1000mg/L of standard stock solution was prepared and a series of standards at various dilution (20mg/L, 40mg/L, 60mg/L, 100mg/L and 140mg/L) were also prepared from the stock solution to produce a calibration graph. The same procedures as described for carbohydrate analysis were done on the standard

series and a standard graph was plotted according to the standard results obtained and the carbohydrate composition for every sample was determined.

3.6: Statistical analysis:

The effects of nitrogen on the growth and proximate composition of *Nannochloropsis*sp. was analyzed by using one- way analysis of variance (ANOVA) as well as Tukey and Duncan multiple comparisons test.

Chapter 4

Results

4.1 Growth Curve experiment:

4.1.1 Mean Cell Density:

The mean cell count of *Nannochloropsis* sp. cultured in normal Conway medium for the growth curve experiment displayed in figure 4. The growth of *Nannochloropsis* sp. in the curve showed a particular pattern. There was the lag phase from Day 0 to Day 3. Day 3 to Day 4 was the transition of lag phase to exponential phase. From Day 4 to Day 9 the exponential growth occurred. The transition to early stationary growth was happened between Day 9 to Day 10. The growth was in the stationary from the Day 10 to Day 11. The Day 11 to 12, death of the microalgae occurred. So the ultimate early stationary phase was Day 9. The growth was in the late stationary phase at Day11.

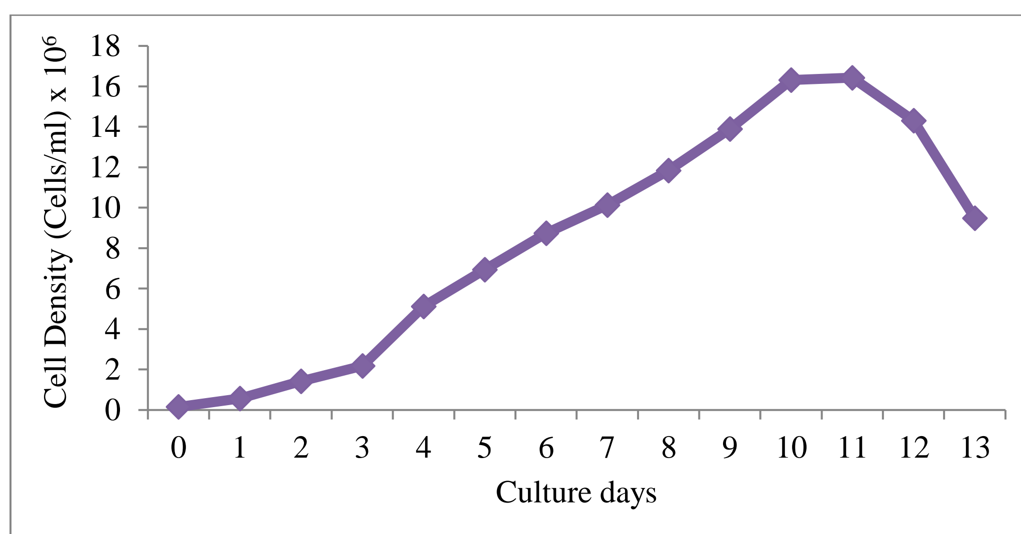


Fig: 4: Cell density (cells/ml x 10^6) of *Nannochloropsis* sp. in Conway medium

4.1.2: Cell density:

The cell count for different treatments including a control (100g/L KNO_3) and 4 stresses like stress 1 (50g/L KNO_3), stress 2 (75g/L KNO_3), stress 3 (125g/L KNO_3) and stress 4 (150g/L KNO_3) are shown in Figure 4c. The cell counts were done according to their (control and stresses) stationary phases and two declining days. From that point, stress 1, stress 2 and control were counted from Day 0 to Day 13. Stress 3 and stress 4 were counted from Day 0 to Day10.

The lag phase of stress 1 to stress 4 including control were same in terms of duration. From Day0 to Day4, the lag phase noticed from the figure: for all of the treatments. The exponential phase appeared after the Day 4. But there was a transition from lag to exponential phase showed between Day4 to Day5. The exponential was different in duration for different treatments. Stress 1 and Stress 2 had the exponential phase from almost Day4 to Day10 for Stress 1 and Day4 to Day9 for Stress9. The control showed the exponential phase from Day4 to Day10. The Stress 3 and Stress 4 showed narrow exponential phase. It was existed from Day4 to Day7. The stationary phase showed in the stress 1, stress 2 and control were from Day 10 to Day11, Day 9 to Day 10 and Day 10 to Day 11 respectively. In case of Stress 3 and Stress 4, the Stationary phase was from Day8 to day 9.

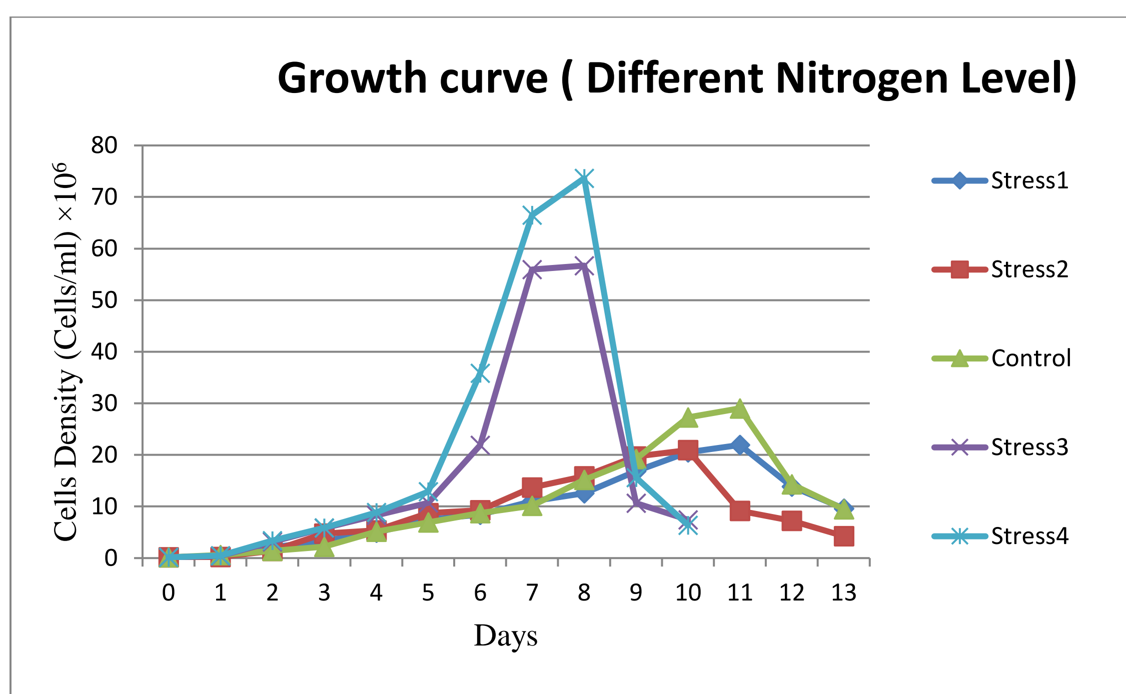


Fig: 5: Mean cell density (cells/mL)x10^6 versus culture period (days) for *Nannochloropsis* sp. cultured in different nitrogen stress. Values are mean ± standard error

4.1.3: Specific Growth Rate (SGR):

The specific growth day (SGR) for different treatments including a control and 4 stresses like stress 1, stress 2, stress 3 and stress 4 are shown in Table.2. The SGR was obtained from the cell concentration data of the treatments.

Table2: Specific Growth Rate (SGR) of *Nannochloropsis* sp. cultured in different nitrogen stress

Treatments	**Specific Growth Rate (10^6cells/ day)**
Stress 1	0.451
Stress 2	0.491
Control	0.525
Stress 3	0.739
Stress 4	0.772

The stress 1 and stress 2 and control showed almost same SGR. For Stress 1, the SGR value was 0.451 and stress 2 was 0.491 and the control showed the SGR was 0.525. Every day the growth rate for stress 1 was 0.451×10^6 cells, for stress 2; it was 0.491×10^6 cells and for control it was 0.525×10^{6}. The stress 3 and stress 4 had higher SGR than the other treatments. For Stress 3, it was 0.739 and stress 4 showed its SGR 0.772. So their everyday growth rate was 0.739×10^6 and 0.772×10^6 cells respectively.

4.1.4: Cell doubling time (td):

The Cell Doubling Time (td) for different treatments including a control and 4 stresses like stress 1, stress 2, stress 3 and stress 4 are shown in Table 3. The Cell Doubling Time (td) was obtained from the specific growth (SGR) data of the treatments.

Table.3: Cell Doubling Time (td) of *Nannochloropsis* sp. cultured in different nitrogen stress

Treatments	**Cell Doubling Time (td) (in Day)**
Stress 1	1.536
Stress 2	1.409
Control	1.321
Stress 3	0.937
Stress 4	0.898

The stress 1 and stress 2 and control showed almost same cell doubling time. For Stress 1, the td value was 1.536 and stress 2 was 1.409 and the control showed the Td was 1.321. The stress 1 doubled its cell density in 1.536 days and for stress 2 took 1.409 days. The stress 3 and stress 4 had lower td than the other treatments. For Stress 3, it was 0.937 and stress 4 showed its td 0.898. So every 0.937 day, the stress 3 doubled its cell density and at every 0.898 day stress 4 doubled its cell density.

4.2: Proximate Composition in *Nannochloropsis* sp.:

4.2.1: Protein Content:

The protein content for different treatments including a control and 4 stresses like stress 1, stress 2, stress 3, and stress 4 are shown in Figure 6. The protein content was obtained by following the protein determination procedure mentioned in the methodology section.

The stress 1 showed its protein content was 35% and 41% protein was found in the stress 2 treatment. But the control treatment had higher protein percentage than the stress 1 and stress 2. The control had 47% protein. The control treatment and the stress 3 showed similar protein content. The stress 3 had 48% protein content. 54% protein was found in the stress 4 cells. Higher protein percentage was found in the higher nitrogen concentration.

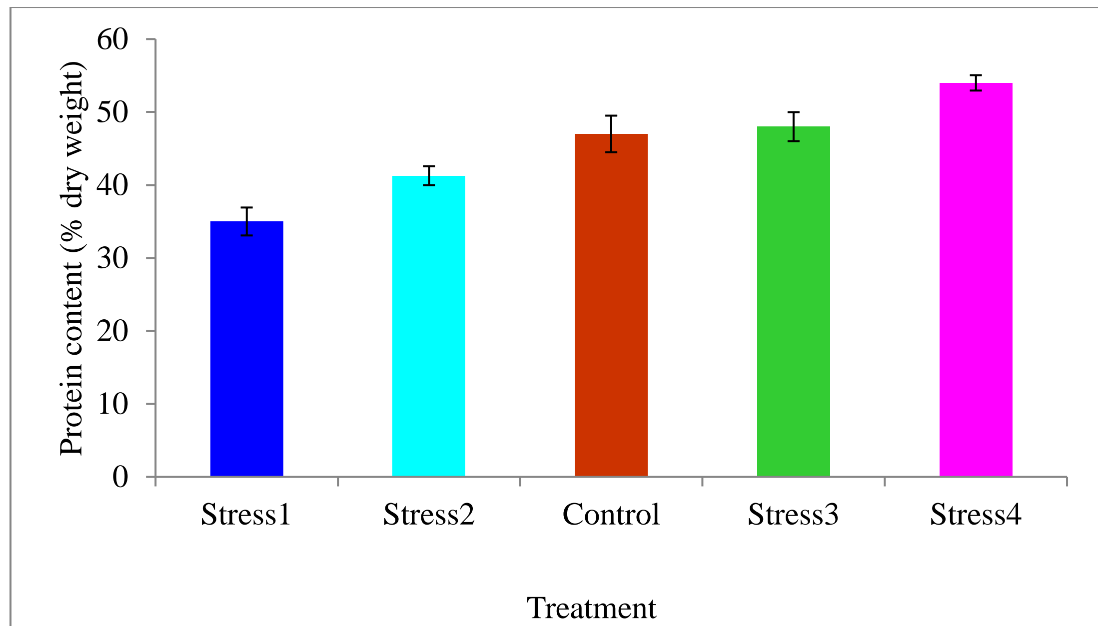


Fig: 6: Protein Content of *Nannochloropsis* sp. cultured in different nitrogen stress

4.2.2: Lipid content:

The lipid content for different treatments including a control and 4 stresses like stress 1, stress 2, stress 3 and stress 4are shown in Figure 7. The lipid content was obtained by following the lipid determination procedure mentioned in the methodology section.

The lipid content was much higher in stress 2 than control treatment. Stress 1, stress 3 and stress 4 were similar in the lipid content. But these (stress 1, stress 3 and stress 4) were also higher than the control treatment.

The lipid content in control treatment was 22%. The stress 2 showed higher lipid content and it was 36%. The other three stresses; stress 1, stress 3 and stress 4 lipid content was 32%, 31% and 31% respectively.

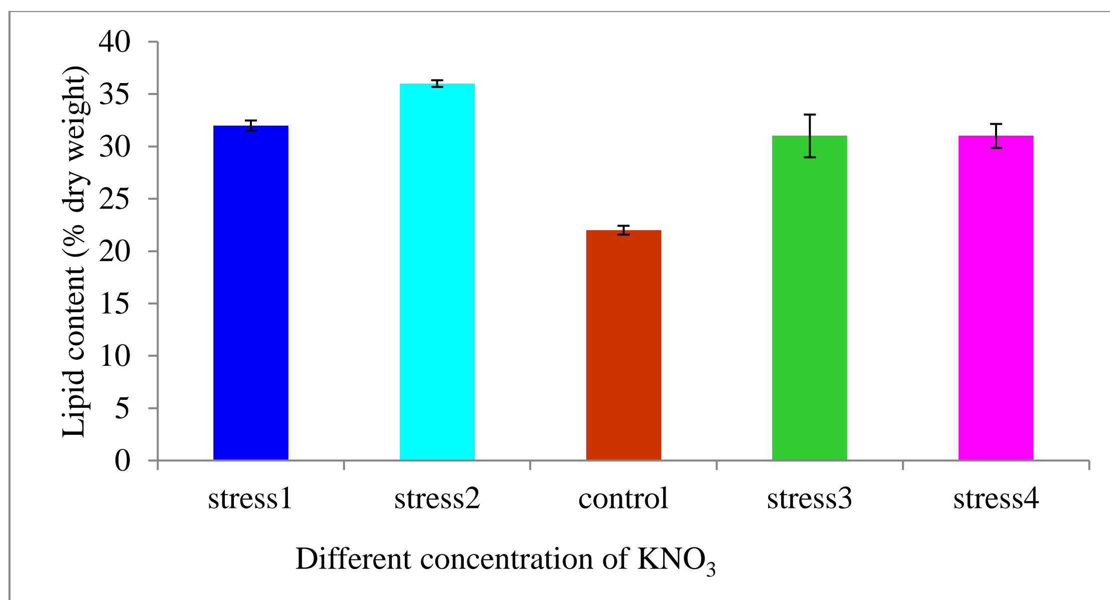


Fig: 7: Lipid content of *Nannochloropsis* sp. cultured in different nitrogen stress

4.2.3: Carbohydrate Content:

The Cell carbohydrate content for different treatments including a control and 4 stresses like stress 1, stress 2, stress 3, and stress 4 are shown in Figure 8. The Carbohydrate content was obtained by following the carbohydrate determination procedure mentioned in the methodology section.

The carbohydrate content was much different in terms of higher nitrate content. The stress 1 showed the carbohydrate content was 23% and the stress 2 showed the carbohydrate content 20%. The Control showed its carbohydrate content 24%. The stress 3 showed carbohydrate content 20% whereas the stress 4 showed 13%.

So the highest carbohydrate content was in control (24%) treatment. The lowest carbohydrate content was in the highest nitrate content of the treatments (13%).

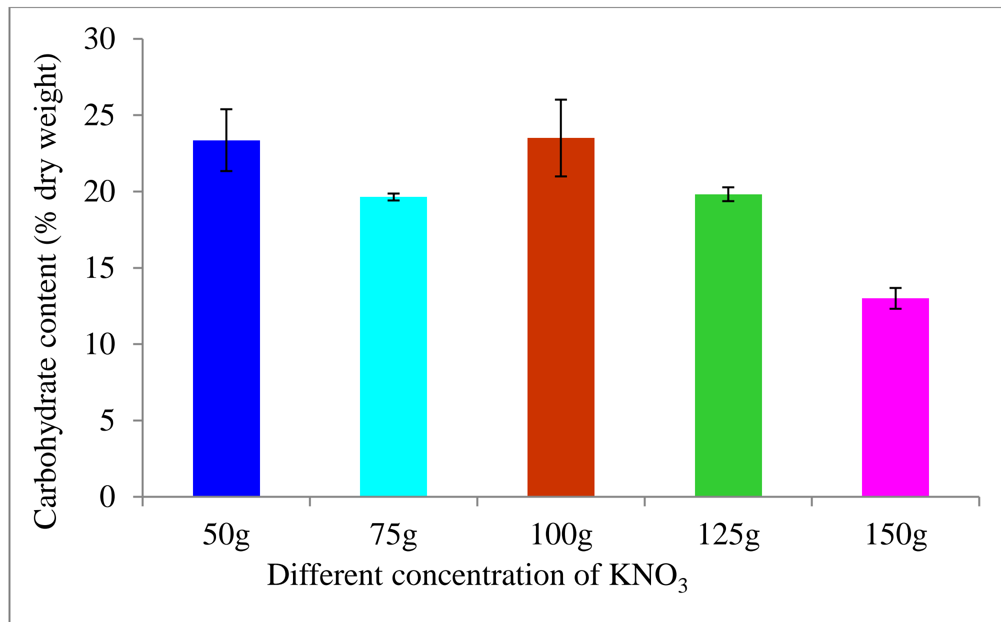
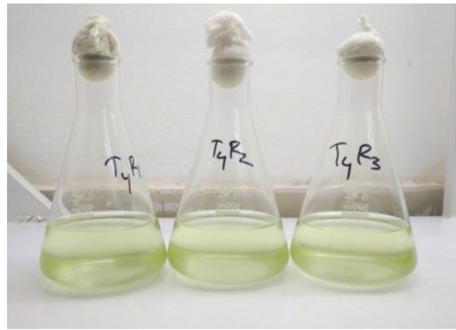


Fig: 8: Carbohydrate content of *Nannochloropsis* sp. cultured in different nitrogen stress

4.3: Color variation of nitrogen depleted and augmented treatments:

Treatment	Day 1	Stationary Day
Stress 1		
Stress 2		
Control		

Stress 3



Stress 4

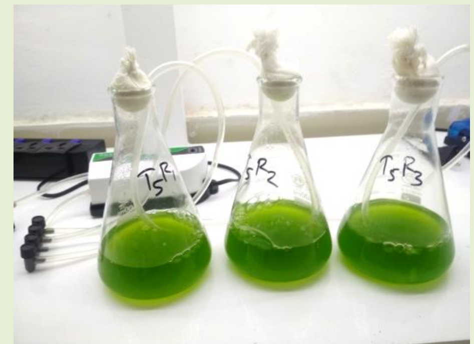


Fig: 9: The color difference of *Nannochloropsis* sp. cultured in different nitrogen stress

Chapter 5

Discussion

5.1: *Nannochloropsis* sp. growth performance under different nitrogen concentration:

Cell density and optical density are the two factors from which the growth performance could be evaluated for this experiment. The experiment was maintained in different nitrogen concentration mainly potassium nitrate (KNO_3) as a source. KNO_3 concentration was depleted and augmented in different treatments in a particular pattern like 25% and 50% depletion, and 25% and 50% higher KNO_3 in concentration from the standard KNO_3 concentration. Several studies suggested that, nitrogen- limitation consequences the lower biomass productivity and nitrogen concentration changes affect the growth rate which can be obtained through cell density by determining specific growth rate (SGR) (Pancha et al., 2014) (El-Kassas, 2013).

Previous study demonstrated that although nitrogen limitation enhances lipid accumulation, it leads to lower biomass productivity (El-Kassas, 2013). In the present study, the treatment with 50g/L KNO_3 showed lower cell density than the control (100g/L KNO_3). The treatment with highest KNO_3 concentration showed highest cell density. The cell density was 73.5×10^6 cells/ml for 150 g/L KNO_3. The higher the nitrogen concentration, the higher the cell density. In the growth curve of cell density, it was observed that, the lag phase (Day 0–3) for all of the treatments remain similar in terms of duration. That means whatever the nitrogen was higher, lower or normal in concentration, the cells had to be adapted with the environment in a similar shape from the view of their adaptation duration with the culture environment. The exponential phase was much shorter (Day 3–7) in the higher nitrogen concentration than the normal concentration. On the other observation, stress 1 and stress 2 had almost same exponential phase like control treatment. At the exponential phase, the population growth altered the surroundings of the cells and the cells may have begun to shade one another as their concentration tend to increase (Laura Barsanti, 2006).

The specific growth rate (SGR) and cell doubling time (td) are the other growth performance indicators which were produced from the cell density data of the

treatments. The SGR from the result section showed that, the treatment with higher nitrogen concentration (stress-4, 150g/L KNO_3) had higher SGR than the others. The SGR of the control treatment was 0.468 ($\times10^6$ cells/ml) and the stress 4 was 0.772 ($\times10^6$ cells/ml). So the growth rate of stress 4 (150g/L KNO_3) was 65.1% higher than the control (100g/L KNO_3). The Stress 1 (50g/L KNO_3) had the SGR value 0.451 ($\times10^6$ cells/ml). So, the treatment with the lowest nitrogen concentration of the experiment had 3.47% lower growth rate than the control treatment. This finding is directly similar with the previous study of Xie et al., (2017) where he showed that, when the nitrogen concentrations were decreased, it acted as a restriction factor for green microalgae.

The cell doubling time (td) of the experiment was generated from the SGR. From the result section, it was observed that, td was the highest in the lower nitrogen treatment. The td for 50g/L KNO_3 was 1.54 days and the td of the control was 1.48 days. So the lowest nitrogen treatment of the experiment was 3.6% higher than the control. So, to double the cell density of the previous day, the stress 1 (50g/L KNO_3) required 3.6% more time than the control treatment. The td of the highest nitrogen treatment was 0.898 day. In stress 4, the highest nitrogen treatment was 39.43% less in td value than the control treatment. So, the stress 4 cell doubling time was 39.43% rapid than the control treatment.

5.2: *Nannochloropsis*sp. proximate composition under different nitrogen concentration:

Changes in nitrogen concentration can affect growth rate as well as protein, lipid, and carbohydrate synthesis in microalgae (Pancha et al., 2014). In this experiment,when the nitrogen content was higher in a culture media the carbohydrate content was lower. The stress 4, which had 50% more nitrogen than the control but 40.9% lower carbohydrate than the control treatment. The carbohydrate content was almost half of the normal value. Rest of the treatments showed negligible differences from the control treatment. This study reveals that when the nitrogen concentration in the culture media was increased the protein content was increased significantly. Contrarily, decreased nitrogen concentration in the culture media showed decreased protein concentration. It was observed from the result that, when the nitrate content decreased 25% from the

control condition, the protein content decreased 12.77%, and subsequently, 50% nitrate concentration depletion showed 25.54% protein content decrease. On the other hand, when the nitrate content increased 25% from the control level the protein content increased only2.13%, and subsequently, when 50% nitrate content increased, protein content increased 14.89%. This outcome relates the findings of previous study of the protein and nitrogen concentration relationship. The Günerken et al., (2016)&Benvenuti et al., (2016) observed that, nitrogen deprivation induced the protein content of green algae and thus the cell growth also retarded.

Nitrogen deprivation (N-deprivation) is a proven strategy to maximize the concentration of TAG in microalgae (Hu et al., 2008b;Rodolfi et al., 2009;Thompson, 1996).Supporting the results of this study, Converti et al. (2009)reported that the major effects of nitrogen deficiency in algal culture include the enhanced biosynthesis and accumulation of lipids. In this study, there were significant differences in the lipid content of the treatments with nitrogen deprivation and augmentation against the control treatment. The lower nitrogen content treatment (stress 2) showed 63.36% more lipid than the control treatment. The control treatment was determined with 22% lipid content that was the lowest among the treatments.So the lower nitrogen treatment in the culture environment to achieve higher lipid content was suitable consideration.

Since chlorophyll has four nitrogen atoms per molecule, it was considered to be abundant with compounds of nitrogen and was one of the most favorable sources of intracellular nitrogen pool for the cells to utilize under deficient conditions of the external nitrogen pool (Li et al., 2008). Chlorophyll was the important element for the photosynthesis of *Tetraselmis* sp. that was involved in cell growth metabolism and lipid buildup(Li et al., 2008). In this study the nitrogen abundant condition (125 g/L and 150g/L KNO_3) showed higher biomass as well as growth rate, and cells also multiplied rapidly in that condition. That happened just because there was available chlorophyll forming materials in the media. For nitrogen depleted condition, the reverse results were observed. Due to the comparatively lower chlorophyll forming content (nitrogen molecule) in the environment of 50g/L and 75g/L KNO_3 treatments, the growth rate was not usual and cell density was below normal range.

According to Hu et al.(2008a) and Mai et al.(2017),when microalgae are N-deprived and unable to produce new proteins for growth, biosynthetic metabolism is directed towards producing and accumulating TAG as a stored form of carbon and energy. In this study the lipid content was increased with decreasing nitrogen content in the culture media. Treatment with 50g/L and 75g/L KNO_3 showed higher lipid content as lowered nitrogen level put the microalgae cells in a stress situation and for that they gather more lipid content in their cell walls and body. (El-Kassas, 2013) also had the same observation of nitrogen limitation on the lipid accumulation in the microalgae cells.

Chapter 6

Conclusions

Marine microalgae *Nannochloropsis* sp. is a great source of biofuels. Various studies have been conducted on the special features that are explained detail in the literature review section of this study. In this study, the different concentration of nitrogeneffect on the growth and proximate composition was studied. Growth phase showed that, when the nitrogen concentration was higher, the cell density was also higher and growth rate was more rapid. Depleted nitrogen concentrations have showed slower growth rate and extended exponential phase. The proximate composition phase showed that, depleted nitrogen concentration in the culture environment induced the cells to accumulate more lipid content than the higher nitrogen concentration. But the protein content was higher when the culture *Nannochloropsis* sp. cultured in higher nitrogen concentration. So as a supplement of protein source, the aquaculture industry may use the strategy of culturing *Nannochloropsis* sp. in higher nitrogen concentration as the cell density and protein content was higher simultaneously. In the bio-oil or biofuel industries, the *Nannochloropsis* sp. could be cultured in the lower nitrogen concentration. But the cell density and growth rate was much lower at lower nitrogen concentration. Therefore,further study needed to be done to optimize lower nitrogen concentration that could provide comparatively higher growth rate and lipid content.

Chapter7

Recommendation and Future Perspectives

Present study showed that the higher growth rate, biomass, and protein content were directly related with the higher nitrogen concentration in the culture condition. Hence, it is recommended that *Nannochloropsis* sp. should be cultured in higher nitrogen concentration for achieving higher biomass in terms of growth and protein content. Present study also revealed that the lower nitrogen concentration in the culture media induced the accumulation of higher lipid in the cell. Therefore, *Nannochloropsis* sp. need to culture in the depleted nitrogen concentration to get higher lipid. Though protein content was higher in higher nitrogen concentration, the quality of protein cannot predict from this study. In addition, the higher lipid content cannot be an indicator of good quality lipid. The protein quality and lipid quality could be assured by the determination of amino acid and fatty acid profile. Future research need to be done on the determination of fatty acids and amino acids profile.

References

Abadia, J. 1986. Function of iron in chloroplasts. Journal of Plant Nutrition. 9(3–7): 609–646.

Agarwal, A., Xu, T., Jacob, M., Feng, Q., Li, X., Walker, L., & Clark, A. 2012. Genomic and Genetic Approaches for the Identification of Antifungal Drug Targets. Infectious Disorders - Drug Targets.8(1): 2–15.

Angles, E., Jaouen, P., Pruvost, J., & Marchal, L. 2017. Wet lipid extraction from the microalga Nannochloropsis sp.: Disruption, physiological effects and solvent screening. Algal Research, 21, pp. 27–34.

Bartley, M. L., Boeing, W. J., Corcoran, A. A., Holguin, F. O., & Schaub, T. 2013. Effects of salinity on growth and lipid accumulation of biofuel microalga Nannochloropsis salina and invading organisms. Biomass and Bioenergy, 54, pp. 83–88.

Béchet, Q., Shilton, A., Fringer, O. B., Munoz, R., & Guieysse, B. 2010. Mechanistic modeling of broth temperature in outdoor photobioreactors.Environmental Science and Technology, 44(6), 2197–2203.

Bellou, S., Triantaphyllidou, I. E., Aggeli, D., Elazzazy, A. M., Baeshen, M. N., & Aggelis, G. 2016. Microbial oils as food additives: Recent approaches for improving microbial oil production and its polyunsaturated fatty acid content. Current Opinion in Biotechnology, 37, pp. 24–35.

Benvenuti, G., Lamers, P. P., Breuer, G., Bosma, R., Cerar, A., Wijffels, R. H., &Barbosa, M. J. 2016. Microalgal TAG production strategies: Why batch beats repeated-batch. Biotechnology for Biofuels, 9(1), 1–17.

Brown, M. R., Jeffrey, S. W., Volkman, J. K., & Dunstan, G. A. 1997. Nutritional properties of microalgae for mariculture. Aquaculture, 151(1–4), 315–331.

Bruland, K. W., Donat, J. R., & Hutchins, D. A. 1991. Interactive influences of bioactive trace metals on biological production in oceanic waters.Limnology and Oceanography, 36(8), 1555–1577.

Bryant, H. L., Richardson, J. W., Wickersham, T. A., Maisashvili, A., Bryant, H., Richardson, J., Anderson, D., Wickersham, T., & Drewery, M. 2001.The values of whole algae and lipid extracted algae meal for aquaculture. 27, pp. 218-222

Chauton, M. S., Reitan, K. I., Norsker, N. H., Tveterås, R., & Kleivdal, H. T. 2015. A techno-economic analysis of industrial production of marine microalgae as a source of EPA and DHA-rich raw material for aquafeed: Research challenges and possibilities. Aquaculture, 436,pp. 95–103.

Chisti, Y. 2007. Biodiesel from microalgae.Biotechnology Advances, 25(3), 294–306.

Converti, A., Casazza, A. A., Ortiz, E. Y., Perego, P., & Del Borghi, M. 2009. Effect of temperature and nitrogen concentration on the growth and lipid content of Nannochloropsis oculata and Chlorella vulgaris for biodiesel production. Chemical Engineering and Processing: Process Intensification, 48(6), 1146–1151.

Cuhel, R. L., Ortner, P. B., & Lean, D. R. S. 1984. Night synthesis of protein by algae. Limnology and Oceanography, 29(4), 731–744.

D'Souza, F. M. L., & Kelly, G. J. 2000. Effects of a diet of a nitrogen-limited alga (Tetraselmis suecica) on growth, survival and biochemical composition of tiger prawn (*Penaeus semisulcatus*) larvae.Aquaculture, 181(3–4), 311–329.

El-Kassas, H. Y. 2013. Growth and fatty acid profile of the marine microalga Picochlorum Sp. grown under nutrient stress conditions. Egyptian Journal of Aquatic Research, 39(4), 233–239.

Greene, R. M., Geider, R. J., Kolber, Z., & Falkowski, P. G. 1992. Iron-induced changes in light harvesting and photochemical energy conversion processes in eukaryotic marine algae. Plant Physiology, 100(2), 565–575.

Günerken, E., D'Hondt, E., Eppink, M., Elst, K., & Wijffels, R. 2016. Influence of nitrogen depletion in the growth of N. oleoabundans on the release of cellular components after beadmilling. Bioresource Technology, 214, pp. 89–95.

Harrison, P. J. 2014. Marine Culture Media. February 2005, pp. 21–33.

Ho, S. H., Chen, C. Y., & Chang, J. S. 2012. Effect of light intensity and nitrogen starvation on CO 2 fixation and lipid/carbohydrate production of an indigenous microalga *Scenedesmus obliquus* CNW-N. Bioresource Technology, 113, pp. 244–252.

Ho, S. H., Chen, C. Y., Lee, D. J., & Chang, J. S. 2011. Perspectives on microalgal CO2-emission mitigation systems - A review.Biotechnology Advances, 29(2),

189–198.

Hu, H., & Gao, K. 2003. Optimization of growth and fatty acid composition of a unicellular marine picoplankton, *Nannochloropsis* sp., with enriched carbon sources. In *Biotechnology Letters* 25, pp.89-92

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., & Darzins, A. 2008a. Microalgal triacylglycerols as feedstocks for biofuel production: Perspectives and advances. Plant Journal, 54(4), 621–639.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., & Darzins, A. 2008b. Microalgal triacylglycerols as feedstocks for biofuel production: Perspectives and advances.Plant Journal , 54(4), 621–639.

Huo, Y. X., Cho, K. M., Rivera, J. G. L., Monte, E., Shen, C. R., Yan, Y., & Liao, J. C. 2011. Conversion of proteins into biofuels by engineering nitrogen flux.Nature Biotechnology, 29(4), 346–351.

Illman, A. M., Scragg, A. H., & Shales, S. W. 2000. Increase in *Chlorella* strains calorific values when grown in low nitrogen medium. Enzyme and Microbial Technology, 27(8), 631–635.

Ishika, T., Bahri, P. A., Laird, D. W., & Moheimani, N. R. 2018. The effect of gradual increase in salinity on the biomass productivity and biochemical composition of several marine, halotolerant, and halophilic microalgae. Journal of Applied Phycology, 30(3), 1453–1464.

Kagan, M. L., West, A. L., Zante, C., & Calder, P. C. 2013. Acute appearance of fatty acids in human plasma - A comparative study between polar-lipid rich oil from the microalgae *Nannochloropsis oculata* and krill oil in healthy young males. Lipids in Health and Disease, 12(1), 1–10.

Kobayashi, M., Kakizono, T., & Nagai, S. 1993. Enhanced carotenoid biosynthesis by oxidative stress in acetate-induced cyst cells of a green unicellular alga, *Haematococcus pluvialis.* Applied and Environmental Microbiology, 59(3), 867–873.

Lam, M. K., Yusoff, M. I., Uemura, Y., Lim, J. W., Khoo, C. G., Lee, K. T., & Ong, H. C. 2017. Cultivation of *Chlorella vulgaris* using nutrients source from domestic wastewater for biodiesel production: Growth condition and kinetic studies.

Renewable Energy, 103, pp.197–207.

Larned, S. T. 1998. Nitrogen- versus phosphorus-limited growth and sources of nutrients for coral reef macroalgae. Marine Biology, 132(3), 409–421.

Laura Barsanti, P. G. 2006. Algae: Anatomy, Biochemistry, and Biotechnology, Second Edition - 2nd. Algae - Anatomy, Biochemistry and Biotechnology,.

Leyva, L. A., Bashan, Y., Mendoza, A., & de-Bashan, L. E. 2014. Accumulation fatty acids of in *Chlorella vulgaris* under heterotrophic conditions in relation to activity of acetyl-CoA carboxylase, temperature, and co-immobilization with *Azospirillum brasilense*. Die Naturwissenschaften, 101(10), 819–830.

Li, Q., Du, W., & Liu, D. 2008. Perspectives of microbial oils for biodiesel production. Applied Microbiology and Biotechnology, 80(5), 749–756.

Liu, Z. Y., Wang, G. C., & Zhou, B. C. 2008. Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. Bioresource Technology, 99(11), 4717–4722.

Ma, S., Han, B., Huss, V. A. R., Hu, X., Sun, X., & Zhang, J. 2015. *Chlorella thermophila* (Trebouxiophyceae, Chlorophyta), a novel thermo-tolerant Chlorella species isolated from an occupied rooftop incubator. Hydrobiologia, 760(1), 81–89.

Mai, T., Nguyen, P., Vo, T., Huynh, H., Tran, S., Nim, T., Tran, D., Nguyen, H., & Bui, P. 2017. Accumulation of lipid in *Dunaliella salina* under Nutrient Starvation Condition. American Journal of Food and Nutrition, 5(2), 58–61.

Mata, T. M., Martins, A. A., & Caetano, N. S. 2010. Microalgae for biodiesel production and other applications: A review.Renewable and Sustainable Energy Reviews, 14(1), 217–232.

Mühlroth, A., Li, K., Røkke, G., Winge, P., Olsen, Y., Hohmann-Marriott, M. F., Vadstein, O., & Bones, A. M. 2013. Pathways of lipid metabolism in marine algae, co-expression network, bottlenecks and candidate genes for enhanced production of EPA and DHA in species of chromista.Marine Drugs11(11),4662–4697).

Mar Drugs.Navarro-Peraza, R. S., Piña-Valdez, P., Cuevas-Rodriguez, E. O., Nieves-Soto, M., Soto-León, S., Contreras-Andrade, I., & Viveros-García, T. 2017.

Effects of temperature and nitrogen limitation on growth kinetics, proximate composition and fatty acid profile of *Nannochloropsis* sp. Revista Mexicana de Ingeniera Quimica, 16(2), 359–369.

Norambuena, F., Hermon, K., Skrzypczyk, V., Emery, J. A., Sharon, Y., Beard, A., & Turchini, G. M. 2015. Algae in fish feed: Performances and fatty acid metabolism in juvenile Atlantic Salmon. PLoS ONE, 10(4), 1–17.

Pancha, I., Chokshi, K., George, B., Ghosh, T., Paliwal, C., Maurya, R., & Mishra, S. 2014. Nitrogen stress triggered biochemical and morphological changes in the microalgae *Scenedesmus* sp. CCNM 1077. Bioresource Technology, 156, pp.146–154.

Rao, A. R., Dayananda, C., Sarada, R., Shamala, T. R., & Ravishankar, G. A. 2007. Effect of salinity on growth of green alga *Botryococcus braunii* and its constituents. Bioresource Technology, 98(3), 560–564.

Reitan, K. I., Rainuzzo, J. R., Øie, G., & Olsen, Y. 1997. A review of the nutritional effects of algae in marine fish larvae. Aquaculture, 155(1–4), 207–221.

Reitan, K. I., Rainuzzo, J. R., & Olsen, Y. 1994. Effect of Nutrient Limitationn on Fatty Acid andd Lipid Content of Marine Microalgae. Journal of Phycology, 30(6), 972–979.

Rismani-Yazdi, H., Haznedaroglu, B. Z., Bibby, K., & Peccia, J. 2011. Transcriptome sequencing and annotation of the microalgae Dunaliella tertiolecta: Pathway description and gene discovery for production of next-generation biofuels. BMC Genomics, 12(1), 148.

Rodolfi, L., Zittelli, G. C., Bassi, N., Padovani, G., Biondi, N., Bonini, G., & Tredici, M. R. 2009. Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnology and Bioengineering, 102(1), 100–112.

Rosli, S. S., Lim, J. W., Uemura, Y., Lam, M. K., Isa, M. H., Oh, W. Da, & Sakidin, H. 2018. PH optimization to promote attached growth of microalgae biomass onto polyurethane foam material. AIP Conference Proceedings, 2016, pp.1–6.

Sabaté, J., & Soret, S. 2014. Sustainability of plant-based diets: back to the future. The American Journal of Clinical Nutrition, 100,pp.476S-482S.

Sato, N., Hagio, M., Wada, H., & Tsuzuki, M. 2000. Environmental effects on acidic lipids of thylakoid membranes. Biochemical Society Transactions.*28*(6): 912–914.

Seto A, Kumasaka K, Hosaka M, Kojima E, Kashiwakura M, K. T. 1992. Production of eicosapentaenoic acid by a marine microalgae and its commercial utilization for aquaculture. In Kyle DJ, Ratledge C (eds), Industrial Applications of Single Cell Oils. American Oil Chemical Social Champaign, Illinois.pp. 219–234.

Shetty, P., Gitau, M. M., & Maróti, G. (2019). Salinity Stress Responses and Adaptation Mechanisms in Eukaryotic Green Microalgae. *Cells*, *8*(12), 1–16.

Siaut, M., Cuiné, S., Cagnon, C., Fessler, B., Nguyen, M., Carrier, P., Beyly, A., Beisson, F., Triantaphylidès, C., Li-Beisson, Y., & Peltier, G. 2011. Oil accumulation in the model green alga *Chlamydomonas reinhardtii*: Characterization, variability between common laboratory strains and relationship with starch reserves. BMC Biotechnology, 11(1), 7.

Solovchenko, A. E., Khozin-Goldberg, I., Didi-Cohen, S., Cohen, Z., & Merzlyak, M. N. 2008. Effects of light intensity and nitrogen starvation on growth, total fatty acids and arachidonic acid in the green microalga Parietochloris incisa. Journal of Applied Phycology, 20(3), 245–251.

Spilling, K., Brynjólfsdóttir, Á., Enss, D., Rischer, H., & Svavarsson, H. G. 2013. The effect of high pH on structural lipids in diatoms. Journal of Applied Phycology, 25(5), 1435–1439.

Sukenik, A., &Carmeli, Y. 1990. Lipid synthesis and fatty acid composition in Nannochloropsis sp. (Eustigmatophyceae) grown in light-drak cycle.Journal of Phycology, 26(3), 463–469.

Thompson, G. A. 1996. Lipids and membrane function in green algae. Biochimica et Biophysica Acta. 1302, pp. 114-117.

Turpin, D. H. 1991. Effects of inorganic N in availability on algal photosynthesis and carbon metabolism.Journal of Phycology, 27(1), 14–20.

Van Leeuwe, M. A., & Stefels, J. 1998. Effects of iron and light stress on the biochemical composition of antarctica Phaeocystis (Prymnesiophyceae)pigment composition.Journal of Phycology, 34(3), 496–503.

Vymazal, J. 1988. The use of periphyton communities for nutrient removal from polluted streams. Hydrobiologia, 166(3), 225–237.

Wells, M. L., Potin, P., Craigie, J. S., Raven, J. A., Merchant, S. S., Helliwell, K. E., Smith, A. G., Camire, M. E., & Brawley, S. H. 2017. Algae as nutritional and functional food sources: revisiting our understanding. *Journal of Applied Phycology* 29(2), 949–982.

Westhoek, H., Lesschen, J. P., Rood, T., Wagner, S., De Marco, A., Murphy-Bokern, D., Leip, A., van Grinsven, H., Sutton, M. A., & Oenema, O. 2014. Food choices, health and environment: Effects of cutting Europe's meat and dairy intake. Global Environmental Change, 26(1), 196–205.

Williams, P. J. L. B., & Laurens, L. M. L. 2010. Microalgae as biodiesel & biomass feedstocks: Review & analysis of the biochemistry, energetics & economics. Energy and Environmental Science 3(5),554–590.

Xie, T., Xia, Y., Zeng, Y., Li, X., & Zhang, Y. 2017. Nitrate concentration-shift cultivation to enhance protein content of heterotrophic microalga *Chlorella vulgaris*: Over-compensation strategy. Bioresource Technology, 233, pp.247–255.

Xin, L., Hong-ying, H., Ke, G., & Ying-xue, S. 2010. Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp. Bioresource Technology, 101(14), 5494–5500.

Appendices (Statistical Analysis of Data)

a) One-way Analysis of Variance examining the effect of nitrogen concentration on the protein content of *Nannochloropsis* sp.

ANOVA					
Protein% DW					
	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	630.000	4	157.500	17.477	.000
Within Groups	90.120	10	9.012		
Total	720.120	14			

Protein% DW						
	Treatment	N	Subset for alpha = 0.05			
			1	2	3	4
TukeyHSD[a]	T1	3	35.0000			
	T2	3	41.0000	41.0000		
	T3	3		47.0000	47.0000	
	T4	3		48.0000	48.0000	
	T5	3			54.0000	
	Sig.		.180	.098	.098	
Duncan[a]	T1	3	35.0000			
	T2	3		41.0000		

	T3	3			47.0000	
	T4	3			48.0000	
	T5	3				54.0000
	Sig.		1.000	1.000	.692	1.000
Means for groups in homogeneous subsets are displayed.						
a. Uses Harmonic Mean Sample Size = 3.000.						

b) One-way Analysis of Variance examining the effect of nitrogen concentration on the lipid content of *Nannochloropsis* sp.

ANOVA					
Lipid%					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	786.121	4	196.530	21.031	.000
Within Groups	93.446	10	9.345		
Total	879.568	14			

Lipid%					
	Treatment	N	Subset for alpha = 0.05		
			1	2	3
TukeyHSD[a]	T3	3	2.05067E1		
	T4	3		2.96460E1	
	T5	3		3.21187E1	
	T1	3		3.24133E1	
	T2	3			4.31859E1
	Sig.		1.000	.799	1.000
Duncan[a]	T3	3	2.05067E1		
	T4	3		2.96460E1	
	T5	3		3.21187E1	
	T1	3		3.24133E1	
	T2	3			4.31859E1
	Sig.		1.000	.315	1.000
Means for groups in homogeneous subsets are displayed.					
a. Uses Harmonic Mean Sample Size = 3.000.					

C) One-way Analysis of Variance examining the effect of nitrogen concentration on the lipid content of *Nannochloropsis* sp.

ANOVA					
Carbohydrates%					

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	217.962	4	54.490	12.601	.001
Within Groups	43.243	10	4.324		
Total	261.205	14			

Carbohydrates%				
	Treatment	N	Subset for alpha = 0.05	
			1	2
TukeyHSD[a]	T5	3	13.00000	
	T2	3		19.64333
	T4	3		19.82333
	T1	3		23.36667
	T3	3		23.50000
	Sig.		1.000	.231
Duncan[a]	T5	3	13.00000	
	T2	3		19.64333
	T4	3		19.82333
	T1	3		23.36667
	T3	3		23.50000
	Sig.		1.000	.060
Means for groups in homogeneous subsets are displayed.				
a. Uses Harmonic Mean Sample Size = 3.000.				

Brief Biography of the Student

Md. Shahadat Hossain is the 3rd son of Late Abdul Gofur and Mrs. Hurar Nahar Begum, was born and grown up in Chattogram city. He has completed SSC from Bangladesh Bank Colony High School and HSC from South Asia College Chittagong. He has also achieved his BSc degree in Fisheries from Chattogram Veterinary and Animal Sciences University. He is now a candidate of Master's degree of the same institute from the Department of Aquaculture. He has expertise on both field and laboratory works. He has done many farm works in Cox's Bazar district and microalgae laboratory research. Internship in Bangladesh's various fisheries related organizations and also in University Malaysia Terrenganu, UMT is his advanced qualification besides academic study. He has a lot of experience on co-curricular activities. He was a proud member of Bangladesh Scouts and achieved President's Scouts Award in 2010. His research interest areas include, microalgae, fish breeding, microbiology, fish genetics, bio-floc technology, fish disease, ecology, and advanced aquaculture technologies. He is determined to make him a competent researcher and wants to reach in the apex of research world of fisheries in the world.