EFFECT OF NITROGEN, IRON AND TEMPERATURE ON YIELD AND COMPOSITION OF MICROALGAE

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CHAPTER I

INTRODUCTION

Anthropogenic activities such as industrialization and deforestation combined with rapid population growth have led to increased use of fossil fuels. Increase in fossil fuel consumption due to growing economies and individual wealth in those economies around the world is making fossil fuels a scarce resource. Enhanced consumption of fossil fuels in both developed and growing economies has contributed to significant elevation in concentration of Earths greenhouse gases, such as carbon dioxide (CO_2), nitrous oxide (N_2O), sulphur dioxide (SO_2), and methane (CH_4). The scarcity for energy and rapid change in Earth's climate is prompting governments to look for alternate sources of energy that are renewable, sustainable, environmentally friendly, and at the same time result in decrease of greenhouse gas concentrations. Renewable energy sources being considered include solar thermal plants, wind farms, hydroelectricity, geothermal and use of biomass (Brennan and Owende, 2010). When biofuels are obtained from biomass, dedicated energy crop are often used as feedstocks which could be in form of solid biomass i.e pellets, liquid fuel or biogas (McKendry, 2003; Amin, 2009). Biofuels can replace fossil fuels (Mata et.al 2010), since biofuel are produced using atmospheric CO_2 and their use does not lead to addition of CO_2 into the atmosphere as they are mainly photosynthetic organisms that recycle CO_2.

To address the issues of climate change and its effects, legislations were enacted in 2007 by the United States Congress via the Energy Independence and Security Act (EISA). The primary objective of this legislation was to propose road map(s) to reduce dependence on fossil fuel, reduce United States energy consumption and create alternative fuel sources through research and implementation of strategy to reduce greenhouse gas emission (EISA section 204). Recently (April 2010), the EISA requires the Environmental Protection Agency to revise the Renewable Fuel Standards (RFS) program created under the energy policy act of 2005 to increase the volume of renewable fuel blended into transportation fuel from 9 billion gallon per year in 2008 to 36 billion gallon per year by 2022 (US EPA 2010b). Thus RFS1 was amended by EISA, leading to the creation of RFS2, which proposed new annual volume standards for four categories of renewable fuel [cellulosic biofuel, biomass-based diesel, advance biofuel and total renewable fuel]. This proposal suggests that transportation fuel from biomass-based diesel comprising of soybeans, algae, rendered fats, greases, corn oil and other virgin vegetable oil should account for not less than 1 billion gallons per year. EISA estimated that 54% of biomass based biodiesel produced in 2009 came from soya bean, while a large chunk came from other sources (crops like corn, canola etc) creating competition between biodiesel production and human consumption. However, biodiesel from micro algae has been recognized as a high potential source without any effect on food availability, and thus has been projected to be produced in larger volume in the near future.

As the third generation feedstocks have the highest advantage in terms of biofuel production, but not much is known about the optimal growing conditions for the

feedstock i.e. microalgae. Similar to terrestrial plants, the microalgae respond to nutrients and temperature by altering their biochemical and physiological structure to resist or tolerate stress conditions. The altering of their biochemical structure can however lead to accumulation or depletion of certain chemical compound like protein, carbohydrates or lipids, which would enable their physiological structure, adapt or endure stress conditions. The microalgae also differ in their cell size, biomass production, fatty acid content and compositions depending on the species. The interaction between the species and growing environment needs to be evaluated and the optimum conditions need to be identified for enhanced microalgae production.

Therefore, the hypothesis is that microalgae fatty acid concentration is increased and fatty acid composition is modified under stress conditions. To test this hypothesis, a study was conducted to evaluate response of microalgae *Nannochloropsis salina*, *Chlorella sp*., and *Pleurochysis carterae* to nutrient deficiency and temperature stress with the following objectives:

Research Objectives

1. To assess the effect of nitrogen, iron and temperature on the biomass and fatty acid composition of the three microalgae.
2. To develop a microalgae growth rate function in response to nitrogen, iron and temperature.

CHAPTER II

LITERATURE REVIEW

Biofuel Feedstock Types

Feedstocks used for biofuel production can be categorized into three generations: (1) First generation feedstocks, (2) Second generation feedstocks and (3) Third generation feedstocks. First generation feedstocks are mainly arable crops like maize, sugar beet, sugarcane and oil seed crops such as rapeseed, soybeans as well as animal fats (FAO, 2008). The production and overall consumption of liquid fuel from this category of feedstocks cannot attain the projected demand of biofuel to replace fossil fuel. There also are the attendant problem of cultivation of these feedstock on prime farmland used for food and fiber production (FAO, 2007) because of pressure on water usage especially freshwater, input requirements i.e. fertilizers and pesticides and the overall impact on food supply which would increase food price, the issue of food security and availability becomes a debate especially in developing countries (Amin, 2009).

Second Generation Feedstock are lignocellulosic feedstocks obtained from energy crops like switchgrass, sweet sorghum, miscanthus, wood/forest residue, agricultural residue etc. Although they were considered as alternatives to arable crops (first generation

feedstock), as these are obtained from non-food source, the issue of land use and required land use changes are obstacles with these category of feedstocks (Amin, 2009).

Third Generation Feedstocks are oil derived from microalgae which can be used for biofuel and co-products. Microalgae address the issues associated with previous feedstocks because it can be cultivated all through the year in a closed photobioreactor system culture technique, while most algae selected for biofuel production has oil content as much or higher than rapeseed and other oil seed crops with high oil content. (Amin, 2009; Brennan and Owende, 2010). It is also cultured without adding pressure on freshwater because it can be cultured in any form of water (saline, brackish), as well as on any land form such as marginal, desert, saline land etc with limited effect on the arable farmland ensuring food security and availability (Amin, 2009).

Characteristics of Algae

Algae are primeval organisms found virtually in every ecosystem in the biosphere. The species have changed over years due to evolution, and various modification in species i.e. paraphyletic occurrence due to environmental variation which plays an important role in the habitat different species are found. This variation is also present within species of the same habitat as well as strains of the same species (Nabora, 2004; Graham and Wilcox, 2000).

Algae are thallophytes lacking stems, mesophyll cells, bundle sheaths, roots or leaves and exist as either macroalgae or microalgae (Graham and Wilcox, 2000). Macroalgae are mainly seaweeds used in fish tanks and processed to gelling substances while microalgae have a broad range of usage. The choice of specific microalgae proposed for biofuel

depends on biofuel products which could be biodiesel, ethanol, propanol etc. while the residue obtained from any of the biofuel production process can be used for animal feed, cosmetics, pharmaceutical products, and biofertilizer (Brennan and Owende, 2010) (Becker 2004).

Microalgae are microscopic unicellular or multicellular organisms, usually with a maximum diameter of 50μm. They thrive in vast environmental conditions, such as saline or alkaline habitats, cold arctic regions, hot springs and arid soils. Most are free living. Significant proportions, however, live parasitically or in symbiotic relationship with other organisms (Nabora, 2004).

Microalgae cell structures are either prokaryotes or eukaryotes. Prokaryotic cell algae lack membrane-bound organelles such as plastids, nuclei or mitochondria thus conduct photosynthesis in the cytoplasm instead of the organelles. Prokaryotic microalgae of this class are categorized as cyanobacteria (cyanophyceae). Eukaryotic cell algae have organelles in which photosynthesis occur. Majority of the microalgae possess a nucleus which aid in cell function allowing the cells to metabolize, survive and reproduce. Eukaryotic microalgae are classified into variety of classes based on their life cycle, basic cellular structure, and pigmentation.

Classes of Algae

Algae are classified into different division or phyla based on the pigmentation and specific food reserves and cell wall constituents in each species. Pigmentation determines habitat each genera or species are found apart from the varying adaptability potential (Richmond, 2004).

Microalgae Used in Biofuel Production:

- Chlorophyta (green algae): This group is diverse and posses variability in morphology, they are aquatic or marine, unicellular or multicellular organisms. They have chlorophyll *a* and *b* and several cartenoids. Cell walls are mainly cellulosic with some species having hemicelluloses and calcium carbonate. The food reserve of green algae are starch and oil, they are mostly used for biofuel research because of their stored products which could be used for either ethanol or biodiesel (Richmond 2004; Round et. al 1990; Stoermer et.al 1999).
- Bacillariophyta (diatoms): The color of the strains and species varies from yellow to brown due to the masking green color of chlorophyll *a*, c_1 and c_2 with brown and yellow of fucoxanthin and β- carotene (Richmond, 2004). They exist in freshwater, moist soil and moist surfaces; they are unicellular organisms, with high silica embedded in the cell wall which aid in resistance to decay (Round et. al 1990; Stoermer et.al 1999), Diatoms deposited most of the limestones on earth as well as fossil fuel. The stored products of this organism are chrysolaminarin (polysaccharide) and oil. Diatoms also contents significant amount of PUFAs especially eicosapentaenoic acid which can be used as food additives and the oil found in diatoms for biofuel.
- Chrysophyta (Golden-brown algae): They occur mainly in freshwater especially oligotrophic water column with low calcium concentration (Richmond 2004; Bengtson et.al 2009). Cellwall is cellulosic with large deposition of silica and consists of photosynthetic pigments chlorophyll *a* and *c*. The stored products of this alga are chrysolaminarin and oil (Ben-Amotz 1983; Bengtson et.al 2009).

- Rodophyta (Red algae): They occur mainly in multicellular and filamentous forms, and consist of chlorophyll *a* and *d*, phycobiliproteins and starch as stored products (Richmond 2004). The stored products accumulate in the cytoplasm outside the chloroplast.
- Cyanobacteria (Blue-green algae): This phylum includes prokaryotic aquatic bacteria that obtain their energy through photosynthesis. They are often referred to as *blue-green algae*, even though it is now known that they are not related to any of the other algal groups, which are all eukaryotes. Cyanobacteria may be single-celled or colonial. Depending upon the species and environmental conditions, colonies may form filaments, sheets or even hollow balls and blooms i.e. dense masses on the surface of a body of water (Kneeling, 2004). Despite their name, different species can be red, brown, or yellow. There are two main sorts of pigmentation which enables them photosynthesize, chlorophyll *a* together with various proteins called phycobilins, which give the cells a typical blue-green to grayish-brown color. A few genera, however, lack phycobilins and have chlorophyll *b* as well as *a*, giving them a bright green color (Murray, 2004).
- Phaeophyta (Brown algae): Their brown color is due to dominance of fucoxanthin over chlorophyll *a* and *c*. With few exceptions they are mainly marine algae and grow in colder region of the world. Cell wall consist of an inner cellulose microfibrillar and outer amorphous layer of alginates (Nabora, 2004), stored products are carbohydrates and oil (Becker, 1994).
- Xanthophyta (yellow-green algae): They exist mainly as freshwater or terrestrial species, their color is due to the presence of both chlorophyll *a* and cartenoids, while chlorophyll *c* is minute and fucoxanthin is absent. They are mostly unicellular or

colonial species, cell wall are cellulosic composed of two overlapping halves. The storage products are chrysolaminarin and oil (arachidonic and eicosapentaenoic acids (Richmond 2004 ; Pohl 1982).

Table 1 - Major microalgae classes considered for biofuel production

Class/ Division	Algae (examples of some species)	Color	Habitat	Stored Products
Chlorophyta	*Botrycoccous braunii* *Chlorella sp* *Dunaliella sp* *Haemotococcus pluvilalis* *Tetraselmis suecica* *Neochloris oleoabundans* *Nannochloris sp*	All green algae	Fresh water Fresh/Salt water Fresh/marine Fresh water Saline water Fresh water	Starch, Oil
Bacillariophyta or *Diatoms*	*Phaeodoctylum tricornutum* *Asterionella glacialis*	Golden yellow or Brown algae	Marine/ fresh water Marine	Chrysolaminarin, Oil
Rodophyta	*Graciliaria spp* *Porphyridium cruentum*	Red algae	Fresh/ marine water	Floridean, Starch, Oil
Cyanobacteria	*Arthrosipra spirulina* *Synechocystis sp*	Blue-green algae	Saline water	Starch
Phaecophyta	*Sargassum spp*	Brown algae	Saline water	Laminarin soluble carbohydrate, Oil
Xanthophyta	*Monallanthus sp*	Yellow algae	Saline water	Starch,Oil
Eustigmatophyta	*Nannochloropsis sp*		Fresh water	Starch,Oil
Prymnesiophyta	*Pleurocrhysis carterea* *Isochrysis, Pavlova salina*	Greenish brown Brown	Marine	Starch,Oil

Microalgae Used for this Research

Nannochloropsis salina

This microalga belongs to the class Eustigmatophyceae, genus *Nannochloropsis*, specie *salina* (CCMP 538). The genus of this alga consists of six species which are not different morphologically and it cannot be differentiated with either electron or light microscope (Hibberd, 1981). Species are identified via DNA sequence analysis, by isolating and characterization of the 18S rDNA sequences (Fawley et.al 2007). The genus was collected and isolated by Bayer (1964), identified by Anderson (1967) , deposited at the center for collection of marine phytoplankton by Guillard (1965), the genus in the current taxonomic classification was first termed by Hibberd (1981), The collection site for this specie was 35.03N 76.00W Pamlico sound, North Atlantic ocean, North Carolina USA (Center for Marine Phytoplankton).

Most of the species were found in marine habitat, some were collected from fresh and brackish water. The species of *Nannochloropsis* are small, unicellular, pale green flagellates with a cell length and width less than 5 µm. Culture medium used is either L1 or F/2 enriched sea media. This strain can be cultured autotrophically in laboratory, open outdoor or closed bioreactor at temperatures ranging from 11-16ºC. These organisms use chlorophyll *a* as their primary photosynthetic pigment and caroteniods as their accessory pigment, which makes it different from other related green microalgae that uses chlorophyll *b* and *c* as accessory pigments. It is considered as potential feedstock for biodiesel because of its rapid reproduction, short lifecycle, and ability to accumulate high levels of fatty acids when cultured at optimal condition.

Chlorella specie

This microalga belongs to class Chlorophyceae, genus Chlorella (CCMP 2333). It was collected and stored in the algae bank of the Culture Center for Marine Phytoplankton by Andersen and kawachi (2002) and isolated by Andersen (2003). The collection site is 24.8791N 125.2934E, Shimajiri Mangrove, Miyako-jima, North Pacific Ocean. Okinawa Japan (Center for Marine Phytoplankton).

These organisms have either a single nucleus or multi-nucleus. Multi-nucleus cell organizations (coenocytic) have peripheric cytoplasm containing nucleus and chloroplast as the vital plastid (Richmond, 2004). Most of these organisms thrive in marine environments however; some survive or have the ability to live in fresh/ aquatic water, terrestrial especially moist soil, rock, snow banks or places favored by fern or mosses. They are green algae with small globular cells, have cell length and width between 8-16 µm. The optimal temperature range for their growth is 22-26 ºC. However, some strains can tolerate temperature of up to 40ºC. The commonly used culture medium is called K medium (Natural oligotrophic ocean water) (Masojodek and Torzillo, 2008). Chlorophyceae cell walls are mainly cellulosic incorporated with calcium carbonate and hemicelluloses. Like terrestrial plants, they have chlorophyll *a* and *b* and store starch in plastids as food reserves which can be altered via N-deficiency to accumulate lipids (Nabora, 2004; Masojodek et.al., 2008).

Pleurochrysis carterae

This microalga belongs to class Prymnesiophyceae, genus Pleurochrysis, species carterae (CCMP 647). It was collected and stored in the algae bank of the Culture Center for

Marine Phytoplankton by Lewin (1986), at site 33.30N 115.8000W, Salt lake Ocean, California USA (Culture Center for Marine Phytoplankton).

Prymnesiophyceae is also referred to as Haptophyta because of the presence of a thin filamentous appendage between two smooth flagella (Richmond, 2004). They are mainly marine algae with cell length and width 8-19 µm. Each cell has two disc shaped chloroplast, outer cell covering has small flat scales which could be calcium carbonate or cellulose. The presence of fucoxanthin in the plastids gives it a brown or golden-yellowish color. The culture media commonly used are F/2, Si, F/2 agar, or blue star agar. The optimal temperature range for growth is between 22-26 ºC. Lipids are their main storage product (Nabora, 2004; Sheehan et.al., 1998).

Types of Microalgae Growth Process

Microalgae growth processes can be categorized into three primary methods, which are autotrophic, heterotrophic or mixotrophic.

Autotrophic also referred to as photoautotrophic process involves producing complex organic compound from simple inorganic molecules by photosynthesis. Thus, they are organisms that obtain elements required for growth from basic inorganic compounds like salts and light energy for growth (Brennan and Owende, 2010). Heterotrophic microalgae do not carry out photosynthesis but they use organic substrates like glucose or acetate as the carbon and energy source to stimulate growth, while mixotrophic microalgae derive energy from both photosynthesis and chemical oxidation. They combine both photoautotrophic and heterotrophic mechanism to sustain growth (Khan et.al., 2009).

All photosynthetic microalgae use chlorophyll *a* as their primary photosynthetic pigment, with a secondary photosynthetic pigment which could be chlorophyll *b* in chlorophyta (green algae) chlorophyll *d* in rhodophyta and other algae uses chlorophyll *c* as well as accessory pigments which varies with algae species (Graham and Wilcox, 2000).

Photosynthetic microalgae possess single or multiple chloroplasts in individual cells, and like terrestrial plants utilize light reaction and Calvin cycle to produce chemical energy which are stored as lipids, proteins or carbohydrates in plastids as food reserve (Graham and Wilcox, 2000). Because many of the microalgae species can be induced to accumulate substantial quantity lipid, thus high oil yield between 70-85% of biomass dry weight (Mata et.al, 2010), their ability to complete their entire growth cycle in a few days/weeks, which is not possible with terrestrial oil seed crops, they are considered as the most viable option for renewable alternative energy (Amin, 2009).

Abiotic Requirements for Algae Culture

Light

Light is an important requirement for microalgae growth. The quantity of photon energy absorbed by each cell is a combination of several factors such as cell density, length of optical path, thickness of layers, photon flux density and rate of agitation (Masojodek and Torzillo, 2008). Photoadaptation and photo-acclimation are responsible for the effect of light on biochemical composition of autotrophically cultured microalgae (Parson et.al., 1961; Dubinsky et.al., 1995). The procedure involves dynamic changes in cell composition, modification in physiological, biophysical and ultrastructural properties to augment photosynthesis and algal growth.

The spectral quality and photoperiod are important light source for culturing microalgae. Although light intensity is vital for growth, the cell density and culture depth determines the quantity of light required for culture growth as more light penetration would be required at higher culture depth and dense cell concentration. Light intensity required for growth varies from 75 $\mu E.m^{-2}\ s^{-1}$ to 500 $\mu E.m^{-2}\ s^{-1}$ depending on culture method which could be either laboratory or large scale production (Boussiba, 1987). Alternative source of light apart from solar radiation is via the fluorescent tubes especially those emitting either blue or red light spectrum because they are the most active light spectrum for photosynthesis. When natural or artificial illumination are used for cell culture, excess light intensity should be avoided because it results in photo-inhibition of cell which affects cell generation time. The duration of illumination or photo-period varies and it ranges from 18 hours light: 6 hours dark, 12 hours light: 12 hours dark, or 16 hours light: 8 hours dark.

Cell structure respond to high light intensity by reducing chlorophyll *a* and pigments involved in photosynthesis to boost photoprotective agents like secondary caroteniods (zeaxanthin, beta-carotene, astaxanthin). At low light intensity, it increases chlorophyll *a* and other harvesting pigments i.e chlorophyll *b*, chlorophyll *c*, phycobiliproteins and primary carotenoids. The carotenoids build up occur due to disruption in carbon and nitrogen flow inside the cells under stressed conditions, they are found in significant quantity in unique structures like plastoglobuli of plastids (Ben-Amotz et.al., 1982; Vechtel et.al., 1992; Hu et.al.,1997) or cytoplasmic lipid bodies (Vechtel et.al., 1992; Hu et.al., 1997), thus preventing the absorption of surplus light energy by the photosynthetic machinery.

Temperature

Although some microalgae strains thrive at extreme temperature, temperature affects the biochemical composition of strains which makes it a vital parameter to consider and control in microalgae culture (Masojodek and Torzillo, 2008; Hu et. al 1997). Optimal temperature range for most microalgae strains either freshwater or saline are 23-28ºC. However, some survive extremes of -5ºC and above 90ºC.

Temperature in a physiological tolerant rate (i.e optimal temperature require for growth, although the range depends on strains) might influence the quantity of cellular lipid and fatty acid classes. Temperature reduction below an optimal range often lead to increase in unsaturation of lipids in the membrane, improves stability and fluidity of cell membranes especially thylakoid membrane via increase in unsaturated fatty acid in the membrane, thus prevent the photosynthetic machinery from photoinhibition at low temperature (Nishida et.al., 1996; Thompson et.al., 1992) because reduction in temperature activates enzymes as an adaptive mechanism to maintain respiration and photosynthesis. However, increase in temperature from the required range for cell growth can either increase carotenoid content or total carotenoid (Tjahjono et.al., 1994; Liu and Lee, 2000; Simon, 1971), this leads to the formation of active oxygen radicals in algal cells, which might lead to oxidative stress inducing carotenogenesis (Tjahjono et.al., 1994; Liu and Lee, 2000) as well as enhancing temperature dependent enzymatic reaction involved in carotenogenesis.

Environmental change such as temperature fluctuations, which enables organisms to acclimatization and responses to stimulus are natural attribute in living organisms. Therefore a change in environmental condition is based on the response and acclimation

of the organism to the stress factor (limiting or excess) that induced change. A stress factor would affect the growth and biochemical reaction in organisms, thus when organisms are able to adapt or acclimatize to environmental change it results in metabolic imbalance that require physiological adjustments for growth to be established.

Four decades of research (Parson et.al., 1961) on eleven different species of marine phytoplanktons representing six taxonomy cultured in similar physical and chemical conditions, shows resemblance in cell composition particularly total organic carbon in cells because microalgae of different origin with few exceptions tend to have almost similar cell biochemical composition especially the amount of crude protein, lipids and carbohydrates when grown in approximately optimal culture conditions. However, for single species of chlorophyta *chlorella sp. Botryococcus braunii, and Dunaliella salina*, with estimated biochemical composition of 30-50% protein, 20-40% carbohydrate and 8-15% lipids under favorable environmental conditions but different culture media shows variations in cell biochemical composition. However, under stressed or unfavorable conditions these species accumulate 80% fatty acid, 80% hydrocarbon and 40% glycerol biomass dry weight respectively (Richmond, 2004). Therefore, environmental conditions predominantly temperature, nutrient supply, salinity and light, affect biomass production and photosynthesis as well as cellular metabolism, composition, synthesis pathway and activity.

The effect of temperature and nitrogen on cell division and lipid productivity was observed on *Nannochloropsis oculata* and *Chlorella vulgaris* (Attilio et. al., 2009), optimal temperatures for the two strains were 20ºC and 30ºC respectively. Temperatures

tested were 15ºC and 25ºC for *Nannochloropsis oculata* and 25ºC, 35ºC and 38ºC for *chlorella vulgaris.*

Table 2 - Effect of temperature on *Nannochloropsis oculata* growth rate and lipid productivity.

Temperature (ºC)	Specific growth rate (day^{-1})	Lipid production ($mg_{lipids}L^{-1}$ day^{-1})
15	0.06	9.11
20	0.13	10.01
25	0.07	10.10

(Source: Converti et al. 2009)

In Table 2, variation in temperature from the optimal of 20ºC shows a significant change in cell division of the strain tested. At temperatures below and above the optimal of 20ºC growth rate was slow, measuring about half of the optimal.

Table 3 - Effect of temperature on *Chlorella vulgaris* growth rate and lipid productivity.

Temperature (ºC)	Specific growth rate (day^{-1})	Lipid production ($mg_{lipids}L^{-1}$ day^{-1})
25	0.14	20.22
30	0.14	8.16
35	0.12	8.21
38	-0.01	-2.72

(Source: Converti et al. 2009)

The effect of temperature in this strain is only significant at temperature above 30ºC (Table 3). At 35ºC, 17% decrease in the growth of the strain was recorded as compared to the threshold of 30ºC, while at 38ºC, a drastic decline in cell growth was observed which resulted in death of cells, with a visible color change from green to brown and the slow cell doubling time.

Water

Culturing of freshwater algae is ideal with spring water due to heavy metal contamination of natural tap water and distilled water (Anderson, 2004), The source of seawater is

however vital for marine algae productivity. In some species, oligotrophic water is ideal because of its low concentration of trace metal and nutrient which can be supplemented via enriched medium, less phytoplankton's and sediments. Alternatively, aged natural water or nano-pure water (water purified using a Barnstead/Thermolyne Nanopure lab water system) can be used, natural seawater is aged for six months in the dark at 5°C, while spring and lake water are aged for two months in a dark room at temperature of approximately 20°C (Anderson, 2004). Freshly collected natural water can also be treated by adding 2grams of activated charcoal per liter of water, agitated for a hour and filtered without aging for a long period. The various source of water mentioned above can be used for microalgae cultivation based on availability and suitability for culture purpose.

pH Range and Optimal

Tris (2-amino-2-[hydroxymethyl]1-1-propanediol) and glycylglycine are commonly used to buffer pH in culturing marine algae to avoid or reduce precipitation of salts contained in stock solution (McLachalan, 1973). However, Tris is quite toxic in some species while glyclyglycine is non-toxic but acidic.Both pH buffers are adjusted with HCl or 1N NaOH to obtain the desired pH of the medium (Anderson, 2004). pH buffer use extensively in fresh water algae are HEPES (N-[2-hydroeyethyl] piperazine-N´-[2-ethanesulphonic acid) and MOPS (3-N-morpholino propane sulfonic acid) (McFadden and Melkonian, 1986).

Nutritional Requirement for Microalgae Culture

Nitrogen

The quantity of nitrogen in microalgae culture is important since it is a fundamental factor in culture media preparation (Becker, 1994), particularly the form in which nitrogen is made available i.e nitrate, nitrite, ammonia or urea. The most widely used source of nitrogen supply for microalgae culture is ammonia and urea because they are readily assimilated and are economical. Nitrate and nitrite source of nitrogen supply are quite expensive and requires high metabolic energy for its absorption because it requires reduction into organic molecules (ammonia) before incorporation by cell.

Nitrogen is a vital component/ element for composition, formation and functionality of protein and DNA in algal cells (Becker 1994; Simon, 1971). The average nitrogen requirement for most green algae is between 5-10% or 5-50mM (Becker, 1994; Hardie et.al.,1983). When nitrogen supply is sufficient, majority of microalgae are unable to produce nitrogen storage materials, however, few are found with nitrogen storage compounds like cyanophycin and phycocyanin (Simon, 1971; Boussiba and Richmond, 1980), Microalgae grown in nitrogen limited conditions demonstrate a vigorous and precise degradation of phycobilisomes (Collier and Grossman, 1992).

Photosynthesis in nitrogen starved microalgae continues, although at a reduced rate until cell nitrogen decrease below its threshold. The flow of carbon fixed by photosynthesis in such situation is however diverted from the path of protein synthesis, to either lipid or carbohydrate synthesis, the formation of either lipid or carbohydrates is specie specific and varies even within same species (Borowitzka and Borowitzka, 1988). Nitrogen starvation can either improve the biosynthesis and accumulation of lipids or reduce the

process in some taxonomy. When the lipid production process is enhanced in nitrogen starved conditions, neutral lipids in form of triacylglycerols are predominant composition of lipids in the depleted cells (Thompson 1996).

Nitrogen starved algae accumulate secondary caroteniods and reduce chlorophyll content in cells. Studies conducted have shown that nitrogen starved microalgae produces favorable products mostly oil and protein but has reduced biomass. Thus, increase in lipid production when strains are deficient in nitrogen increase the duration required to yield same amount of product from un-starved microalgae (Becker, 1994; Hu et.al., 1997).

Microalgae respond differently to nitrogen limitation and excess. Nitrogen stress on *Nannochloropsis oculata* was tested with the concentration of nitrate in the form of $NaNO_3$ in the media doubled and halved. The $NaNO_3$ required for *Nannochloropsis oculata* is $75g/L^{-1}$, reduced $NaNO_3$ supply is $37.5g/L^{-1}$ and excess is $150g/L^{-1}$, while *chlorella vulgaris* is $150g/L^{-1}$, reduced $NaNO_3$ is $75g/L^{-1}$ and excess is $300g/L^{-1}$. The composition was selected according to Guillard component measures.

Table 4 - Growth rate and lipid productivity of *Nannochloropsis oculata* at different nitrate concentration

NaNO3 (g/L^{-1})	Specific growth rate (day^{-1})	Lipid Production ($mg_{lipids}\ L^{-1}\ day^{-1}$)
37.5	0.13	20.30
75	0.14	20.44
150	0.14	8.16

(Source: Converti et al. 2009)

Table 5 - Growth rate and lipid productivity of *Chlorella vulgaris* at different nitrate concentration

NaNO3 (g/L^{-1})	Specific growth rate (day^{-1})	Lipid Production ($mg_{lipids}\ L^{-1}\ day^{-1}$)
75	0.10	16.41
150	0.10	13.61
300	0.13	10.01

(Source: Converti et al. 2009)

The strains were grown in batch cultures with light intensity 70.0 µE.m^{-2} s^{-1} , temperature 20ºC injected via pump M2K3 (Schego, Offenbach, Germany) (Converti et al., 2009). The result of the experiments indicated that low nitrogen concentration in the growth medium led to increased lipid production because higher nitrogen concentration extends growth cycle which resulted in lower lipid production per day in the strain tested.

Cultures grown under N-stressed condition produces favorable products, but the overall productivity is significantly reduced. High lipid production when algae are starved with nitrogen results in longer duration than the required time to yield the same amount from non-starved microalgae because the cell division process in delayed and biochemical structure of cell are altered from the original chemical composition due to stress and converted to oil or starch to survive stress conditions. However, the N-stress condition is favorable if carbohydrates are the product considered (Becker, 1994; Boussiba and Richmond, 1980).

A similar study on effect of nitrogen starvation on monoalgae grown in artificial sea water (ASW), using 100ml of medium cultured in 250ml flask arranged on a shaker at illumination of 75 µE.m^{-2} s^{-1} at 27ºC or in 500ml flask positioned in water bath at temperature of 28ºC with light intensity of 75 µE.m-2.s-1 (Boussiba, 1987). The cultures were centrifuged in the mid-logarithmic stage, washed once and re-inoculated into a nitrogen free medium. The cell concentration, temperature, light intensity and pH were kept constant during the starvation period of 20days (Boussiba, 1987). The lipid content of cells changed with growth phase. Cell at the early stationary phase (25mg chlorophyll.l^{-1}) had almost doubled the lipid content when compared with cells in the

early logarithmic phase (5mg chlorophyll.l^{-1}). The increase occurred as a result of stored accumulated lipids when growth was disrupted by low nutrients.

Table 6 - Effect of cell growth on lipid content of *Nannochloropsis salina*

Lipid content (% AFDW: Ash Free dry weight)	
Chlorophyll (mg.l^{-1})……Laboratory	Outdoor(steady state)
3	----	16.6
5	10.0	16.0
10	----	18.0
15	13.0	----
20	----	21.0
25	20.0	----

(Source: Boussiba et al. 1987)

Table 6 shows the ash free dry weight of laboratory and outdoor cultures of *Nannochloropsis salina*. The highest lipid content in the laboratory was obtained at the highest cell concentration while outdoor cultures differ in cell concentration and lipid content.

The effect of nitrogen concentration on cell growth and biomass was examined in chlorophyceae (green algae) and cyanobacteria (blue-green algae) (Becker, 1994). Increasing N-level increased biomass, protein and chlorophyll content. At low N, chlorophyceae contains high level of total lipids which is about 45% of dry weight. 70% of these total lipids are neutral lipids containing 16:0 and 18:0 fatty acid, while at high N-level total lipid dropped to about 20% with polyunsaturated fatty acid dominating. Cyanobacteria did not however show any significant change in lipid and fatty acid composition. Chlorophyceae in mass culture can be manipulated to yield biomass with desired lipid content, but other growth parameter (light, pH) should vary to get a significant lipid production (Becker, 1994).

CO_2 as a carbon source

The CO_2 concentration in natural air used to aerate culture medium during strain culture is quite low to sustain optimal growth and biomass productivity of the culture media. Therefore, CO_2 supply to the medium could be from waste gas from industrial combination process, diesel engine, cement plants or fermentation. It requires an inorganic carbon source to perform photosynthesis especially cultures growing in freshwater with low salinity and nearly neutral pH (Becker 1994). Intensive mixing or agitation of culture media also helps to improve CO_2 concentration in the media, but CO_2 enriched air is mostly supplied to the media. The assimilation of CO_2 and bicarbonate by a rapidly growing alga could result in elevated pH due to discharge of OH^- by algae in the system. Hence in mass culture the pH should be maintained to keep it in an optimal range for the cultivated species and prevent carbon depletion.

Phosphorus

It is a macronutrient essential for cellular metabolic process via formation of structural and functional constituent necessary for normal growth, biosynthesis of nucleic acid, and energy transfer (Becker 1994; Goldman and Mann 1980). Phosphorous concentration is often growth limiting in natural aqueous environment, because phosphorous occur as either orthophosphate or organic phosphate combination, orthophosphate is incorporated into organic components via several phosphorylation process. Inorganic phosphate could occur in cells as polyphosphate, accumulating distinct polyphosphate granules which emerge in cell when phosphorus is sufficient but invisible in phosphorous deficient cells (Healey 1982).

Microalgae absorb phosphorous in inorganic forms like H_2PO_4 or $HPO^2_{4,}$ concentration and ability to absorb phosphorous is species dependent even if supply is sufficient. The optimal absorption rate of phosphorous concentration for most strains are 50µg P/l -20mg P/l. Deficient phosphorous is similar to nitrogen starved, because it leads to increase in lipid and carbohydrate content, decrease in protein, nucleic acid, chlorophyll *a* content (Becker, 1994; Healey, 1982). Distinctions occur in phycobilisome degradation in phosphorous deficiency as little degradation occurs when compared to nitrogen deficiency. Degradation of phycobilisome in cell occurs during cell division when new phycobilisome synthesis stops (Collier, 1992). When phosphorus is supply to reduce deficiency, the absorption of phosphorus is higher than unstarved phosphorous culture media.

Sulfur

It is vital to all cells because it is a constituent of essential amino acids (methionine, cysteine and cystine), vitamins and sulphur-lipids. It is provided as inorganic sulphate in the culture medium. Certain species utilize organic sulphur sources like sulphur containing amino acids in some situations. Sulfur absorption is an active phosphorylation-driven requiring light energy and temperature sensitive process. It also requires reduction like nitrate before it is absorbed or incorporation into cells (Becker, 1994; Richmond, 2004).

Calcium

The physiological role of calcium is implicit, but when required it should be in minute ratio or supplied in composition with other nutrient. Ions of calcium protect the cytoplasmic membrane, aids salt formation with colloids and precipitation of $CaCO_3$. It is

responsible for calcite or aragonite formation in or on cell wall of some strains, which prevents decay in some species (diatoms) (Becker, 1994; Richmond, 2004).

Sodium

It is particularly required in marine and halophilic microalgae in trace concentration, toxic at high concentration especially to freshwater microalgae. It aids nitrogen fixation during conversion of molecular nitrogen to ammonia (Becker, 1994; Richmond, 2004). However, because of the tight correlation in chemical properties with potassium, sodium can replace potassium in any culture media.

Potassium

Insufficient supply of potassium consequently leads to reduced photosynthesis, growth, development and high respiration. Deficient cells can be revived by addition of potassium or rubidium, potassium is involved in protein synthesis and osmotic regulation (Becker, 1994; Richmond, 2004).

Magnesium

It is the fundamental atom of chlorophyll molecules required for photosynthesis, ribosomes aggregation in cell functional units and catalyst formation. Mg deficiency interrupts cell division, resulting in abnormal large etiolated cells (make pale due to lack of light).

Iron

It is vital for metabolism being a constituent of cytochromes. The redox properties and effective in nitrogen assimilation and fixation, photosynthesis, respiration and DNA synthesis are vital for cell structure and constituent (Hardie et.al., 1983), as a functional

part of ferredoxin it affects the synthesis of phycocyanin and chlorophyll. Bleaching and yellowing of algae culture are often an indication of iron deficiency in the medium. The mode of supply is controversial because the fractions of particulate, colloidal or soluble iron available to algae are not clear (Becker, 1994).

Iron deficiency degrades c-phycocyanin and chlorophyll a (Hardie et.al., 1983), increased iron- deficiency induced protein (isiA), this protein combines with Photosystems 1 to form a complex ring of 18 isiA molecules which increases the light-harvesting capacity of system of PS 1 to compensate for reduces phycobilisome and PS 1 response to iron-deficiency (Bibby et.al., 2001). ferredoxin an important component in photosynthesis electron transport is low during iron deficiency while flavodoxin an electron carrier that does not contain iron increase (Bottin and Lagoutte).

Iron is mostly supplied in form of chelated complexes bound to the ethylene diaminetetraacetic acid (EDTA). These compounds are extremely stable, releases enough iron ions by mass action to satisfy algae requirements. The use of iron as a citrate complex has also been reported where citric acid acts as a complex as well as reducing agent and as a pH buffer

Trace Elements

They are required in small amounts of micro-, nano- or even pictogram per liter. The major trace elements in algae media are manganese, nickel, zinc, boron, vanadium, cobalt, copper, molybdenum (Becker 1994; Richmond 2004).

They are elements that:

a. Influence growth in a representative number of species.
b. Have a positive effect on total growth.
c. Show direct physiological effect in the algae growth.
d. Cannot be replaced by another element.
e. Show reversible signs of deficiency in cultures lacking these elements.

The significance of iron to microalgae growth have been studied for several years via research, a study conducted for 12 years based on fluorescence measurement shows that iron regulates phytoplankton biomass in both HNLC (high-nitrate low-chlorophyll) and oligotrophic water near the equator and further south (Behrenfeld et.al 2006) but if iron deficiency is a limiting factor to algal biomass productivity in batch culture condition is yet to be established. The effect of iron with iron source $FeCl_3$ or EDTA on lipid accumulation and biomass productivity was investigated on marine *chlorella* strain under laboratory conditions. The final cell density increased when chelated Fe^{3+} was added to the culture medium at late exponential growth phase, also total lipid content increased when cells were re-inoculated into a new medium containing high level of iron concentration.

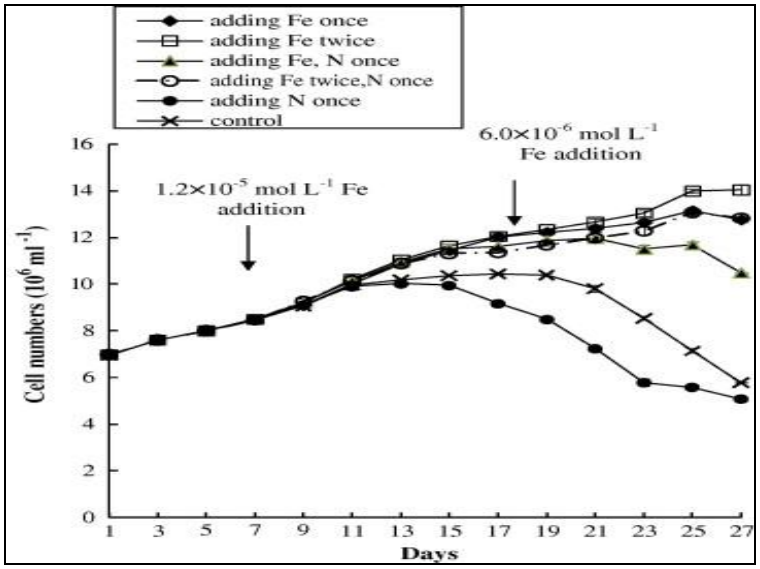

Figure 1 - Effect of nitrogen and iron on growth of *chlorella vulgaris* ***(Source: Liu et al. 2008)***

The experiment in (Fig.1) involves 5 nutrient treatments to evaluate the effect of iron and nitrogen supplement on lipid production and growth of *chlorella* in the late-exponential growth phase using the F/2-Si medium. The growth was measured with a spectrophotometer at an OD_{500nm} every other day and cell count observed with a microscope. Day 7 after the addition of Fe^{3+}, (14 days after culture) the cells of culture supplemented with $1.2*10^{-5}$ mol/L^{-1} Fe^{3+} were significantly higher than control. It was also observed that 15 days after inoculation of culture, the control reached its stationary growth phase, while cells supplemented with $FeCl_3$ were still at the exponential phase, on day 17 the cell with Fe supplements were significantly higher than cultures with Fe/N additions. The growth phases of the culture were best when Fe was supplied twice, this showed the highest cell density, and cultures with N-supplement had the lowest cell density, the cells declined earlier than the control.

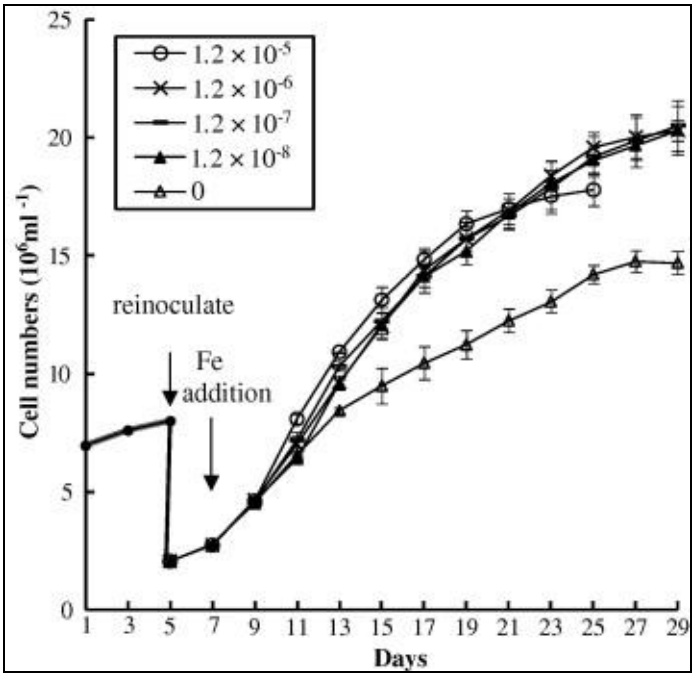

Figure 2 - Effect of iron concentration on the growth and lipid accumulation in *C.vulgaris* ***(Source: Liu et al. 2008)***

Iron concentration was added at a range of $1.2*10^{-8}$ - $1.2*10^{-6}$ mol L^{-1} in a new media with re-inoculated cells of Chlorella strain (Fig. 2). Cultures with $1.2*10^{-5}$ Fe^{3+} had a faster growth rate than the other treatment with a stationary growth at 19 days. The cell density of concentration with high Fe^{3+} concentration ($1.2*10^{-6}$) had lower yield when compared to cell densities of cultures of low Fe^{3+}concentration, but higher than the cultures without Fe^{3+} concentration.

CHAPTER III

MATERIALS AND METHODS

The three microalgae, Nannochloropsis *salina* CCMP 537, *Chlorella sp* CCMP 2333 and *Pleurochyrsis carteae* CCMP 647, were obtained from the Culture Center Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine (https://ccmp.bigelow.org/). The microalgae used are pure unialgal eukaryotes (Axenic culture), grown autotrophically in nano-pure water (deionized). *Nannochloropsis salina* and *Pleurochrysis carterae* were originally cultured in the CCMP laboratory using Guillard F/2 medium while the *Chlorella sp* in K medium.

The study involved three different experiments, with nitrogen, iron and temperature treatments for each strain. For each experiment, there were three levels of each treatment (low, control and high concentration of culture media) and replicated three times. The design was randomized complete design of 27 treatments per experiment in culture media (Hogland, F/2 and K).

The Hoagland (H) stock solution was originally developed to grow plants in hydroponic system, rapid growth of invasive algae was observed in the tank in which the Hoagland stock was store. Thus the three microalgae species used in this study were cultured in Hoagland stock solution to access the yield and fatty acid composition and compared

with F/2 and K media. Since the Hoagland stock is cheaper than the standard K and F/2 media, a yield response better than F/2 and K media might lead to adopting the use of Hoagland for future studies. Details of the stock solutions (culture media) used in this study and its preparation are explained below (Anderson, 2004).

Microalgae Media Compositions

Guillard F/2 Medium

The Guillard and Ryther medium is the most common and widely used enriched seawater medium, designed specifically for growing coastal marine algae, particularly diatoms. The original "F medium" has been reduced to half and the ferric sequestrene has been substituted with $FeCl_3.6H_2O$ and $Na_2EDTA.2H_2O$ (Andersen, 2005).

Salts: $NaNO_3$ 75g, $NaH_2PO_4.H_2O$ 5g, $NaSiO_3.9H_2O$ 30g. The salts are dissolved in 1L distilled water; 1ml of the dissolved salt is used in 1L of prepared stock.

Trace Metal Solution: Initial stock solution contains $MnCl_2.4H_2O$ 180g, $ZnSO_4.7H_2O$ 22g, $CoCl_2$ $6H_2O$ 10g, $CuSO_4.5H_2O$ 9.8g, $NaMoO_4.2H_2O$ 6.3g. These elements were dissolved initially in 1L distilled water and 1ml of the dissolved elements are transferred to a fresh 1L distilled water (primary stock). $FeCl_3.6H_2O$ 3.15g and $Na_2EDTA.2H_2O$ 4.36g were added to the primary stock to bring it to a final trace metal.

Vitamins: Initial stock contains Biotin (Vit H) 1.0g and Cyanocobalamin (Vit B_{12}) 1.0g dissolved in 1L distilled water, 1ml of the primary stock is dissolved with 200mg of Thamine.HCl (Vit B_1) in a final stock of 1L.

K Medium

The K medium was originally designed for oligotrophic marine phytoplankton. The concentration of EDTA chelate is ten-fold higher than other marine microalgae, which reduces metal toxicity due to reduction in trace metal availability (Andersen, 2005).

Salts**:** $NaNO_3$ 75g, NH_4Cl 2.67g, Na.Beta-glycerophosphate 2.16g, $NaSiO_3.9H_2O$ 15.35g, H_2SeO_3 .00129g, tri-base (pH 7.2) 121.10g

Trace metal solution: Initial trace solution $ZnSO_4.7H_2O$ 23g, $CoSO_4.7H_2O$ 14.05g, $NaMoO_4.2H_2O$ 7.26g, $CuSO_4.5H_2O$ 2.5g are dissolved in 1L of distilled water, 1ml of the primary stock are dissolved with $Na_2EDTA.2H_2O$ 37.22g, Fe-Na-EDTA.$3H_2O$ 4.93g, $FeCl_3.6H_2O$ 3.15g, $MnCl_2.4H_2O$ .178g in 1L distilled water bring it to a final stock of trace metal.

Vitamins: Initial stock contains Biotin (Vit H) 1.0g and Cyanocobalamin (Vit B_{12}) 1.0g dissolved in 1L distilled water, 1ml of the primary stock is dissolved with 200mg of Thamine.HCl (Vit B_1) in a final stock of 1L.

Hoagland Medium

The chemical compositions listed below are dissolved in one liter of nano-pure water.

Salts**:** $Ca(NO_3)_2.4H_2O$ 14.19g, KNO_3 5.6g, $MgSO_4.7H_2O$ 5.48g, KH_2PO_4 1.52g.

TRACE METAL: Fe-Na-EDTA 0.62g, NaCl 0.20g, $MnCl_2.4H_2O$ 0.06g, H_3BO_3 0.06g, $CuSO_4.5H_2O$ 0.008g, $ZnSO_4.7H_2O$ 0.01g, $(NH_4)_6Mo_7O_{24}.4H_2O$ 0.003g.

Microalgae Species Selection

Microalgae used for study were selected based on reviewed articles and publication. The selection criteria includes high lipid productivity, ability to survive shear stress, ability to dominate wild strain in open ponds production, high CO_2 sinking capacity, limited nutrient requirement, tolerant to wide variety of varying temperature, fast productivity cycle, high photosynthesis efficiency (Becker, 2004).

Culture Maintenance

Illumination

Illumination is supplied by double 20W cool white fluorescent bulbs, placed at a distance of two feet from the flasks and petri-dish, light intensity was 360 $\mu E.m^{-2}.s^{-1}$. The light source was regulated with an adjustable timer set using a photoperiod of 16 hrs light and 8 hrs dark.

Temperature

The microalgae were cultured at control temperature of 25°C (77°F), the temperature response for low and high were set at 18°C and 32°C. The temperature ranges were set to assess growth and effect temperature variation would have on biomass and fatty acid composition of microalgae.

Water

Nano pure water was used instead of the natural sea water. Nano-pure water is defined as glass distilled water in this study. It is free from metals and trace contaminants and purer than de-ionized water. The use of glass distilled water enables the dissolution of salts, trace and vitamins in the proportion required for experiment.

Air Flow

Atmospheric CO_2 was the source of carbon used by microalgae for growth. During per-culturing (initial cell culture), air was pumped into the medium continuously.

Culture System

The microalgae were pre-cultured in 500 ml beakers, using stock solutions of F/2, K and Hoagland media at their required concentration stated above, while the growth experiments were conducted at different concentrations of nitrogen and iron as well as different temperature regimes.Different salt concentrations of nitrogen and iron were used during the preparation of F/2, Hoagland and K medium to study the effect of these nutrients on the cell growth, biomass and fatty acid composition of the microalgae.

The nitrogen source in the media composition for F/2 and K is $NaNO_3$ supplied at 75g/L (control) (Andersen 2005). Nitrogen concentration in the media composition is 12.35gmol/L. The supply of low $NaNO_3$ to the media was 50% of the original composition, which is 37.5g/L of $NaNO_3$ (low N), thus nitrogen concentration in the media was 6.18gmol/L. High concentration of $NaNO_3$ was 200% of the original supply which is 150g/L of $NaNO_3$ (high N), the final concentration of nitrogen in the media composition was 24.71gmol/L. The nitrogen source in Hoagland media are KNO_3 and $CaNO_3$, KNO_3 was the nitrogen source considered for nitrogen treatment, it was supplied at low KNO_3 concentration of 2.8g/L, control and high KNO_3 salts concentration are 5.6g/L and 11.2g/L respectively(Andersen 2005).

The iron source in F/2 and K medium is $FeCl_3$; the concentration of $FeCl_3$ in the medium is 3.15g (control) (Andersen 2005). It was dissolved with the primary trace metals to have

a final concentration of trace metal. The molarity of the final concentration of iron in the final trace solution is 1.08gmol/L, the supply of $FeCl_3$ in low concentration was 1.57g/L, molarity of iron concentration in media is 0.54gmol/L. High $FeCl_3$ concentration was dissolved at 200% of the original $FeCl_3$ concentration 6.3g/L in final trace solution, molarity of final iron concentration in media is 2.17gmol/L. Iron source in Hoagland is Fe-Na-EDTA at 0.62g/L, low and high Fe-Na-EDTA are at 0.31g/L and 1.24g/L respectively.

The temperature response was conducted in an incubator equipped with artificial lighting. Atmospheric CO_2 was the source of air and carbon, the temperature response for growth was set at 25°C (control), the temperature range were either decreased to 18°C or increased to 32°C, which were altered 7°C from 25°C (control).

The autotrophic batch cultivation of each strain in different media composition (F/2, K and Hoagland) with varying independent variables (nitrogen, iron and temperature) at control, low and high concentration, were stationary cultured in 15 cm Petri dish in culture stock dissolved in 100 ml nano-pure water in three replications each for a minimum of 14 days and maximum of 21 days based on growth rate of microalgae tested at 25°C for comparison. The cell count at day 1 before inoculation is $1.5*10^3$ and final cell count before harvest varies from $8.4*10^5$-$5.4*10^6$. All culturing were done with illumination supplied using twin 20W cool white fluorescent lamp with 16: 8 light and dark cycle. The Petri dishes were placed two feet away to achieve an intensity of $180\mu E.m^{-2}.s^{-1}$ and to prevent overheating of culture. The setting was kept constant throughout each set of experiment conducted.

Cell Growth Measurement

The microalgae cell growth was measured daily at optical density/absorbance of 660nm with the GENESYS 10 Bio spectrophotometer. All measurements were done in triplicates and displayed graphically singly and in means of each replication (absorbance vs duration of culture).

Specific growth rate (h^{-1}) defines the fraction of cell increase over duration of time i.e increase in cell mass per unit time. It is the average growth rate of cell in a particular culture (Richmond, 2004: Caldwell et.al 1981).

Specific growth rate is calculated by the equation (Richmond 2004: Caldwell et.al 1983).

$$\mu = \frac{1}{t} \ln \left(\frac{Xm}{X0}\right) \quad \text{..... [1]}$$

Where $\boldsymbol{X_m}$ and $\boldsymbol{X_0}$ are the concentrations of biomass at the end and at the beginning of a batch run respectively, and $\boldsymbol{t}$ is the duration of the run.

$$\text{Division per day} = \frac{\mu}{\ln 2} \quad \text{..... [2]}$$

$$\text{Generation time} = \frac{1}{Div.day-1} \quad \text{..... [3]}$$

Cell Count

The cell numbers were quantified using the counting chamber of the disposable cyto C haemocytometer under an electron microscope and TC10 Automated cell counter (BIORAD USA). The TC10 Automated cell counter automatically counts cells, displays cell count and cell diameter graphically and gives the total number of dead and live cells when 10µl of trypan blue is added to 10µl of the sub-sampled culture. The cell count and cell growth are displayed graphically (Appendix C).

Culture Harvest

The stationary growth phases of the cultured microalgae cells were harvested using a modified belt filter (Becker 2004). It is a fine weave fabric of pore size less than 10µm; the fabric sieve is folded to filter cells from media. The recovered cells slurry were air dried at room temperature for 24 hours and are stored for fatty acid profile.

Lipid Extraction

Total lipid extraction was conducted using the modified Bligh and Dyer (1959), which is the total lipid extraction and purification method. The procedure uses 2:1:1.8 methanol: chloroform: water with10µg internal standard of 19:0 (10 mg/ml^{-1}) in chloroform. Fatty acid methyl esters (FAME) were formed by saponification in 5% KOH in CH_3OH at 65°C for 90 minutes. It was cooled for 5 minute then methylation with 1ml of 14% BF_3 in methanol and reheated for 30 minutes at 65°C. The extracts were cooled to room temperature, FAME along with other lipids were extracted with hexane-diethyl- ether (9:1), initial separation of FAME from other lipids were performed with chloroform and water and passed through $MgSO_4$ mini-column. The extracts were dried under nitrogen gas to evaporate chloroform. The dried samples were re-suspended in 150µl of hexane and final separation of FAME from other lipids was conducted in column packed with silica gel, because fatty acid binds to silica gel, components that are FAME dissolve in hexane. Finally the fatty acid ester was eluted from the column using 5% ether in hexane. The samples are dried using nitrogen gas and reconstituted in 300µl of chloroform. 100µl of the reconstituted sampled are transferred into the GC vials for FAME analysis.

Fatty acid methyl ester profiles were analyzed using a Hewlett Packard 5890 series II gas chromatography DB23 column (30m x 0.25mm ID and 0.25µm film thickness). The GC had an FID detector set at 300°C and the inlet set at 250°C. It runs a split injection 1:25 split with helium as the carrier gas at 1ml per minute. Oven parameters were as follows: initial temperature was set at 110°C for 1minute, ramp to 182°C at a rate of 22°C per minute, then to 190°C at 0.6 per minutes, then ramped to a temperature of 225°C at 4°C per minute. There was no final hold time at the end of the run. The total run time for FAME analysis under this parameter is 26.36minutes. FAMEs were identified by comparing retention times to known FAME standard (NuChek Prep, Elysian MN).

CHAPTER IV

RESULTS AND DISCUSSIONS

The research assesses the effect of nitrogen, iron and temperature on the biomass and fatty acid composition of the three microalgae, as well as the growth rate of microalgae in response to different treatments of nutrients (nitrogen, iron) and temperature. The study was conducted as batch cultures in the laboratory, cultured species were harvested at their stationary phase and the slurry air dried at room temperature for 24 hours before analyzing the fatty acid composition.

The growth rates of the three microalgae were measured using the spectrophotometer at an absorbance of 660nm. The mean absorbance values from the three replicates recorded for each of the treatments (low, control and high nitrogen and iron concentrations, and temperature) and the cell counts are displayed graphically in plots below. (Appendix A)

Response to nutrients and temperature treatments of microalgae cultured in F/2 media

The mean absorbance (from the three replicates) recorded for nitrogen response in F/2 media considering the three microalgae shows that the *N.salina* and *Chlorella sp* had higher absorbance values and cell growth at both low (37.5g/l) and high (150g/l) of $NaNO_3$ concentrations when compared to the control (75g/l). *P.carterae* peak

absorbance and cell growth trend was the reverse (lower than the control) for the low and high concentration of $NaNO_3$ (Fig.3).

Of the three microalgae tested, *N. salina* and *P. carterae* had higher growth rates compared to *Chlorella sp* when cultured in low $FeCl_3$ (1.58g). *Pleurochyrsis carterae* had the highest growth rate at control $FeCl_3$ (3.15g) (Fig.4), while the three microalgae had similar peak growth when cultured in high $FeCl_3$ (6.30g) (Fig.4). The limited detectable response of *Chlorella sp* at low iron concentration could be attributed to it being green algae; insufficient iron compound in the media would interfere with photosynthesis. Because the main chlorophyll pigment of green algae is *Chl.a* which is also the main pigment for photosynthesis, thus affects cell division but as iron concentration increases the strain responded rapidly to increase in iron (Liu et.al., 2008).

Temperature responses of three microalgae were assessed to determine the ideal temperature the microalgae could be cultured (Fig 5). *Nannochloropsis salina* had a low growth response when cultured at 18°C (low temperature), which is indicative that temperature especially for this strain should be kept at optimum 25°C. Any decrease in the threshold (25°C) for this strain would affect the growth rate although it can be cultured at temperature above the threshold, up to 32°C. When microalgae were cultured at 36°C, to further evaluate growth response at higher temperature none of the tested species survived.

Response to nutrients and temperature treatments of microalgae cultured in Hoagland media

The Hoagland media source of nitrogen is KNO_3, *N. salina* and *Chlorella sp* showed progressive growth similar to original culture media (F/2 and K) in Hoagland media while *P. carterae* did not survive culture in Hoagland media. *Nannochloropsis salina* and *Chlorella sp* had similar growth at low nitrogen (2.8g/L), while *Chlorella sp* had an increased growth rate at control (5.6g/L) and high (11.2g/L) compared to *N. salina* (Fig.6).

The iron source in the Hoagland media is EDTA, the growth rate of the two microalgae compared are similar to the N treatment, with *Chlorella sp* having a higher growth response in the Hoagland media and treatments especially at control and higher treatment than low treatment (Fig.7).

The response of *N. salina* and *Chlorella sp* to temperature is an indication of the fact that these microalgae are best cultured at the control temperature of 25°C. Drastic decrease in growth especially at temperature below the control (18°C) level was observed (Fig.8).

Response to nutrients and temperature treatments of microalgae cultured in K media

Chlorella sp had highest growth rate in K media, which is also the original culture media used by the culture center for marine phytoplankton for the strain preservation and culture. The highest growth response of *Chlorella sp* was in control (75g/L $NaNO_3$) which is the nitrogen source for the treatment. The low N (37.5g/L) growth was the highest response recorded for *Chlorella sp* in all the media cultures so also is the high N treatment (Fig.9). The growth rate of *N.salina* in K media is quite different to Hoagland

media and growth of *N. salina* was higher in F/2 media in all experiments and treatments compared to the other microalgae (Fig.3,4,5).

The iron response of *N. salina* was low compared to low iron concentration in Hoagland (Fig.7) and low and control iron response in F/2 media (Fig.4), but *N. salina* has a similar iron response at control and high iron (3.15 and 6.3 $FeCl_3$) respectively (Fig.10) as *Chlorella sp* in F/2 media (Fig.4), thus *N. salina* can be cultured at high nitrogen and iron concentration in K media as an alternative to F/2 media, but media components (salts) would require double the standard composition which would increase investments especially for large scale production.

N. salina had a reduced temperature response at control in K media compared to other media, this response is attributed to the salt components of the media because the cell seem to be deficient in nutrients even at the recommended amount for growth in K media or the cell of *N. salina* are just not suitable for growth in K media at that composition. *Chlorella sp* however, had the expected growth response to temperature in K media at control temperature (Fig.11).

Specific growth rate

The results from the Tables A7-A15 shows specific growth rate of the three microalgae in different growth media. Growth rate of *Nannochloropsis salina* at different nitrogen concentration, (Table A7) had the highest growth rate during nitrogen starvation, low N in F/2 media. Lack of nutrients or insufficient nutrients makes the cells complete their growth cycle rapidly to maximize the available nutrient for growth. When viewed under the microscope the cell size were not altered (neither increased nor decreased), thus the microalgae might have converted its chemical compositions to stored products like lipids

or starch during the starvation. The growth rate of *Nannochloropsis salina* in K and Hoagland media was rapid at control but it did not reflect in the yield because the rate at which they multiple is proportional to cell decline (death).

However, the other two microalgae, *Chlorella sp* and *Pleurochyrsis carterae* had the highest cell growth when nitrogen was supplied at the required level i.e control (Table 8 and Table 9) in K media and F/2 respectively. *Chlorella sp* and *Pleurochyrsis carterae,* growth phase was longer and slower due to the excess nutrient available at high nitrogen in all culture media. Thus it needs to neutralize the toxicity in the media, which makes the microalgae lag phase slow. This might lead to conversion of viable products to less viable products. Protein and carbohydrates might be the dominant products with less lipids and more polyunsaturated fatty acids (Becker 2004) (Table A7, A8 and A9).

The second phase of the research tests for the effect of iron on the growth rate of the three microalgae. *Nannochloropsis salina* (Table 10) had its highest growth rate at low iron concentration in F2 and K media, while the highest growth rate in the Hoagland media was control. Specific growth rate of *Chlorella sp* in all the three culture media were relatively similar except at control level K media which was higher (Table 11). *Pleurochrysis carterae* also had its highest growth phase at low iron concentration F/2 media. This strain would only grow in its original culture media, trials in K and Hoagland media failed in the three replications.

The specific growth rate for temperature treatment for all microalgae irrespective of media shows that at low temperature the microalgae had a slow cell division especially *N.salina.* However, most of the microalgae had a rapid growth rate at the optimal temperature of 25°C, increase in temperature reduced the growth rate.

Doubling (Generation) Time

The doubling or generation time is the time required to attain double the growth of cells and produce a generation of cells in the culture concentration. The exponential growth phase of the microalgae culture is used to calculate the generation time. During the exponential growth phase, the growth of cells is proportional to the cells biomass (Richmond 2004).

The generation time of microalgae in different culture media range from the shortest generation time of 0.4days in F/2 media *N. salina* low $NaNO_3$ to longest duration of 1.9days in K media *N. salina* high N treatment. Overall, the shortest generation time in Nitrogen treatment occurred when *N. salina* and *Chlorella sp* were cultured in F/2 and K media while the longest generation time in Nitrogen treatment occurred in K media in *N. salina* (Appendix B).

The generation time for iron treatments range from shortest duration in K media in *N. salina* 0.4days iron control level and longest duration in K media in the same microalgae at low Fe treatment 1.7days.The generation time for temperature response was also shortest in K media *N. salina* and *Chlorella sp* control level 0.4 days, while the longest was in H and F/2 media in *Chlorella sp* and *P. carterae* high temperature 1.5 days.

Statistical Analysis

The experimental design is a completely randomize factorial treatment combination. It assesses the effect of different culture media and levels of treatment on biomass of microalgae. General linear model was used to evaluate the statistical significance of main

factors (media, nitrogen, iron and temperature) as well as the interaction of main factors in each experiment.

The effect of different media on the growth of *N.salina* was significant at P <0.05 (Table A16-A17), thus growth of each strain in different culture media indicates difference in growth, final biomass as well as dry weight from output obtained from SAS software. Different nitrogen treatments used during the culture of microalgae were significant at P < 0.01, the interaction of the main factors i.e media and nitrogen was highly significant with P < 0.001. Therefore the culture of *N.salina* in different media combined with different level of nitrogen treatment significantly affects the biomass and yield of *N.salina* across different media and nitrogen level (Table 16).

Chlorella sp significant level was at P < 0.05, when culture in different media, varying nitrogen level as well as its interactions (Table 16).

The effect of media cannot be accounted for in *P.carterae,* because it would only grow in the F/2 media which was the original culture media, but within the same culture media the effect of nitrogen level was significant P < 0.05 so also is the interaction of nitrogen level and media (Table 16).

Media culture of *N.salina* with different iron concentration was significant at P < 0.05, while the interaction of main factors was significant at P < 0.01 (Table 17), thus different media and levels of iron has a significant effect on the biomass of *N.salina*.

Chlorella sp level of significance was higher P < 0.01 when cultured in different media; this might be a result of different iron source in the media which would significantly

influence growth at different concentration of iron (Table 17) *P.carterae* within culture was not significantly different at levels of iron concentration and the interaction of main factors.

The effect of culturing *N.salina* at different temperature range and media had a significance of P <0.001 so also were the interaction. The temperature range considered in this research indicates microalgae growth was best at optimal temperature, but below the optimal it decreases the growth (Table 18).

Chlorella sp media response to varying temperature range was highly significant P <0.001, while temperature response and its interaction with media are significant at P< 0.05. The effect of temperature was also significant P< 0.05 within media in *P.carterae*. Thus, considering temperature regimes are beneficial in microalgae culture, but temperature range well below or above the threshold might not give products desirable with research objective, reduce the survival rate of cells which would affect the chemical composition of biomass, yield and dry weight of microalgae. Appendix C contents a table of respective means and standard deviation.

Fatty Acid Analysis

The total fatty acid profile obtained from the GC was calculated using the peak areas to evaluate the relative composition of each fatty acid. Microalgae required for biodiesel production should have significant amount of palmitic, oleic and linoleic acid (Rashid et.al 2008; Lee et.al., 2010). Considering the fatty acid profile of *N.salina* cultured in different media, F/2 media has high oleic acid at control level of nitrogen (Table 19), the same can be observed in K media when grown without nitrogen starvation.

The quality of biodiesel required from the fatty acid profile especially oleic acid are high cetane number (CN), heat combustion, increased oxidative stability, ignition quality, decreased cold filter plugging point, viscosity and lubricity (Mittelbach 1996). Oleic acid in high proportion increases the oxidative stability which enables longer storage (Knothe 2008) and reduces clogging when temperature is cold (winter) (Stoumas 1995).

Response of *N.salina* to iron treatment has palmitic and linoleic acid at higher percentages in different media with low oleic acid profile (Table 20). Variation of iron is not desirable for quality biodiesel production although it influence the biomass yield and cell density of *Chlorella sp* and *N.salina*. Temperature response for *N.salina* was similar at control culture media F/2 and K, with variation in their fatty acid composition at low and high temperature treatment which would determine the quality of products obtained (Table 21).

The fatty acid profile of *Chlorella sp*, nitrogen response shows K media which is the original culture media for *Chlorella sp* is ideal for biodiesel production, when cultured at the required nutrient concentration because of its high oleic acid content (Lee et.al., 2010) if that is the desired product. Fatty acid composition could not be assessed at low iron

response K media because of the low biomass obtained from the culture (Table 23). The exact effect of iron on fatty acid composition cannot be evaluated, although it increased growth and biomass at high iron concentration, the percentage fatty acid composition is reduced compared to nitrogen response except in K media control. Response to temperature is also similar to previous observations, except the high oleic acid content in F/2 media when *Chlorella sp* was cultured at low temperature (Table 24). The high oleic acid content at low temperature could be investigated further, to assess the potentials of culturing at low temperature in the specified media.

The significant oleic acid present in *P.carterae* (Table 25) was at control N response in F/2 media, which indicates that this particular media just like the other two microalgae would only thrive at the required condition for maximum productivity

CHAPTER V

CONCLUSIONS

Results from the cell growth using the absorbance measurements shows that microalgae are best cultured at control temperature 25ºC and required culture media from which microalgae were obtain. Although Low and high nitrogen treatment increased growth rate and cell count of *N. salina* when cultured in all media (F/2, K and Hoagland media), while the effect of iron on cell growth and cell count increased only at high iron treatment for the same microalgae. *Chlorella sp* growth rate response to nitrogen treatment also increased at low and high nitrogen concentration in F/2 and Hoagland media but not K media, High iron treatment also increased growth rate of *Chlorella sp* in F/2 and K media. The final products from microalgae might be significantly affected by varying nutrient concentration which was observed in the percentage fatty acid composition and Oleic acid content of low and high nitrogen response *N. salina* F/2 media compared to control and *Chlorella sp* control iron response in K media which also had a better fatty acid composition and oleic acid content than the low and high treatment. The highest response for temperature treatment during absorbance and cell count was at control temperature 25ºC for all media growth except in *N. salina* K media. The low response at control could be attributed to a lot of factors one of which would be because K media is

not the original culture for *N .salina*, as such, the salt and trace components of the media might hinder growth. Thus across media culture, F/2 media had the best response to cell growth irrespective of microalgae.

In some cases, lipid and fatty acid content can be enhanced by the imposition of nitrogen starvation or other stress factors. Biochemical studies have also suggested that acetyl-CoA carboxylase (ACCase), a biotin-containing enzyme that catalyzes an early step in fatty acid biosynthesis, may be involved in the control of this lipid accumulation process. Therefore, it may be possible to enhance lipid production rates by increasing the activity of this enzyme via genetic engineering.

It is thus important to understand the productivity of microalgae in terms of chemical composition, culture parameters (light, nutrients temperature etc) as well as DNA sequencing that would improve strain biomass yield before culturing. The selection of microalgae for suitable purpose is important so also are the existence and availability of all apparatus to be used for cultivation.

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APPENDIX A – Growth Response Charts From Results

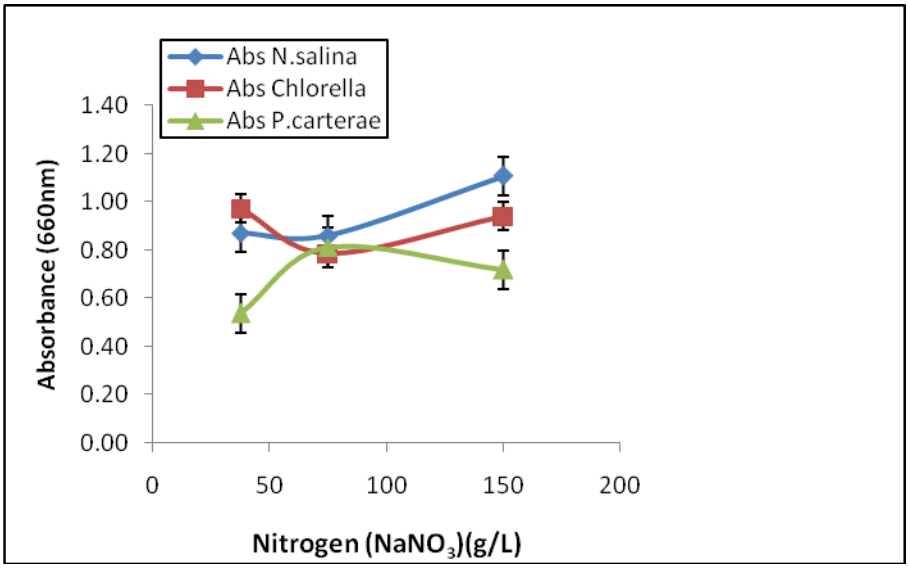

Figure 3- Growth response of the three microalgae to different nitrogen concentrations (NaNO3). The microalgae were cultured in F/2 media at 660nm and temperature of 25ºC. Data are ± SE, n=3

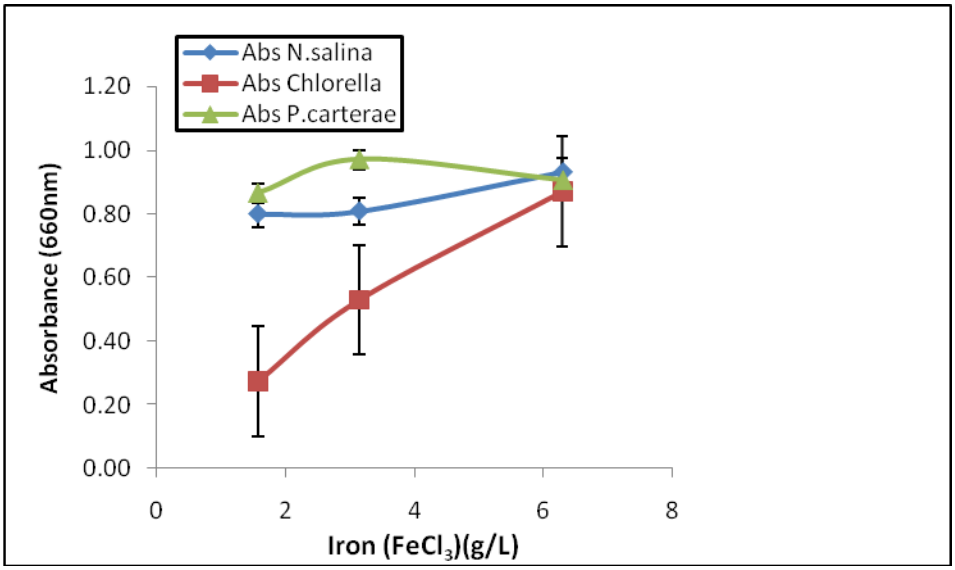

Figure 4- Growth response of the three microalgae to different iron concentration ($FeCl_3$). Microalgae were cultured in F/2 Media at λ 660nm and temperature of 25ºC. Data are ± SE, n=3

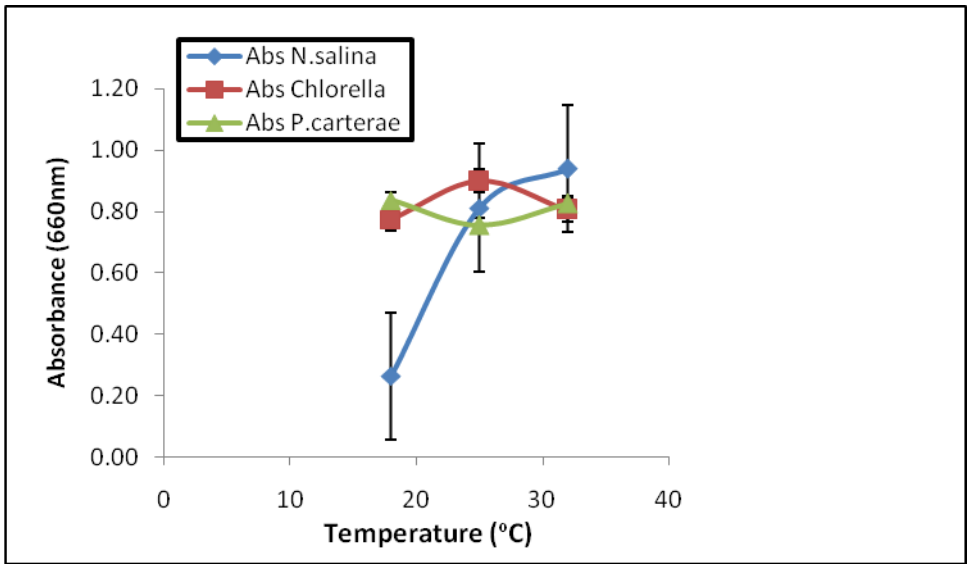

Figure 5 - Growth response of the three microalgae to different temperatures treatments (ºC). Microalgae were cultured in F/2 Media at λ 660nm. Data are ± SE, n=3

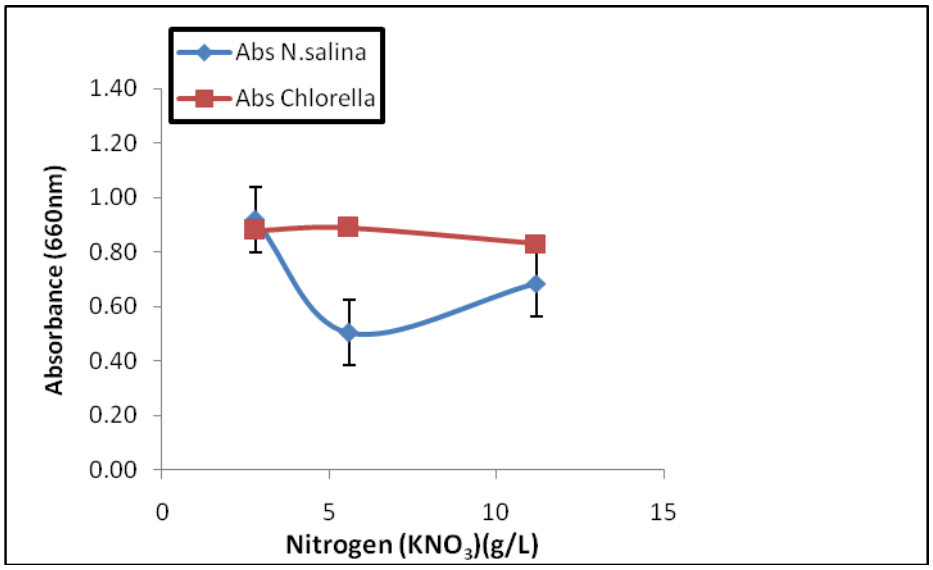

Figure 6 - Growth response of the three microalgae to different nitrogen concentration (KNO_3). Microalgae were cultured in Hoagland Media at λ 660nm and temperature of 25ºC. Data are ± SE, n=3

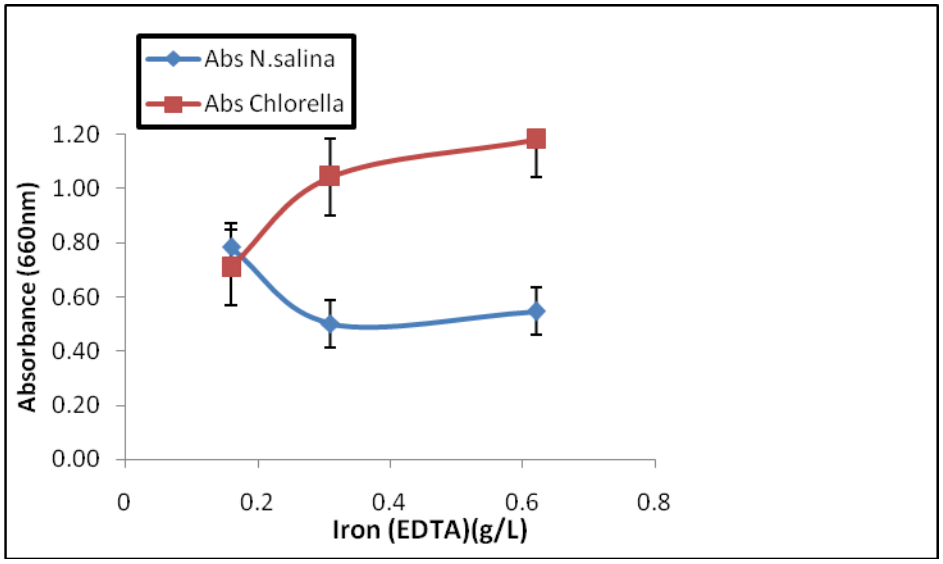

Figure 7 - Growth response of the three microalgae to different iron concentration (EDTA). Microalgae were cultured in Hoagland media at λ 660nm and temperature of 25ºC. Data are ± SE, n=3

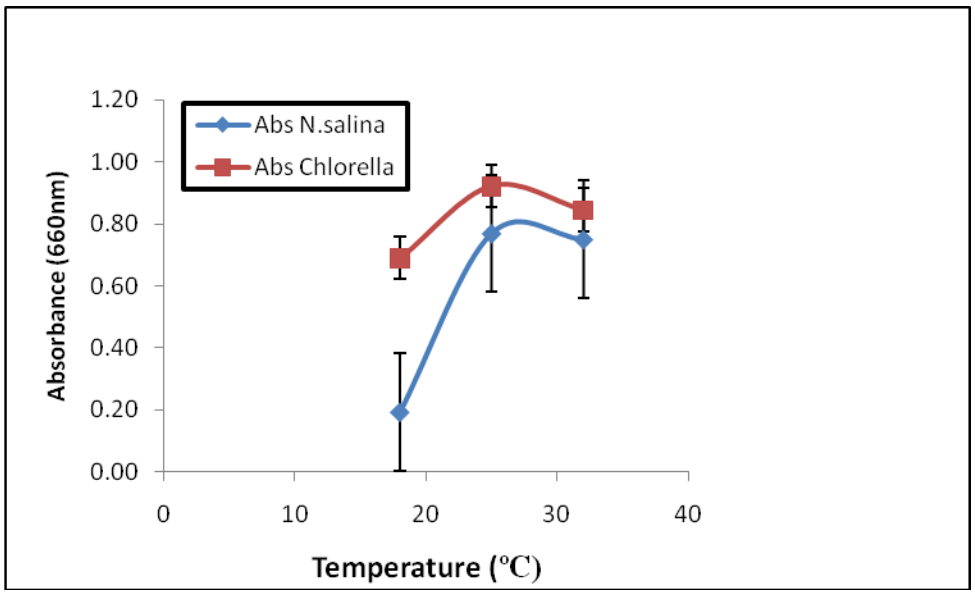

Figure 8 - Growth response of the three microalgae to different temperature treatments (ºC). Microalgae were cultured in Hoagland media at λ 660nm and controlled temperature of 25ºC. Data are ± SE, n=3

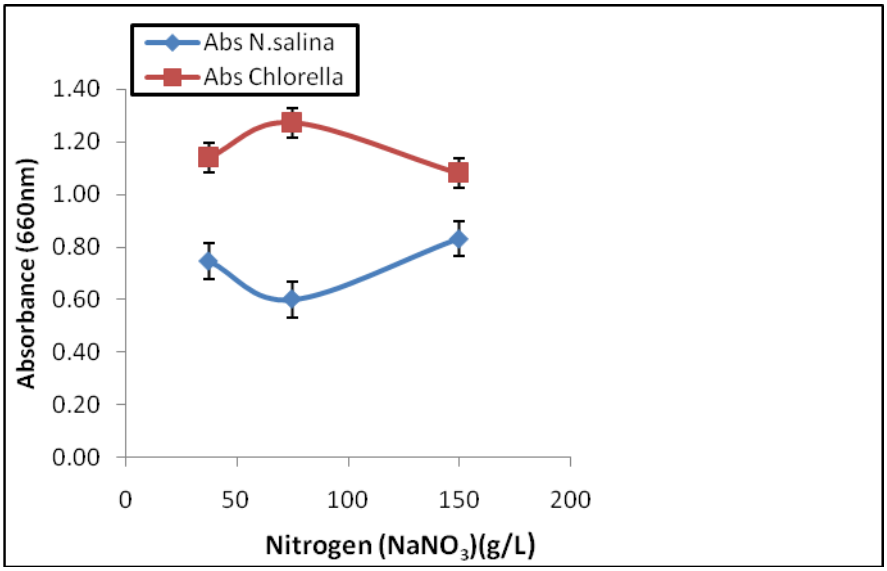

Figure 9 - Growth response of the three microalgae to different nitrogen concentration ($NaNO_3$). Microalgae were cultured in K media at λ 660nm and temperature of 25ºC. Data are ± SE, n=3

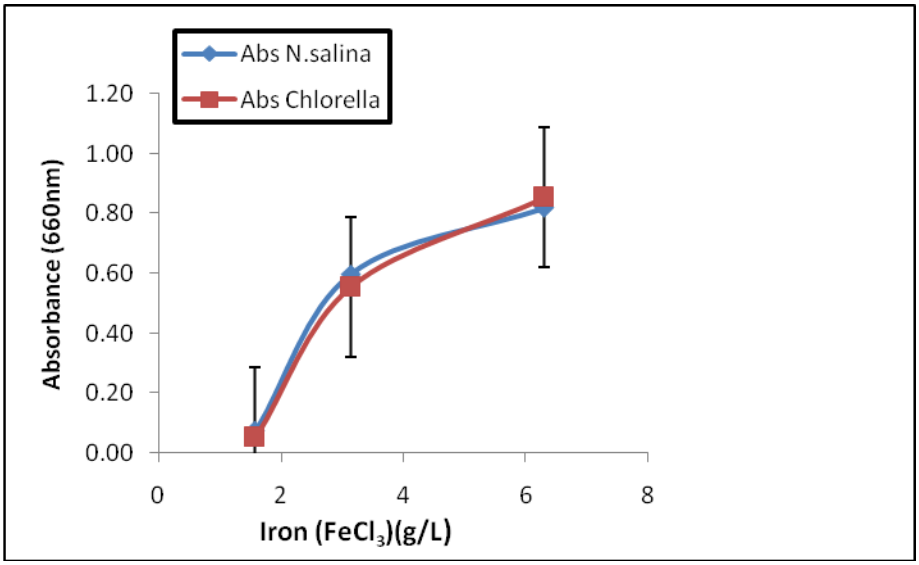

Figure 10 - Growth response of the three microalgae to different iron concentration ($FeCl_3$). Microalgae were cultured in K media at λ 660nm and temperature of 25ºC. Data are ± SE, n=3

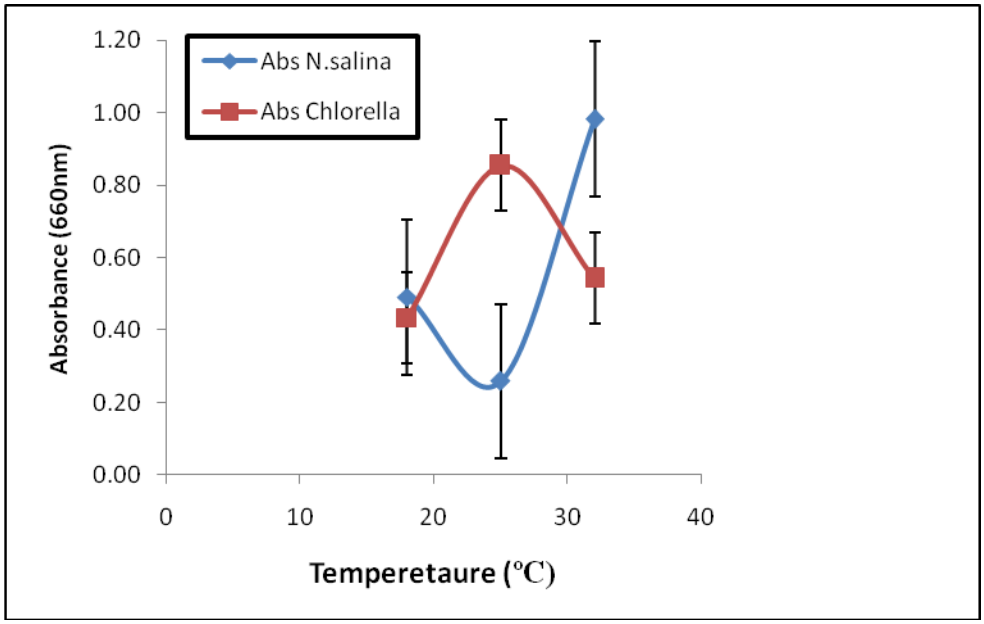

Figure 11 - Growth response of the three microalgae to different temperature treatments (ºC). Microalgae were cultured in K media at λ 660nm with different temperature levels. Data are ± SE, n=3

Table 7 - Specific growth rate of *Nannochloropsis salina* at different $NaNO_3$ concentration in different culture media

$NaNO_3$ ($gL^{-1)}$	F2 [μ^a ($days^{-1}$)]	K [μ^a ($days^{-1}$)]	H [μ^a ($days^{-1}$)]
75 (control)	1.15	1.10	0.70
37.5 (low)	1.70	0.80	0.35
150 (High)	0.90	0.75	0.36

μ^a – *specific growth rate*

Table 8 - Specific growth rate of *Chlorella sp* at different $NaNO_3$ concentration in different culture media

$NaNO_3$ (gL^{-1})	F2 [μ^a ($days^{-1}$)]	K [μ^a ($days^{-1}$)]	H [μ^a ($days^{-1}$)]
75 (control)	0.95	1.70	0.45
37.5 (low)	1.40	1.10	0.70
150 (High)	0.46	0.25	0.350

μ^a – *specific growth rate*

Table 9 - Specific growth rate of *Pleurochrysis carterae* at different $NaNO_3$ concentration in different culture media

$NaNO_3$ (gL^{-1})	F2 [μ^a ($days^{-1}$)]	K [μ^a ($days^{-1}$)]	H [μ^a ($days^{-1}$)]
75 (control)	0.90	-----	-----
37.5 (low)	0.45	-----	-----
150 (High)	0.47	-----	-----

μ^a – *specific growth rate*

Table 10 - Specific growth rate of *Nannochloropsis salina* at different $FeCl_3$ concentration in different culture media

$FeCl_3$ (gL^{-1})	F2 [μ^a ($days^{-1}$)]	K [μ^a ($days^{-1}$)]	H [μ^a ($days^{-1}$)]
3.15 (control)	0.45	0.35	0.75
1.58 (low)	1.51	1.7	0.55
6.30 (High)	1.41	1.35	0.47

μ^a – *specific growth rate*

Table 11 - Specific growth rate of *Chlorella sp* at different $FeCl_3$ concentration in different culture media

$FeCl_3$ (gL^{-1})	F2 [μ^a ($days^{-1}$)]	K [μ^a ($days^{-1}$)]	H [μ^a ($days^{-1}$)]
3.15 (control)	0.80	1.10	0.39
1.58 (low)	0.45	0.35	0.45
6.30 (High)	0.49	0.39	0.36

μ^a – *specific growth rate*

Table 12 - Specific growth rate of *Pleurochrysis carterae* at different $FeCl_3$ concentration in different culture media

$FeCl_3$ (gL^{-1})	F2 [μ^a ($days^{-1}$)]	K [μ^a ($days^{-1}$)]	H [μ^a ($days^{-1}$)]
3.15 (control)	0.48	-----	-----
1.58 (low)	0.65	-----	-----
6.30 (High)	0.28	-----	-----

μ^a – *specific growth rate*

Table 13 - Specific growth rate of *Nannochloropsis salina* at different temperature treatments in different culture media

Temperature (°C)	F2 [μ^a ($days^{-1}$)]	K [μ^a ($days^{-1}$)]	H [μ^a ($days^{-1}$)]
25 (control)	1.45	1.73	1.05
18 (low)	0.60	0.73	0.79
32 (High)	0.45	0.44	0.45

μ^a – *specific growth rate*

Table 14 - Specific growth rate of *Chlorella sp* at different temperature treatments in different culture media

Temperature (°C)	F2 [μ^a ($days^{-1}$)]	K [μ^a ($days^{-1}$)]	H [μ^a ($days^{-1}$)]
25 (control)	0.90	1.55	1.2
18 (low)	0.85	0.79	0.75
32 (High)	0.55	0.55	0.5

μ^a – *specific growth rate*

Table 15 - Specific growth rate of *Pleurochrysis carterae* at different temperature treatments in different culture media

Temperature (°C)	F2 [μ^a ($days^{-1}$)]	K [μ^a ($days^{-1}$)]	H[μ^a ($days^{-1}$)]
25 (control)	1.5	-----	-----
18 (low)	0.9	-----	-----
32 (High)	0.46	-----	-----

μ^a – *specific growth rate*

Table 16 - Two-way ANOVA analysis of effect of media and nitrogen on microalgae dry biomass

Effect of media and nitrogen on biomass of microalgae			
Main Factors	**Level of significance**		
	N.salina	*Chlorella.sp*	*P.carterae*
Media	*	*****	NS
Nitrogen	**	*	*
Interactions of main Factors			
Media*Nitrogen	***	*	*

***, **, * indicate level of significance at 0.001, 0.01, 0.05 NS- not significant

Table 17 - Two-way ANOVA analysis of effect of media and iron on microalgae dry biomass

Effect of media and iron on biomass of microalgae			
Main Factors	**Level of significance**		
	N.salina	*Chlorella.sp*	*P.carterae*
Media	*	**	
Iron	**	*	NS
Interactions of main Factors			
Media*Iron	**	*	NS

***, **, * indicate level of significance at 0.001, 0.01, 0.05 NS- not significant

Table 18 - Two-way ANOVA analysis of effect of media and temperature on microalgae dry biomass

Effect of media and temperature on biomass of microalgae			
Main Factors	**Level of significance**		
	N.salina	*Chlorella.sp*	*P.carterae*
Media	***	*******	*****
Temperature	***	*	*
Interactions of main Factors			
Media* Temperature	***	*	*

***, **, * indicate level of significance at 0.001, 0.01, 0.05 NS- not significant

Table 19 - Percentage of fatty acid composition of *Nannochloropsis salina* as affected by nitrogen treatments in different culture media

Fatty acids		F2 Media			H Media			K Media		
		Low	**Control**	**High**	**Low**	**Control**	**High**	**Low**	**Control**	**High**
Palmitic acid	**16:00**	**33.4**	**26.8**	**22.9**	**34.3**	**33.3**	**33.2**	**26.5**	**23.5**	**27.3**
Stearic acid	18:00	6.7	3.4	2.8	3.0	3.4	2.9	4.1	2.5	3.8
Oleic acid	**18:1 (9c)**	**6.8**	**28.8**	**7.4**	**6.4**	**8.1**	**6.6**	**11.8**	**34.0**	**9.6**
Oleic acid	18:1 (11c)	8.5	2.3	6.5	2.3	7.8	1.4	2.2	1.7	1.8
Linoleic acid	**18:2 n6**	**31.9**	**18.3**	**16.0**	**27.8**	**27.1**	**29.4**	**28.8**	**23.8**	**31.9**
alpha linolenic	18:3 n3	11.0	7.1	0.9	3.0	5.1	3.7	3.1	1.7	3.3

Table 20 - Percentage of fatty acid composition of *Nannochloropsis salina* as affected by iron treatments in different culture media

Fatty acids		F2 Media			H Media			K Media		
		Low	**Control**	**High**	**Low**	**Control**	**High**	**Low**	**Control**	**High**
Palmitic acid	**16:00**	**30.2**	**34.5**	**33.9**	**34.1**	**34.3**	**32.1**	**-**	**30.1**	**28.9**
Stearic acid	18:00	4.3	5.5	9.8	10.8	5.0	10.1	-	4.8	4.0
Oleic acid	**18:1 (9c)**	**4.8**	**29.4**	**5.7**	**3.3**	**4.3**	**14.0**	**-**	**33.5**	**11.6**
Oleic acid	18:1 (11c)	1.8	3.3	4.3	2.9	5.0	2.0	-	4.5	2.2
Linoleic acid	**18:2 n6**	**32.5**	**22.9**	**24.3**	**23.9**	**23.5**	**19.2**	**-**	**20.3**	**29.8**
alpha linolenic	18:3 n3	5.0	4.3	4.1	6.2	6.9	3.8	-	5.3	5.5

Table 21 - Percentage fatty acid composition of *Nannochloropsis salina* as affected by temperature treatments in different culture media

Fatty acids		F2 Media			H Media			K Media		
		Low	**Control**	**High**	**Low**	**Control**	**High**	**Low**	**Control**	**High**
Palmitic acid	**16:00**	**26.4**	**36.3**	**34.7**	**27.8**	**31.3**	**32.5**	**28.8**	**30.4**	**34.2**
Stearic acid	18:00	5.2	3.0	4.1	4.1	5.8	6.6	7.7	7.1	3.0
Oleic acid	**18:1 (9c)**	**12.7**	**23.7**	**5.6**	**4.8**	**3.9**	**8.5**	**5.5**	**31.1**	**4.8**
Oleic acid	18:1 (11c)	6.6	2.8	2.9	3.4	2.8	1.6	3.8	3.5	2.9
Linoleic acid	**18:2 n6**	**12.2**	**28.2**	**32.4**	**28.5**	**26.8**	**27.5**	**16.4**	**24.3**	**30.8**
alpha linolenic	18:3 n3	19.8	6.1	3.0	20.2	4.6	5.1	20.1	3.6	1.1

Table 22 - Percentage fatty acid composition of *Chlorella sp* as affected by nitrogen treatments in different culture media

Fatty acids		F2 Media			H Media			K Media		
		Low	**Control**	**High**	**Low**	**Control**	**High**	**Low**	**Control**	**High**
Palmitic acid	**16:00**	**30.34**	**31.92**	**32.76**	**36.72**	**27.70**	**31.76**	**25.17**	**20.76**	**22.89**
Stearic acid	18:00	28.79	4.06	11.06	9.00	3.69	4.87	6.45	3.29	5.02
Oleic acid	**18:1 (9c)**	**12.42**	**10.39**	**7.03**	**4.78**	**16.29**	**5.01**	**22.99**	**45.88**	**24.11**
Oleic acid	18:1 (11c)	8.21	12.99	4.39	19.76	11.55	23.69	1.15	1.80	3.31
Linoleic acid	**18:2 n6**	**25.31**	**25.06**	**20.31**	**8.87**	**26.80**	**9.96**	**24.86**	**22.97**	**20.99**
alpha linolenic	18:3 n3	13.12	6.45	0.89	10.02	10.02	15.18	18.09	7.65	1 6.48

Table 23 - Percentage fatty acid composition of *Chlorella sp* as affected by iron treatments in different culture media

Fatty acids		F2 Media			H Media			K Media		
		Low	**Control**	**High**	**Low**	**Control**	**High**	**Low**	**Control**	**High**
Palmitic acid	**16:00**	**28.59**	**34.21**	**23.63**	**24.53**	**23.63**	**27.95**	**-**	**25.42**	**20.23**
Stearic acid	18:00	8.35	9 54	8.06	3.24	3.98	4.39	-	5.5	4.01
Oleic acid	**18:1 (9c)**	**6.63**	**8.90**	**17.13**	**5.66**	**2.85**	**11.75**	**-**	**40.21**	**9.98**
Oleic acid	18:1 (11c)	6.54	6.88	4.90	14.46	15.63	5.83	-	7.35	5.13
Linoleic acid	**18:2 n6**	**11.13**	**13.14**	**13.44**	**16.22**	**18.06**	**24.74**	**-**	**13.56**	**24.12**
alpha linolenic	18:3 n3	13.09	13.38	12.65	18.49	15.95	7.72	-	8.13	13.50

Table 24 - Percentage fatty acid composition of *Chlorella sp* as affected by temperature treatments in different culture media

Fatty acid		F2 Media			H Media			K Media		
		Low	**Control**	**High**	**Low**	**Control**	**High**	**Low**	**Control**	**High**
Palmitic acid	**16:00**	**13.14**	**33.16**	**35.13**	**22.61**	**33.65**	**33.82**	**21.32**	**30.81**	**20.63**
Stearic acid	18:00	2.67	7.70	8.42	2.41	3.39	2.73	3.14	5.99	6.06
Oleic acid	**18:1 (9c)**	**56.46**	**7.14**	**4.62**	**6.42**	**7.26**	**8.93**	**5.43**	**27.03**	**13.31**
Oleic acid	18:1 (11c)	2.47	5.64	3.11	29.53	3.31	2.32	12.23	4.64	5.54
Linoleic acid	**18:2 n6**	**8.09**	**14.06**	**26.16**	**5.20**	**28.68**	**30.55**	**5.32**	**22.67**	**11.24**
alpha linolenic	18:3 n3	9.68	11.42	3.49	19.15	1.78	1.51	17.15	5.92	10.52

Table 25 -Percentage fatty acid composition of *Pleurochrysis carterae* as affected by nutrient and temperature treatments in different culture media

		F2 Media								
		Nitrogen response			Iron response			Temperature response		
Fatty acid		Low	Control	High	Low	Control	High	Low	Control	High
Palmitic acid	**16:00**	**31.62**	**28.14**	**33.56**	**32.10**	**36.49**	**31.05**	**32.55**	**36.76**	**34.81**
Stearic acid	18:00	7.95	3.41	5.58	2.28	4.00	9.17	2.43	2.54	2.93
Oleic acid	**18:1 (9c)**	**8.76**	**21.03**	**5.53**	**9.61**	**2.99**	**10.50**	**3.02**	**3.50**	**3.84**
Oleic acid	18:1 (11c)	2.27	1.84	3.02	2.00	1.83	3.43	3.17	2.66	2.59
Linoleic acid	**18:2 n6**	**27.56**	**17.89**	**26.32**	**34.13**	**31.00**	**27.15**	**11.80**	**30.15**	**33.68**
alpha linolenic	18:3 n3	2.13	22.15	6.59	2.77	6.72	2.41	27.29	5.42	3.07

APPENDIX B– Mean absorbance from three replicates for all microalgae

Tables and Figures B1 to B3 – *Nannochloropsis salina* response to Nitrogen in F/2 media.

Table B1

CCMP 537 + F2	
Low N	
Days	Mean Abs
2	0.006
4	0.016
6	0.036
8	0.078
10	0.192
12	0.344
14	0.763
16	1.195
18	1.183
20	1.175
22	1.161
24	0.871

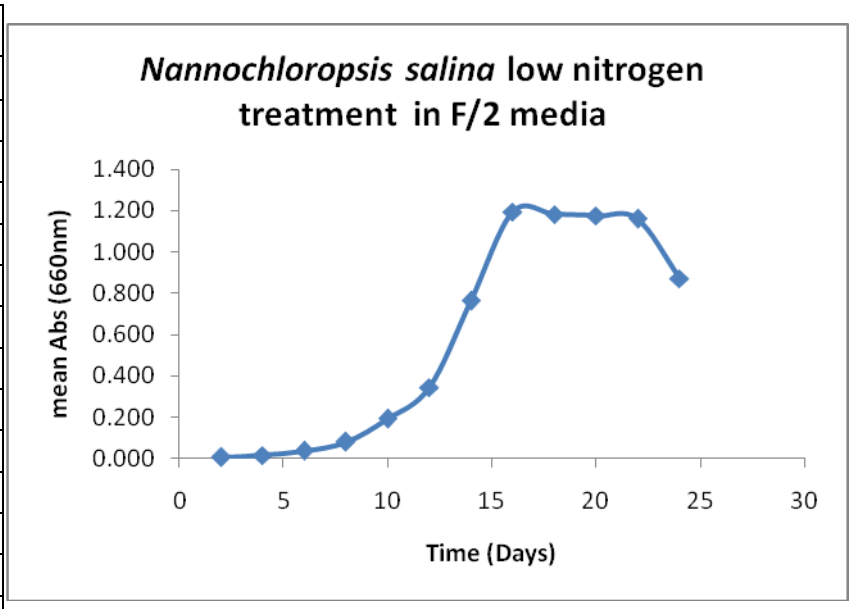

Figure B1

Table B2

CCMP 537 +F2	
CONTROL	
Days	Mean Abs
2	0.005
4	0.015
6	0.026
8	0.060
10	0.143
12	0.280
14	0.520
16	0.631
18	0.858
20	0.861
22	0.861

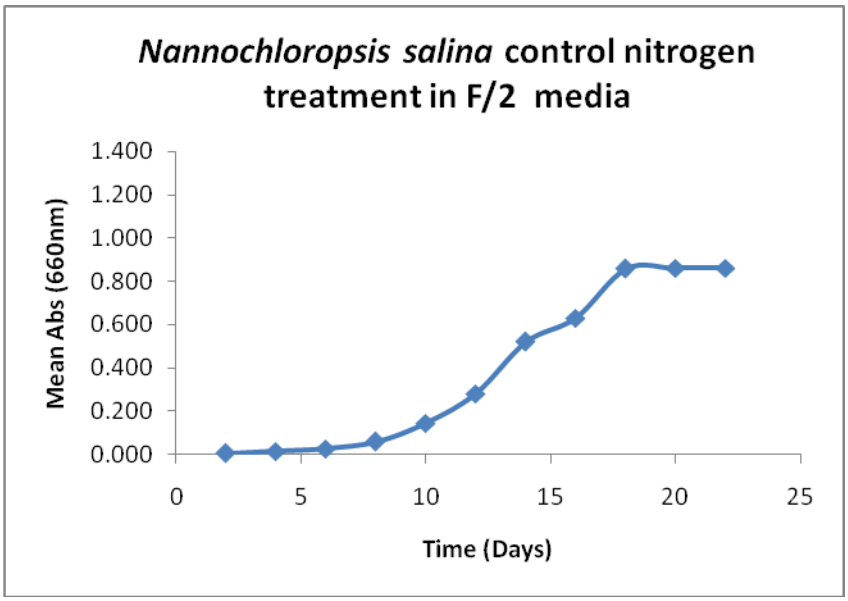

Figure B2

Table B3

CCMP 537 + F2	
High N	
days	mean Abs
2	0.004
4	0.012
6	0.032
8	0.045
10	0.081
12	0.144
14	0.229
16	0.373
18	0.801
20	1.127
22	1.117
24	1.109

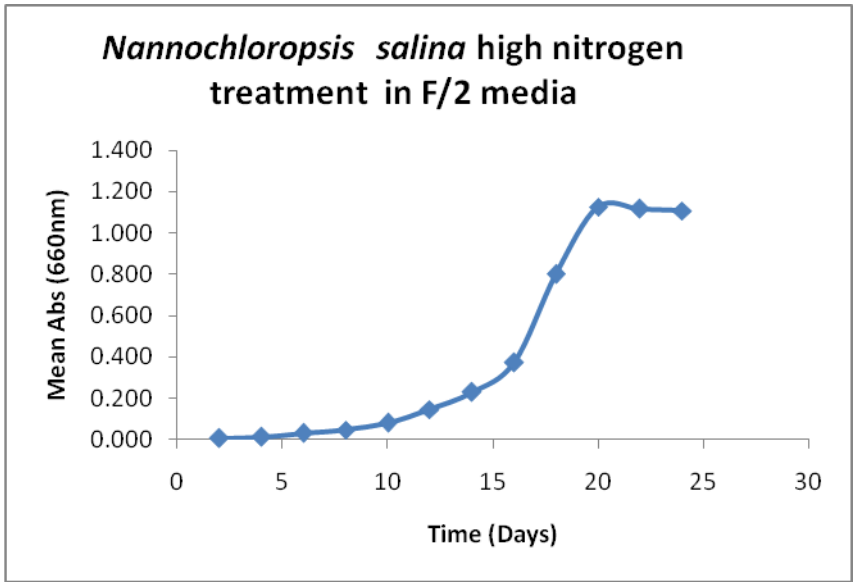

Figure B3.

Tables and Figures B4 to B6 – *Nannochloropsis salina* response to Nitrogen in K media.

Table B4

CCMP 537 + K	
Low N	
days	mean Abs
2	0.001
4	0.003
6	0.014
8	0.006
10	0.007
12	0.008
14	0.017
16	0.076
18	0.209
20	0.388
22	0.697
24	0.747

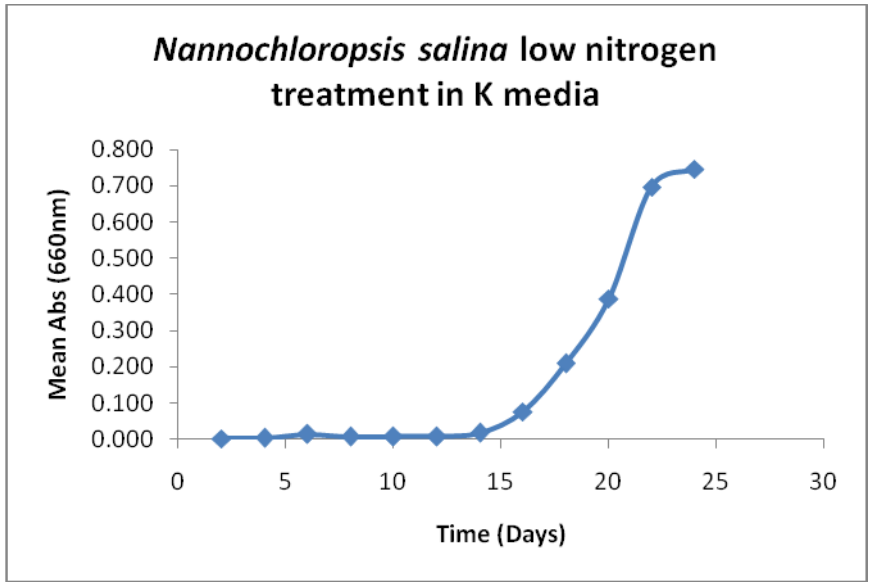

Figure B4.

Table B5

CCMP 537 + K CONTROL	
Days	mean Abs
2	0.002
4	0.004
6	0.008
8	0.010
10	0.097
12	0.177
14	0.269
16	0.355
18	0.450
20	0.564
22	0.604
24	0.601

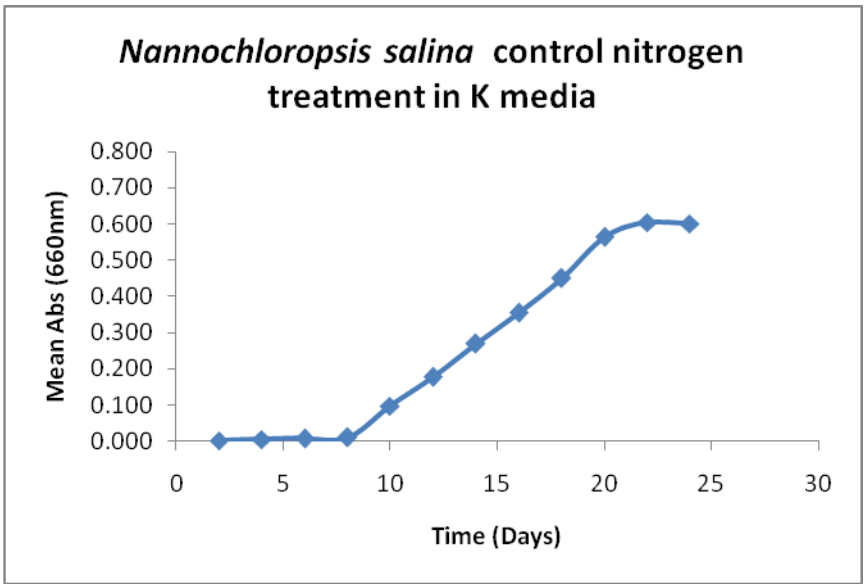

Figure B5

Table B6

CCMP 537 + K (High N)	
Days	Mean Abs
2	0.002
4	0.005
6	0.007
8	0.009
10	0.011
12	0.026
14	0.075
16	0.199
18	0.379
20	0.534
22	0.798
24	0.832

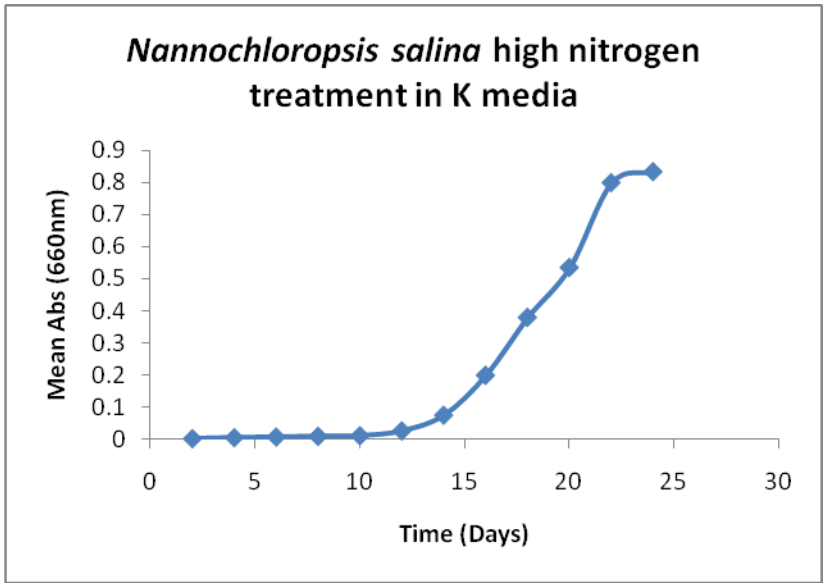

Figure B6

Tables and Figures B7 to B9 – *Nannochloropsis salina* response to Nitrogen in H media.

Table B7

CCMP 537 + H (Low N)	
Days	Mean Abs
2	0.005
4	0.010
6	0.027
8	0.038
10	0.049
12	0.053
14	0.070
16	0.094
18	0.120
20	0.165
22	0.236
24	0.2297

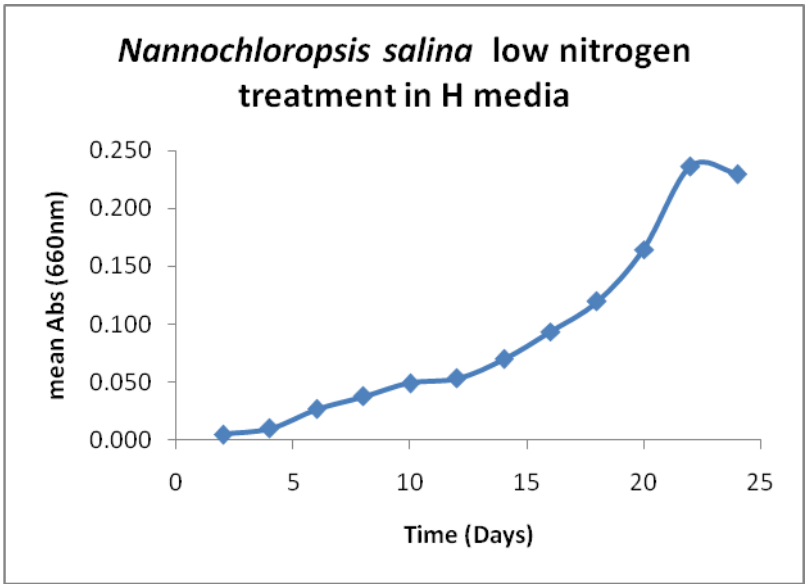

Figure B7

Table B8

CCMP 537 + H Control	
Days	Mean Abs
2	0.001
4	0.003
6	0.006
8	0.012
10	0.019
12	0.026
14	0.037
16	0.044
18	0.067
20	0.096
22	0.123
24	0.203

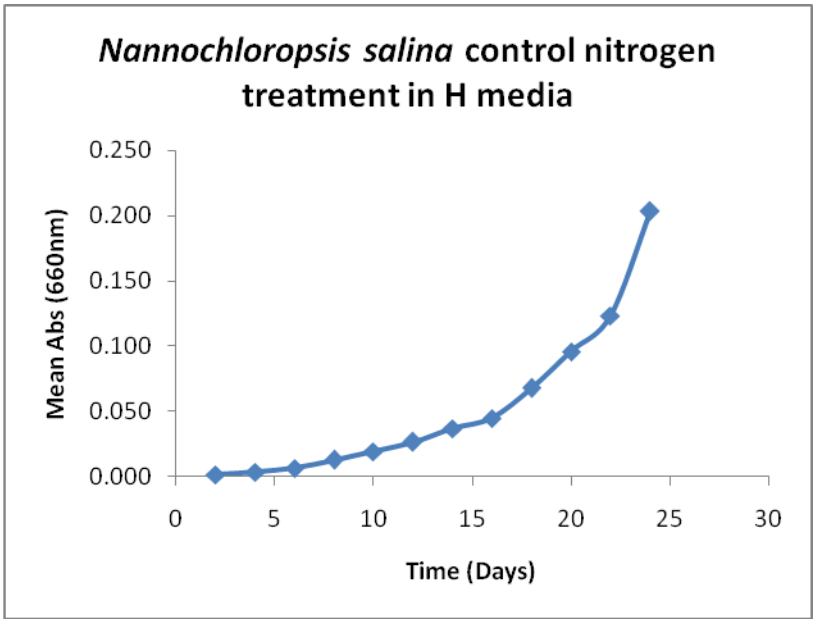

Figure B8

Table B9

CCMP 537 + H	
High N	
days	mean Abs
2	0.003
4	0.006
6	0.012
8	0.015
10	0.022
12	0.049
14	0.052
16	0.063
18	0.094
20	0.131
22	0.217
24	0.215

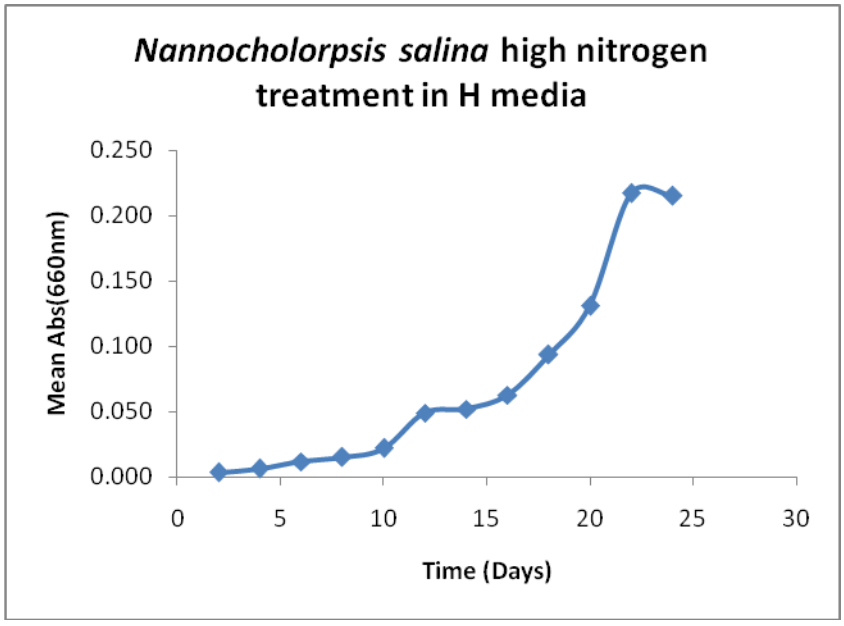

Figure B9

Tables and Figures B10 to B12 – *Chlorella.sp* response to Nitrogen in F/2 media.

Table B10

CCMP 2333+F2	
Low N	
days	Mean Abs
2	0.003
4	0.021
6	0.039
8	0.073
10	0.122
12	0.296
14	0.504
16	0.772
18	0.785
20	0.785

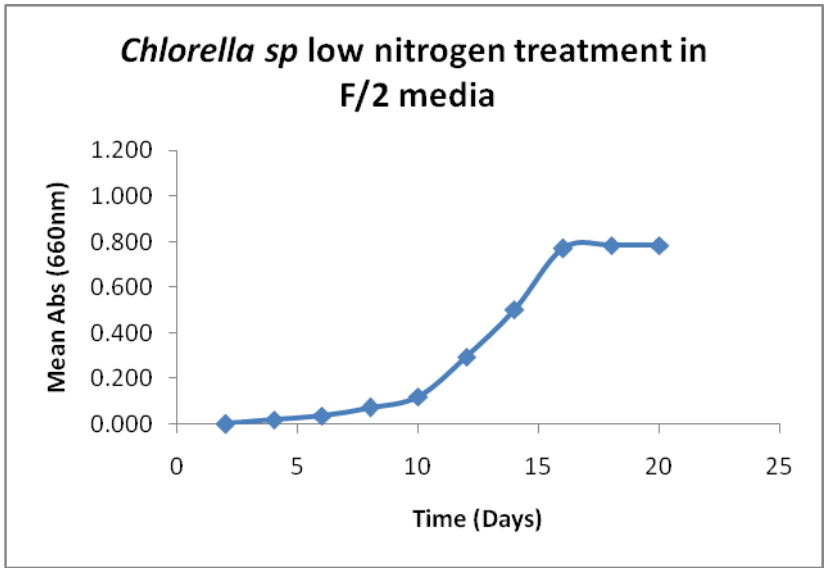

Figure B10

Table B11

CCMP 2333+F2 CONTROL	
days	MEAN
2	0.003
4	0.022
6	0.033
8	0.047
10	0.072
12	0.178
14	0.431
16	0.823
18	0.991
20	0.972

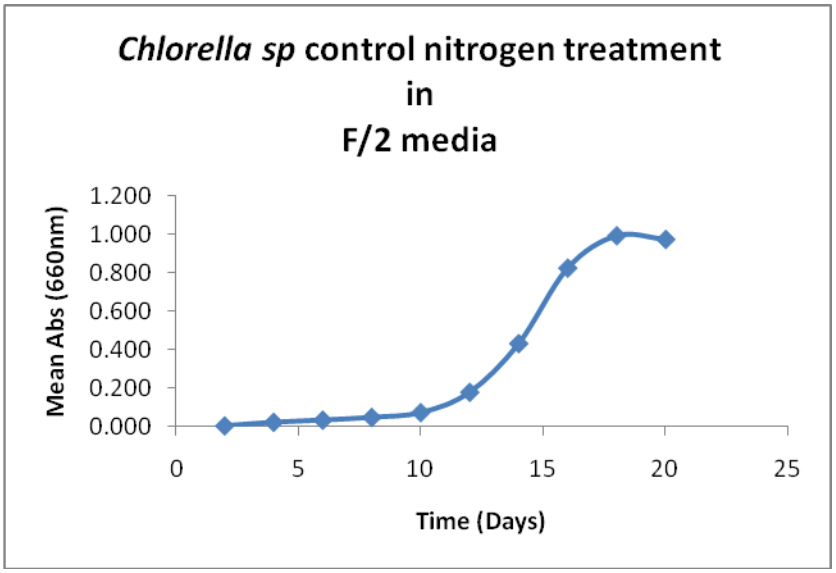

Figure B11

Table B12

CCMP 2333+F2 High N	
days	Mean Abs
2	0.002
4	0.014
6	0.028
8	0.068
10	0.090
12	0.253
14	0.602
16	0.897
18	0.947
20	0.940

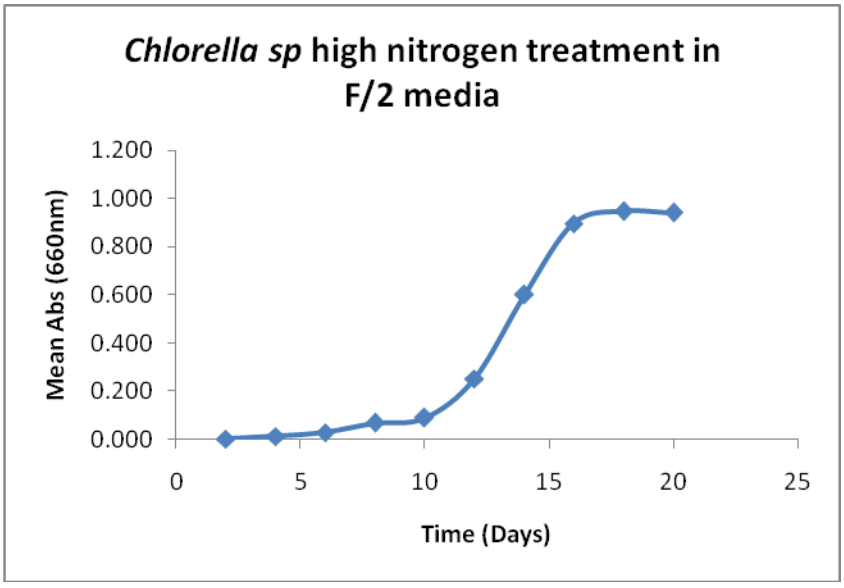

Figure B12

Tables and Figures B13 to B15 – *Chlorella sp* response to Nitrogen in K media.

Table B13

CCMP 2333 + K Low N	
Days	Mean Abs
2	0.003
4	0.017
6	0.023
8	0.027
10	0.031
12	0.025
14	0.051
16	0.186
18	0.550
20	1.141

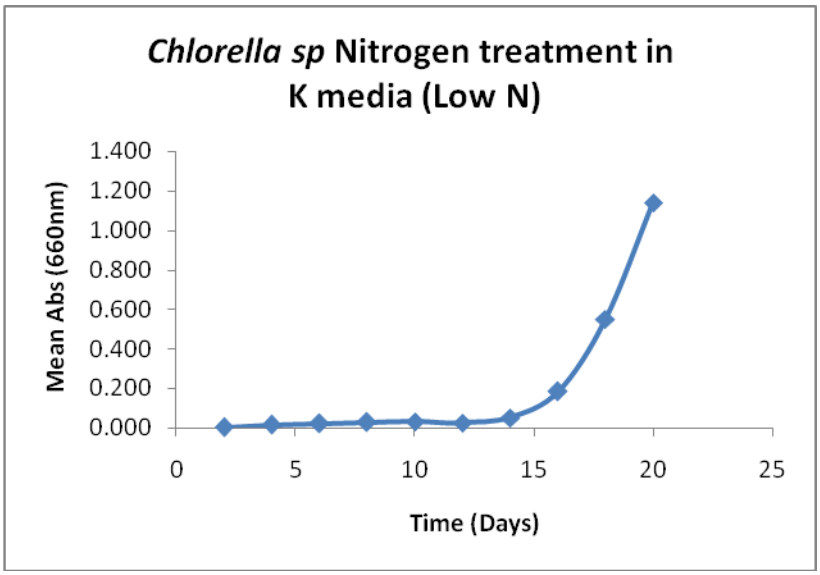

Figure B13

Table B14

CCMP 2333 + K CONTROL	
Days	Mean Abs
2	0.003
4	0.005
6	0.028
8	0.136
10	0.281
12	0.476
14	0.804
16	1.208
18	1.279
20	1.273

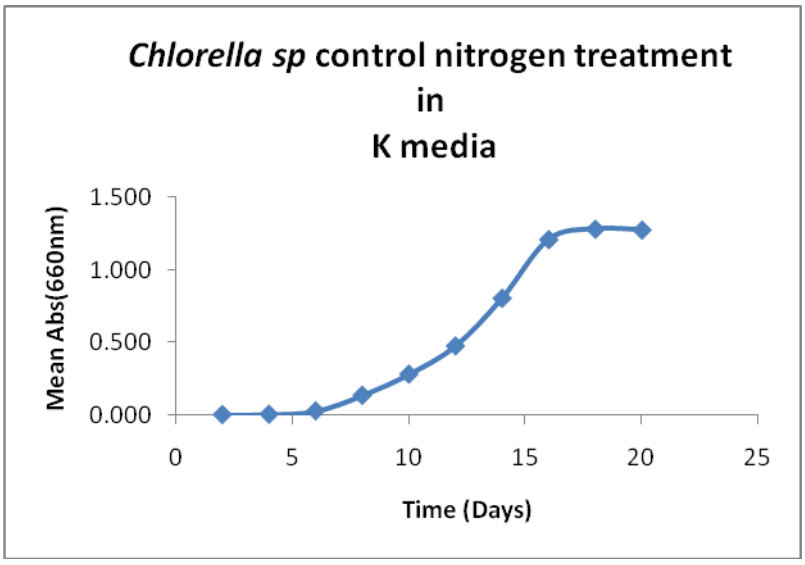

Figure B14

Table B15

CCMP 2333 + K	
High N	
days	Mean Abs
2	0.002
4	0.015
6	0.025
8	0.125
10	0.267
12	0.438
14	0.660
16	0.844
18	1.086
20	1.080

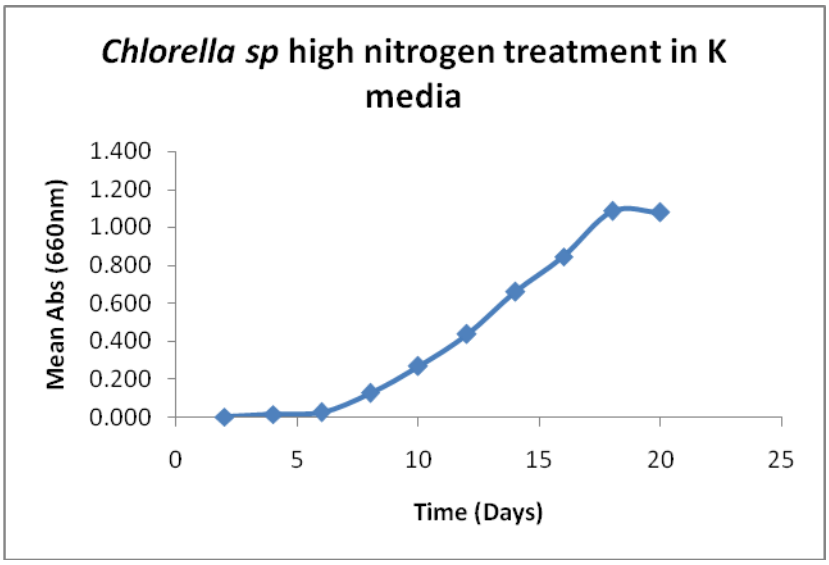

Figure B15

Tables and Figures B16 to B18 – *Chlorella sp* response to Nitrogen in H media.

Table B16

CCMP 2333 +H	
Low N	
Days	Mean Abs
2	0.004
4	0.014
6	0.053
8	0.164
10	0.570
12	0.760
14	0.880
16	0.864
18	0.864

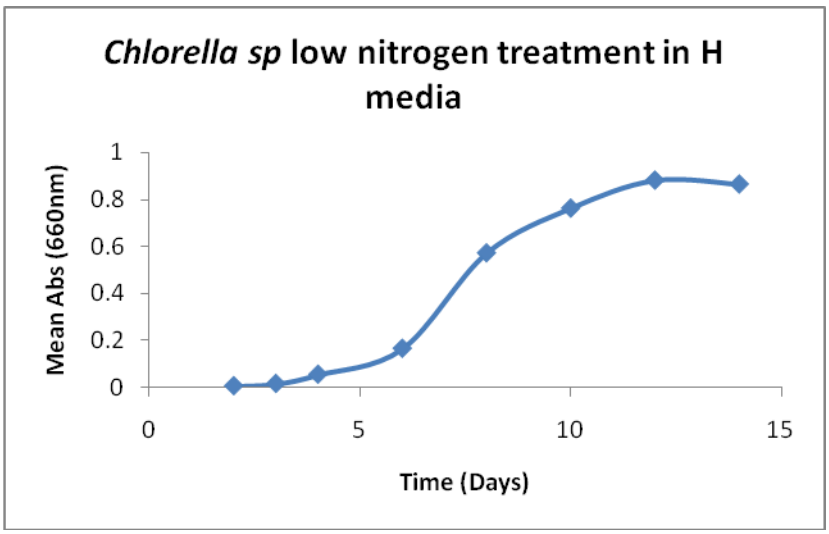

Figure B16

Table B17

CCMP 2333 + H CONTROL	
days	Mean Abs
2	0.002
4	0.010
6	0.015
8	0.024
10	0.030
12	0.068
14	0.125
16	0.245
18	0.350
20	0.368

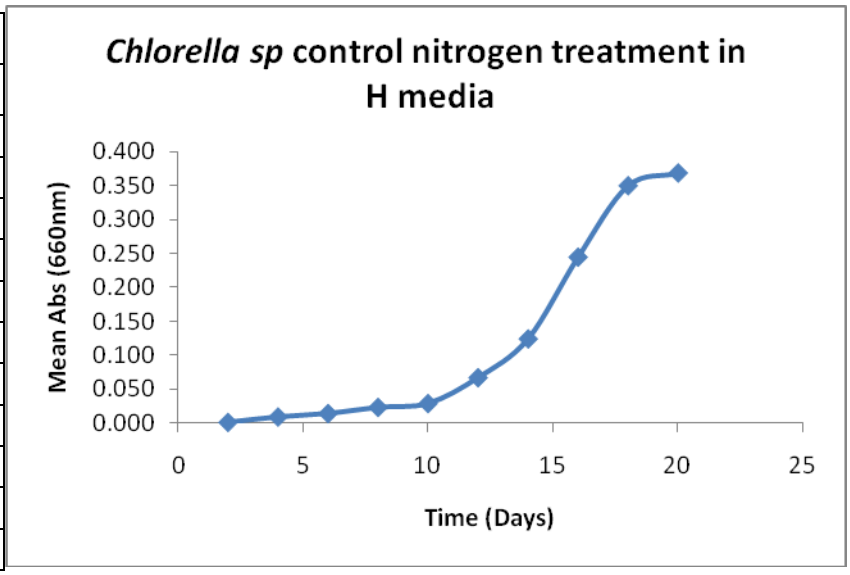

Figure B17

Table B18

CCMP 2333 +H High N	
Days	Mean Abs
2	0.003
4	0.037
6	0.066
8	0.080
10	0.084
12	0.094
14	0.127
16	0.162
18	0.159
20	0.157
22	0.155

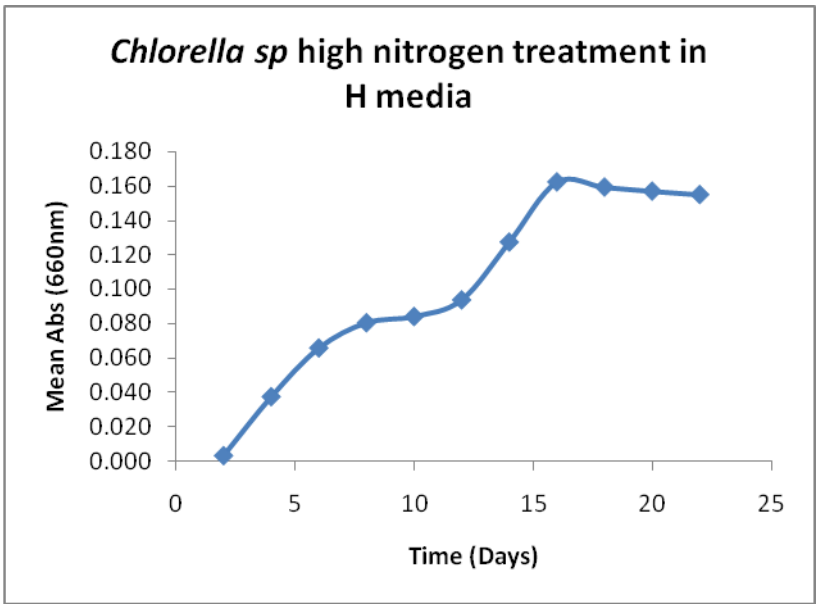

Figure B18

Tables and Figures B19 to B21 – *P.carterae* response to Nitrogen in F/2 media.

Table B19

CCMP 647 +F2 (Low N)	
Days	Mean Abs
2	0.001
4	0.004
6	0.006
8	0.023
10	0.057
12	0.115
14	0.179
16	0.231
18	0.380
20	0.376

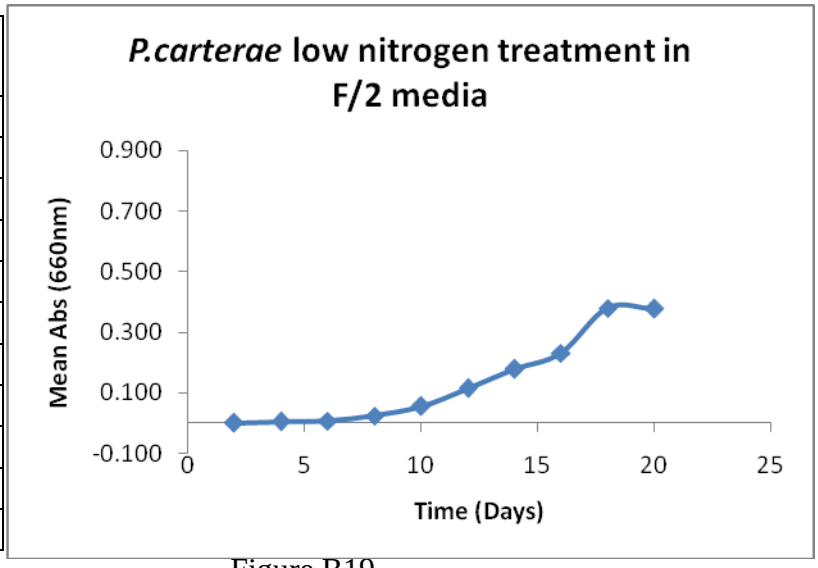

Figure B19

Table B20

CCMP 647 +F2 (Control)	
Days	Mean Abs
2	0.002
4	0.005
6	0.006
8	0.006
10	0.008
12	0.085
14	0.262
16	0.629
18	0.760
20	0.811

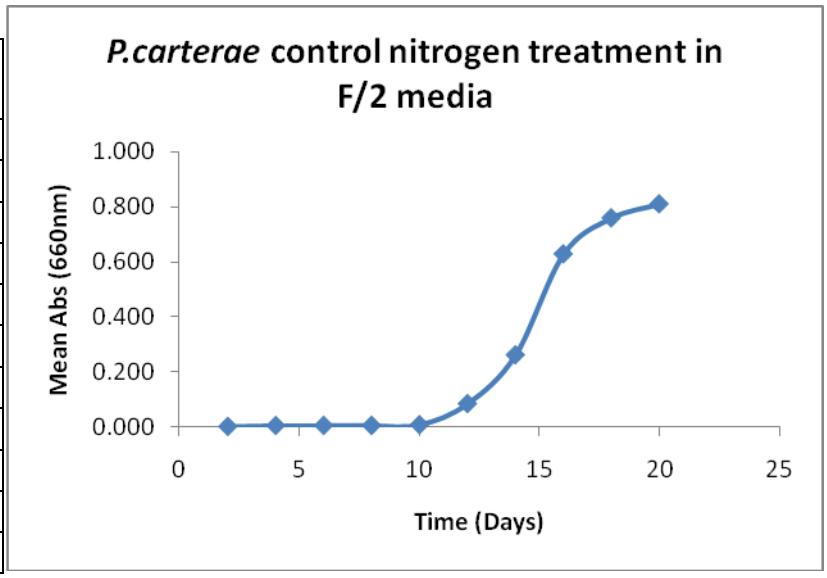

Figure B20

Table B21

CCMP 647 +F2 (High N)	
days	Mean Abs
2	0.001
4	0.005
6	0.018
8	0.051
10	0.082
12	0.118
14	0.229
16	0.335
18	0.496
20	0.718

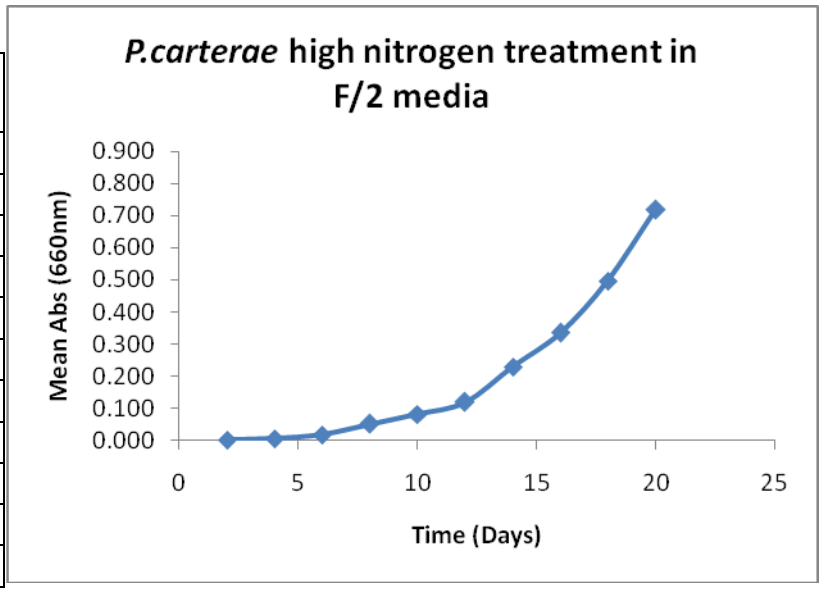

Figure B21

Tables and Figures B22 to B24 – *Nannochloropsis salina* response to iron in F/2, K and H media.

Table B22

Nannochloropsis salina in F2 Medium			
Time (Days)	Mean Absorbance		
	Control	Low	High
2	0.005	0.003	0.010
4	0.011	0.015	0.021
6	0.051	0.044	0.166
8	0.436	0.270	0.369
10	0.748	0.549	0.574
12	0.791	0.694	0.820
14	0.800	0.709	0.923

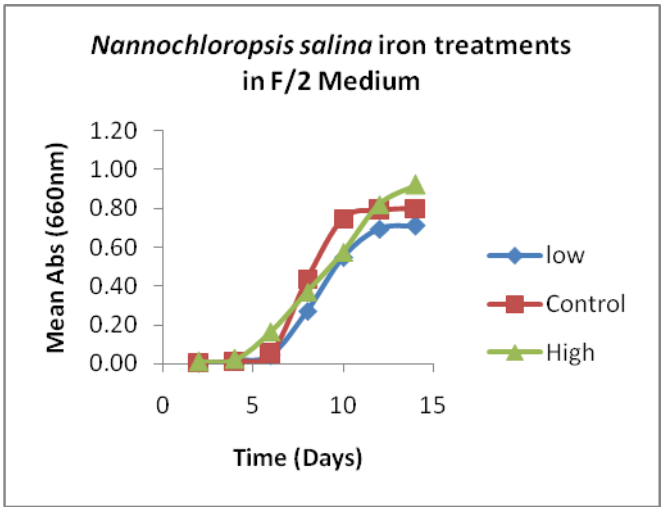

Figure B22

Table B23

***Nannochloropsis salina* in K Medium**			
Time (Days)	Mean Absorbance		
	Control	Low	High
2	0.002	0.001	0.002
4	0.004	0.003	0.004
6	0.008	0.006	0.010
8	0.018	0.013	0.072
10	0.026	0.020	0.287
12	0.033	0.072	0.383

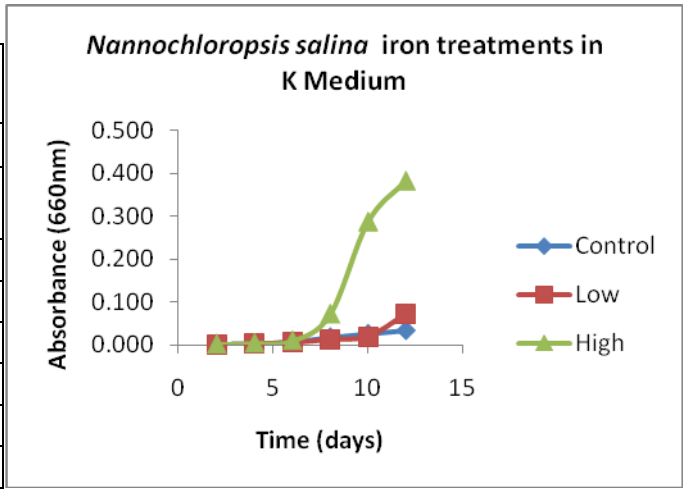

Figure B23

Table B24

***Nannochloropsis salina* in H Medium**			
Time (Days)	Mean Absorbance		
	Control	Low	High
2	0.003	0.002	0.003
4	0.009	0.007	0.031
6	0.016	0.018	0.068
8	0.021	0.104	0.078
10	0.060	0.288	0.099
12	0.189	0.637	0.135
14	0.268	0.765	0.452
16	0.393	0.783	0.519
18	0.401	0.931	0.546

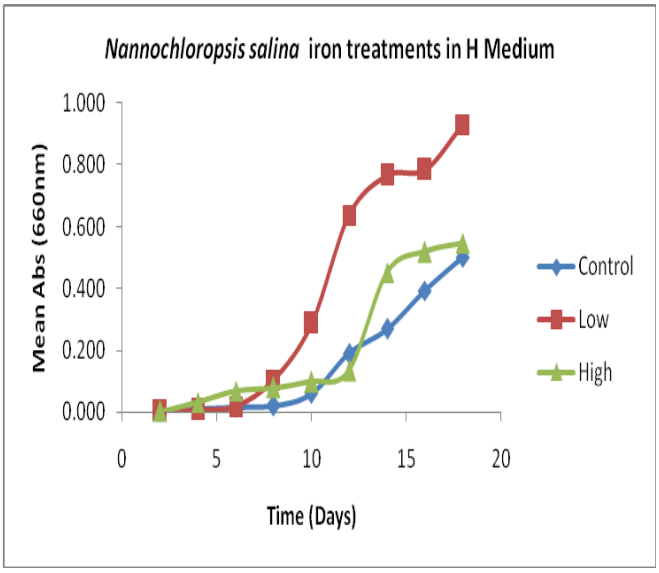

Figure B24

Tables and Figures B25 to B27 – *Chlorella.sp* response to Iron in F/2, K and H media.

Table B25

***Chlorella sp* in F2 Medium**			
Time (Days)	Mean Absorbance		
	Control	Low	High
2	0.002	0.002	0.011
4	0.037	0.031	0.070
6	0.080	0.065	0.129
8	0.160	0.208	0.386
10	0.267	0.322	0.674
12	0.284	0.308	0.834
14	0.300	0.273	0.871

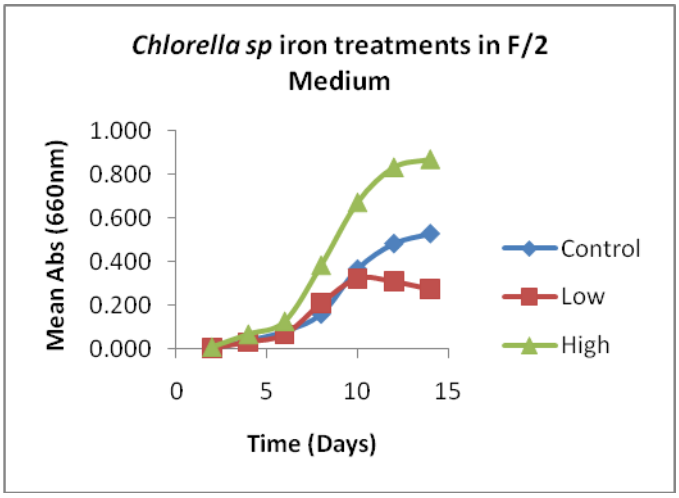

Figure B25

Table B26

***Chlorella sp* in K Medium**			
Time (Days)	Mean Absorbance		
	Control	Low	High
2	0.002	0.002	0.003
4	0.005	0.007	0.013
6	0.012	0.025	0.025
8	0.027	0.046	0.034
10	0.037	0.058	0.054
12	0.042	0.058	0.067
14	0.102	0.059	0.080
16	0.111	0.051	0.084

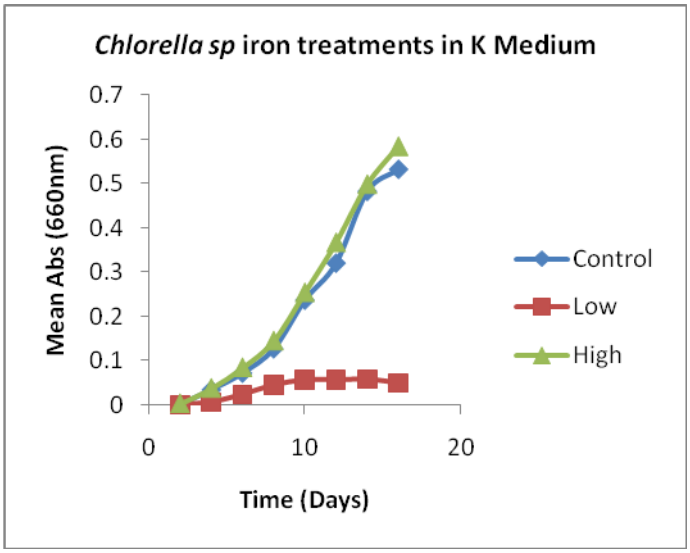

Figure B26

Table B27

Chlorella sp in H Medium			
Time (Days)	Mean Absorbance		
	Control	Low	High
2	0.003	0.003	0.005
4	0.009	0.018	0.039
6	0.032	0.041	0.071
8	0.075	0.062	0.096
10	0.127	0.087	0.118
12	0.241	0.135	0.169
14	0.443	0.161	0.316
16	1.062	0.356	0.594
18	1.443	0.709	1.182

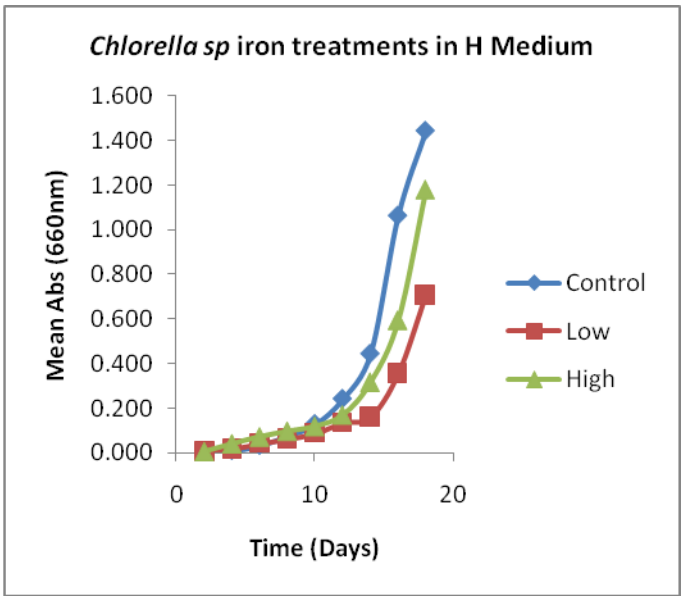

Figure B27

Table and Figure B28 – *P. carterae* response to Iron in F/2 media.

Table B28

P.carterae in F2 Medium			
Time (Days)	Mean Absorbance		
	Control	Low	High
2	0.007	0.002	0.004
4	0.035	0.008	0.015
6	0.082	0.062	0.105
8	0.344	0.182	0.334
10	0.699	0.461	0.564
12	0.901	0.783	0.731
14	0.971	0.866	0.906

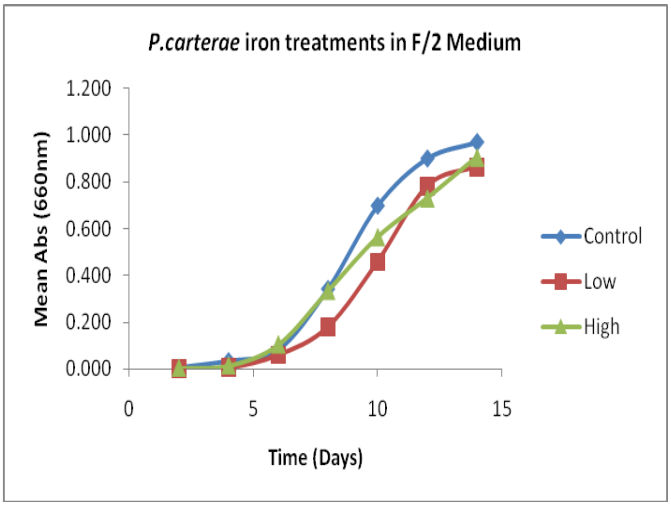

Figure B28

Tables and Figures B29 to B31 – *Nannochloropsis salina* response to temperature (control, low and high = 18, 25 and 32°C respectively)

Table B29

***Nannochloropsis salina* temperature response in F2 Medium**			
	Mean Absorbance		
Time (Days)	Control	Low	High
2	0.006	0.004	0.006
4	0.015	0.007	0.036
6	0.075	0.010	0.077
8	0.241	0.042	0.307
10	0.728	0.097	0.616
12	0.798	0.201	0.780
14	0.798	0.261	0.911
16	0.798	0.254	0.938

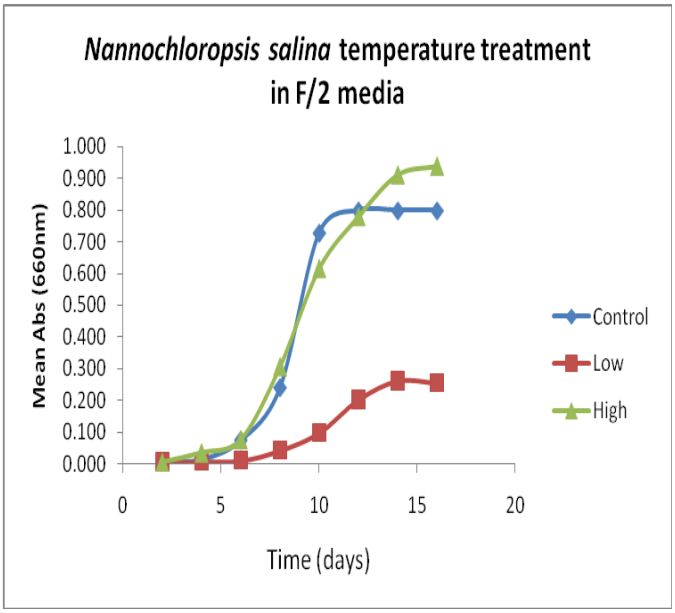

Figure B29

Table B30

***Nannochloropsis salina* temperature response in K Medium**			
	Mean Absorbance		
Time (Days)	Control	Low	High
2	0.003	0.006	0.003
4	0.010	0.018	0.012
6	0.032	0.026	0.099
8	0.059	0.044	0.337
10	0.090	0.084	0.605
12	0.261	0.162	0.903
14	0.259	0.336	0.983
16		0.408	
18		0.389	

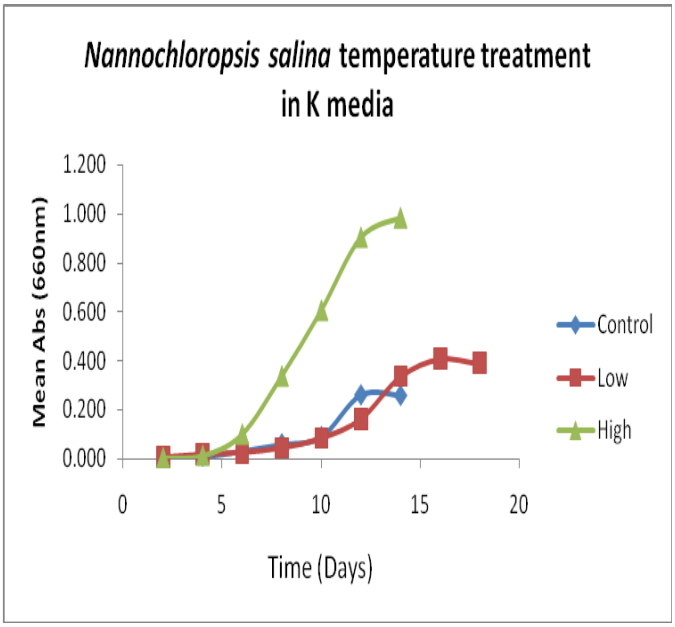

Figure B30

Table B31

***Nanochloropsis salina* temperature response in H Medium**			
	Mean Absorbance		
Time (Days)	Control	Low	High
2	0.003	0.009	0.004
4	0.036	0.021	0.067
6	0.094	0.029	0.118
8	0.239	0.039	0.233
10	0.420	0.075	0.640
12	0.771	0.113	0.755
14	0.733	0.193	0.751
16		0.186	

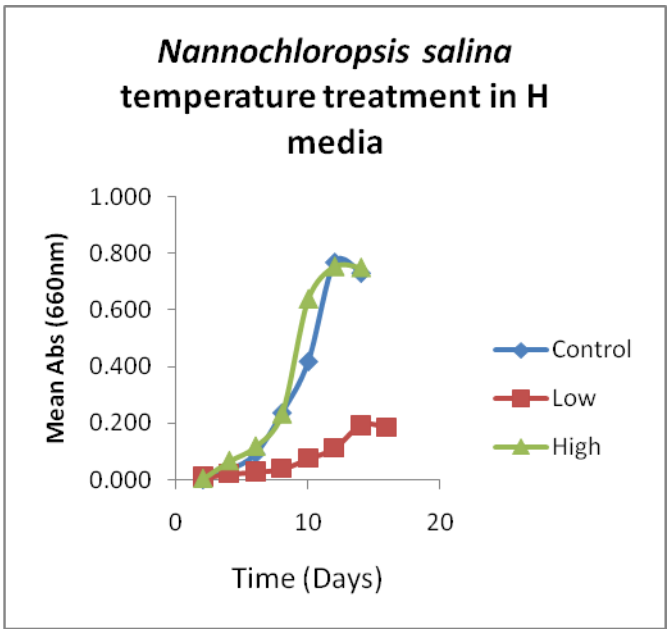

Figure A31

Tables and Figures B32 to B34 – *Chlorella sp* response to temperature (control, low and high = 18, 25 and 32°C respectively)

Table B32

***Chlorella.sp* temperature response in F/2 Medium**			
	Mean Absorbance		
Time (Days)	Control	Low	High
2	0.005	0.009	0.004
4	0.051	0.044	0.040
6	0.119	0.205	0.149
8	0.267	0.444	0.451
10	0.540	0.629	0.693
12	0.848	0.718	0.805
14	0.900	0.736	0.804
16	0.898	0.702	

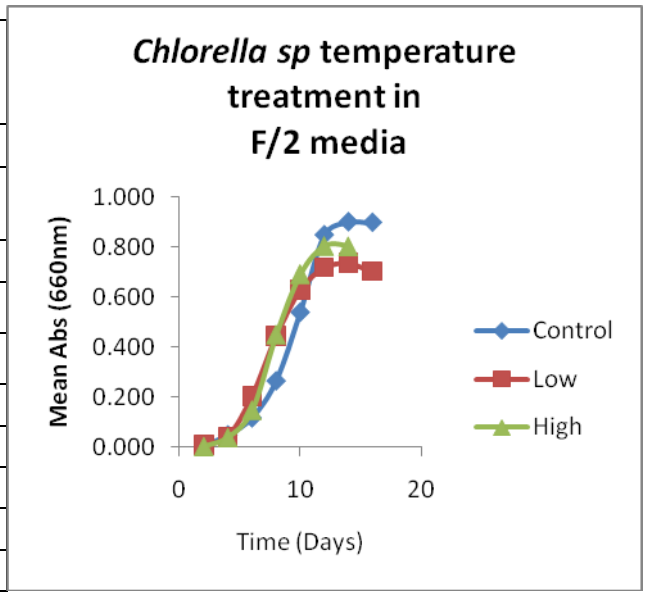

Figure B32

Table B33

***Chlorella sp* temperature response in K Medium**			
	Mean Absorbance		
Time (Days)	Control	Low	High
2	0.004	0.011	0.005
4	0.034	0.030	0.054
6	0.068	0.044	0.127
8	0.134	0.057	0.263
10	0.230	0.065	0.555
12	0.410	0.161	0.546
14	0.435	0.234	0.543
16		0.229	

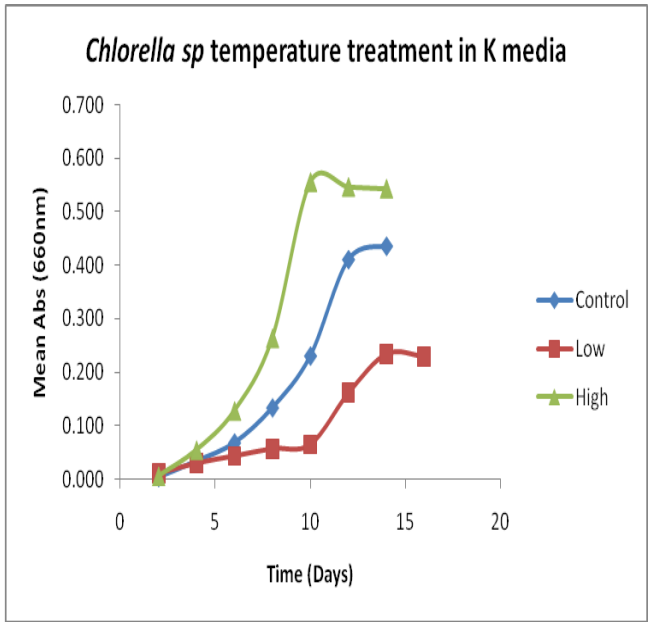

Figure B33

Table B34

***Chlorella sp* temperature response in H Medium**			
	Mean Absorbance		
Time (Days)	Control	Low	High
2	0.004	0.010	0.003
4	0.090	0.031	0.078
6	0.185	0.047	0.230
8	0.290	0.064	0.468
10	0.541	0.115	0.731
12	0.949	0.163	0.839
14	0.923	0.441	0.835
16		0.595	
18		0.584	

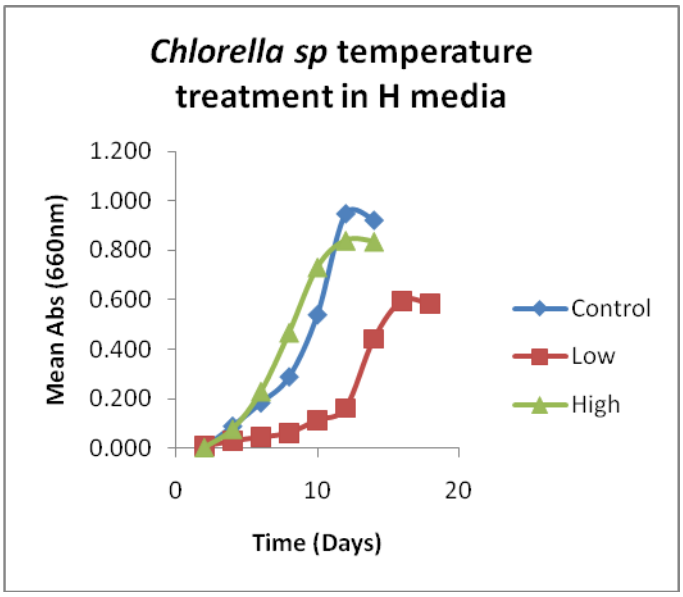

Figure B34

Table and Figure B35 – *P.carterae* response to temperature.

Table B35

***P.carterae* temperature response in F/2 Medium**			
	Mean Absorbance		
Time (Days)	Control	Low	High
2	0.004	0.001	0.002
4	0.012	0.003	0.007
6	0.067	0.014	0.030
8	0.360	0.034	0.217
10	0.623	0.099	0.673
12	0.756	0.317	0.830
14	0.754	0.666	0.825
16		0.697	
18		0.666	

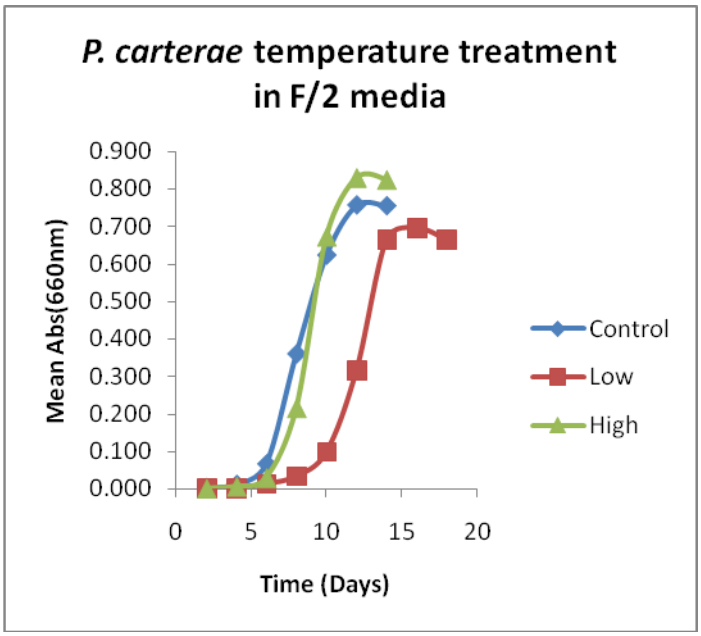

Figure B35

APPENDIX C – Mean statistics and Cell count Plots

Table C1 - Nitrogen treatment.

		Mean dry wt(mg/L)		
Main factors	**Level**	***N.salina***	***Chlorella sp***	***P.carterea***
Nitrogen Concentration	2.8	0.12±0.00	0.17±0.01	0.11±0.01
	5.6	0.13±0.00	0.15±0.01	0.19±0.01
	11.2	0.14±0.01	0.17±0.01	0.11±0.01
	37.5	0.29±0.01	0.31±0.00	
	75	0.23±0.01	0.26±0.00	
	150	0.24±0.01	0.23±0.01	
Media	F/2	0.21±0.01	0.19±0.01	0.13±0.01
	K	0.17±0.01	0.16±0.01	
	H	0.12±0.01	0.19±0.01	
Nitrogen Conc.* media		***N.salina***	***Chlorella sp***	***P.carterea***
H	2.8	0.11±0.01	0.17±0.01	
	5.6	0.13±0.00	0.15±0.00	
	11.2	0.14±0.00	0.15±0.00	
F2	37.5	0.25±0.01	0.18±0.01	0.11±0.01
	75	0.20±0.01	0.14±0.01	0.19±0.01
	150	0.14±0.00	0.13±0.01	0.11±0.00
K	37.5	0.20±0.00	0.23±0.01	
	75	0.12±0.00	0.18±0.00	
	150	0.18±0.00	0.15±0.00	

Table C2 - Iron treatment.

		Mean dry wt(mg/L)		
Main factors	**Level**	***N.salina***	***Chlorella sp***	***P.*carterea**
Iron Concentration	0.31	0.13±0.00	0.14±0.00	
	0.62	0.12±0.00	0.68±0.02	
	1.24	0.11±0.00	0.34±0.02	
	1.58	0.15±0.01	0.01±0.01	0.50±0.00
	3.15	0.12±0.00	0.20±0.01	0.58±0.00
	6.3	0.22±0.00	0.20±0.01	0.66±0.01
Media	F/2	0.20±0.00	0.20±0.01	0.57±0.01
	K	0.11±0.00	0.38±0.01	
	H	0.12±0.01	0.11±0.00	
Iron Conc.* media		***N.salina***	***Chlorella sp***	***P.carterea***
H	0.31	0.13±0.00	0.14±0.00	
	0.62	0.11±0.00	0.68±0.02	
	1.24	0.11±0.00	0.34±0.02	
F2	1.58	0.27±0.00	0.13±0.00	0.50±0.00
	3.15	0.12±0.00	0.13±0.00	0.58±0.00
	6.3	0.22±0.00	0.36±0.01	0.66±0.01
K	1.58	0.02±0.00	0.02±0.00	
	3.15	0.12±0.00	0.27±0.01	
	6.3	0.21±0.01	0.05±0.00	

Table C3 – Temperature treatment.

		Mean dry wt(mg/L)		
Main factors	**Level**	***N.salina***	***Chlorella sp***	***P.carterea***
	18°C	0.26±0.01	0.29±0.01	0.23±0.01
	25°C	0.26±0.01	0.30±0.02	0.45±0.00
	32°C	0.30±0.01	0.24±0.01	0.52±0.00
Media	F/2	0.41±0.00	0.25±0.00	0.40±0.01
	K	0.26±0.00	0.42±0.01	
	H	0.15±0.00	0.15±0.00	
Temperature *media		***N.salina***	***Chlorella sp***	***P.carterea***
H	18°C	0.19±0.00	0.19±0.01	
	25°C	0.13±0.00	0.13±0.00	
	32°C	0.15±0.00	0.15±0.00	
F2	18°C	0.27±0.00	0.25±0.01	0.23±0.01
	25°C	0.51±0.00	0.21±0.00	0.45±0.00
	32°C	0.44±0.00	0.27±0.00	0.52±0.00
K	18°C	0.33±0.00	0.43±0.01	
	25°C	0.15±0.01	0.55±0.01	
	32°C	0.31±0.00	0.29±0.00	

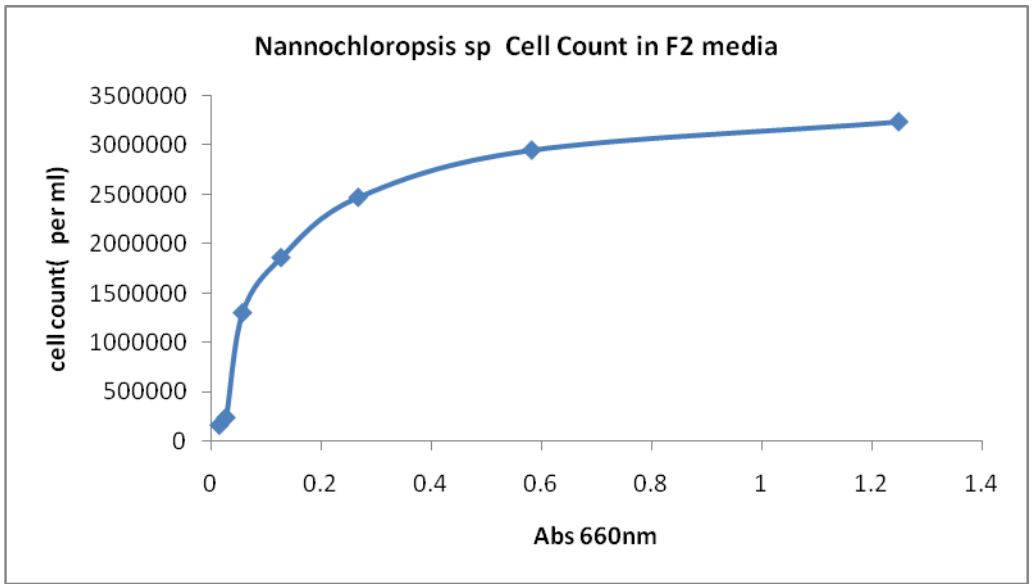

Figure 12 - Cell count of *N.salina* nitrogen treatment cultured in F2 media

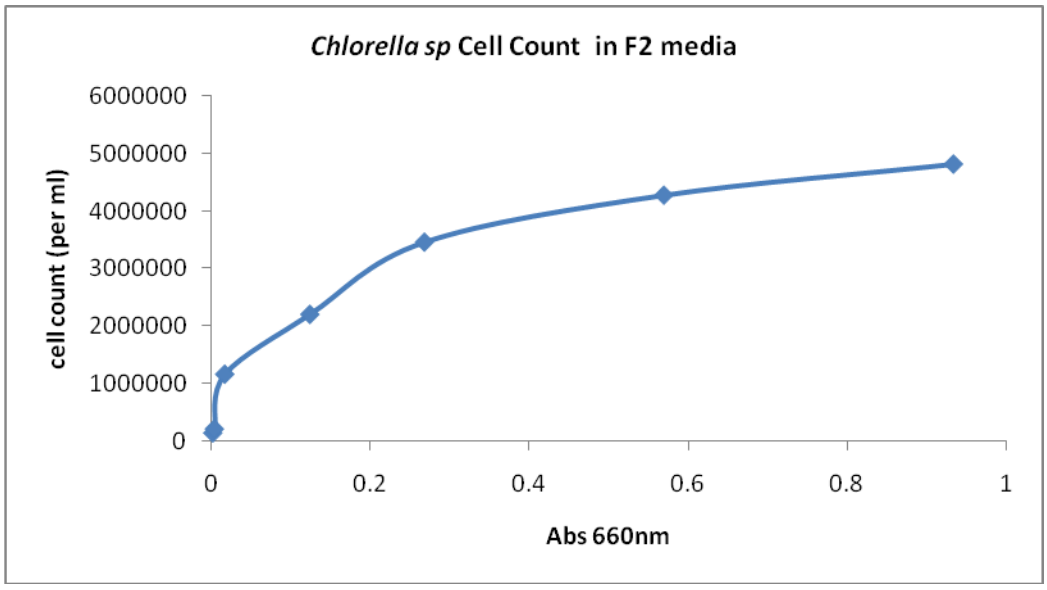

Figure 13 - Cell count of *Chlorella sp* nitrogen treatment in F2 media

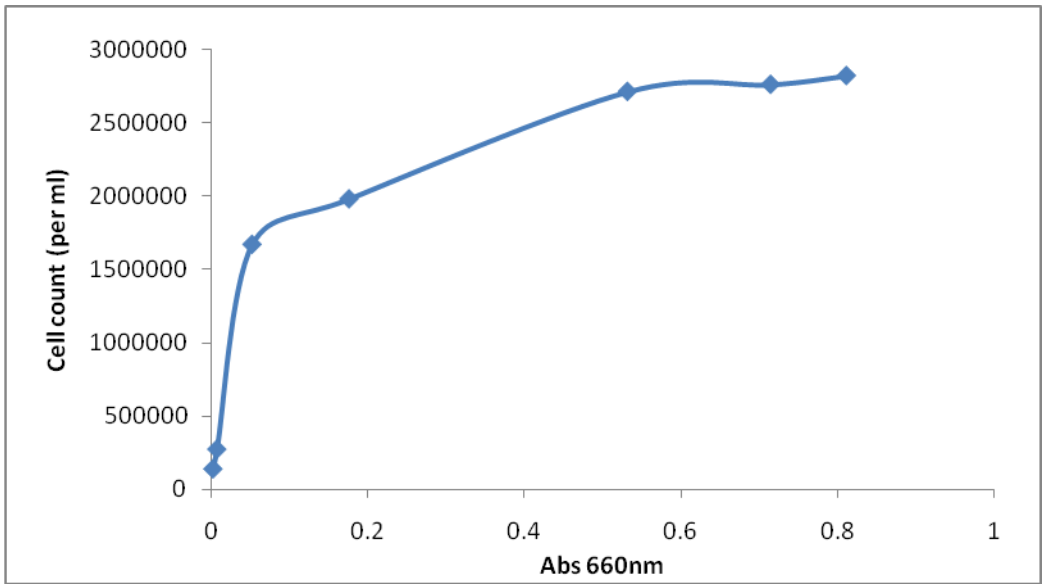

Figure 14 - Cell count of *P.carterae* nitrogen treatment in F2 media

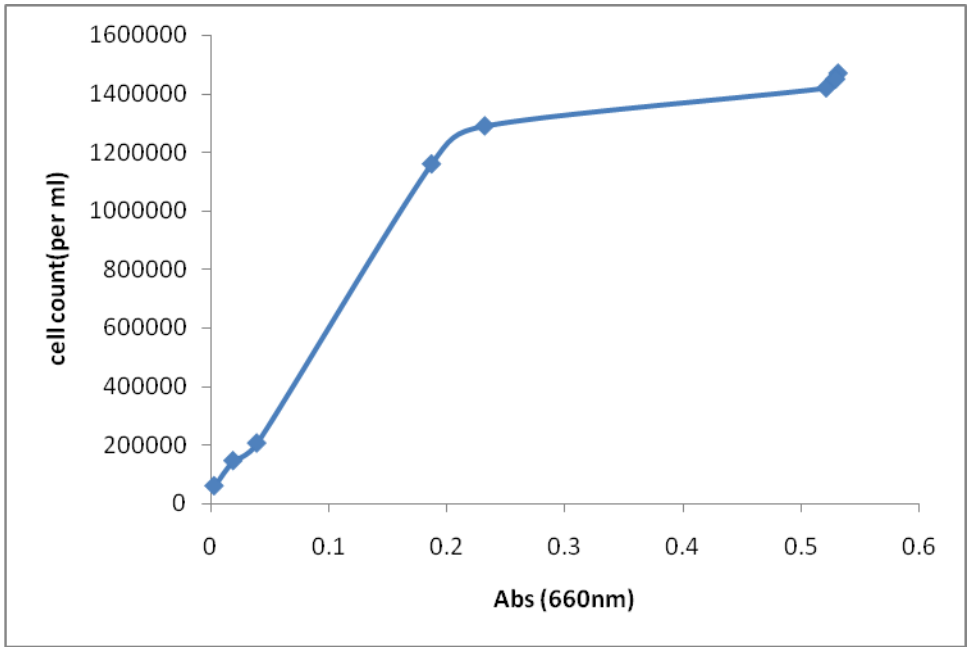

Figure 15 - Cell count of *Nannochloropsis salina* iron treatment in F2 media

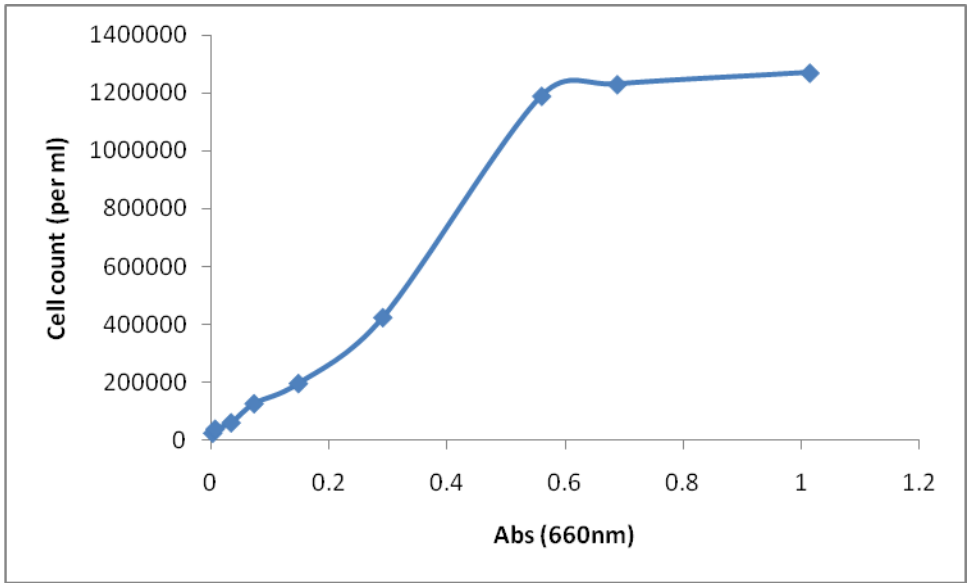

Figure 16 - Cell count of *Chlorella sp* iron treatment in K media

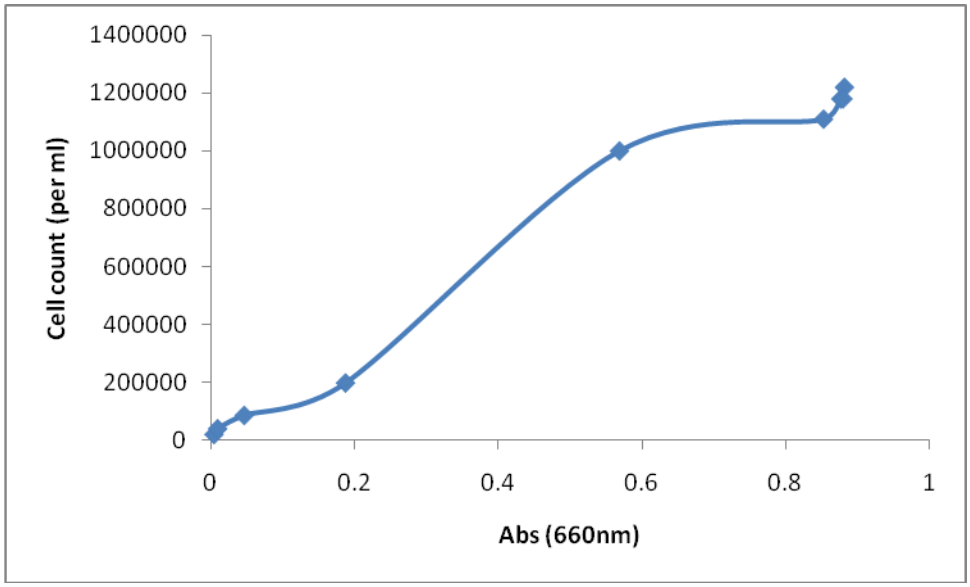

Figure 17 - Cell count of *P.carterae* iron treatment in F2 media

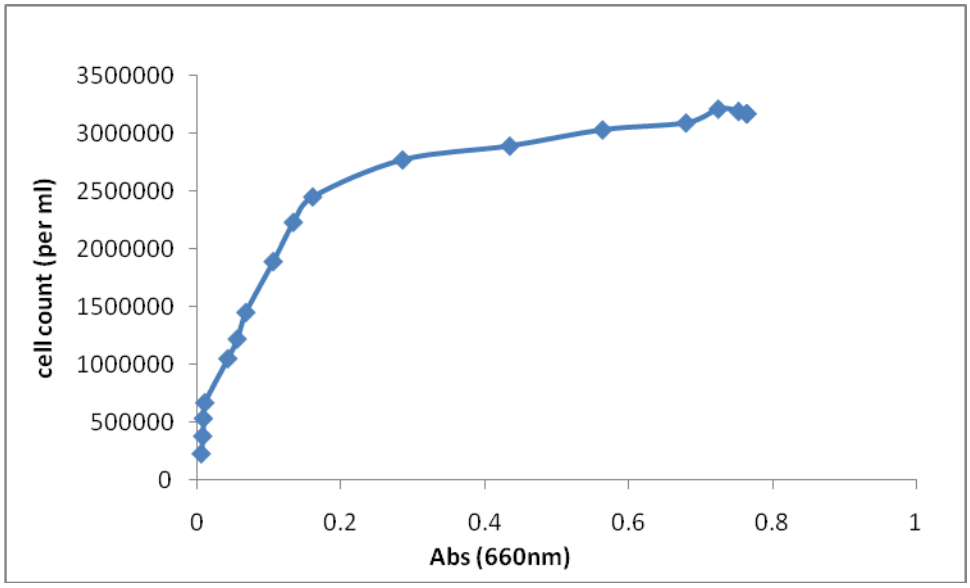

Figure 18 - Cell count of *N.salina* temperature treatment in F2 media

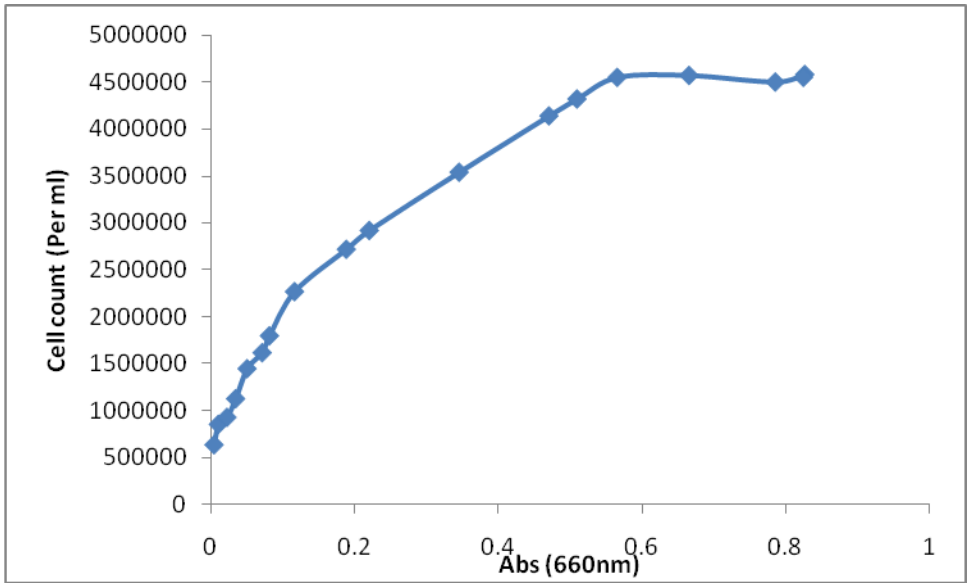

Figure 19 - Cell count of *Chlorella sp* temperature treatment in F2 media

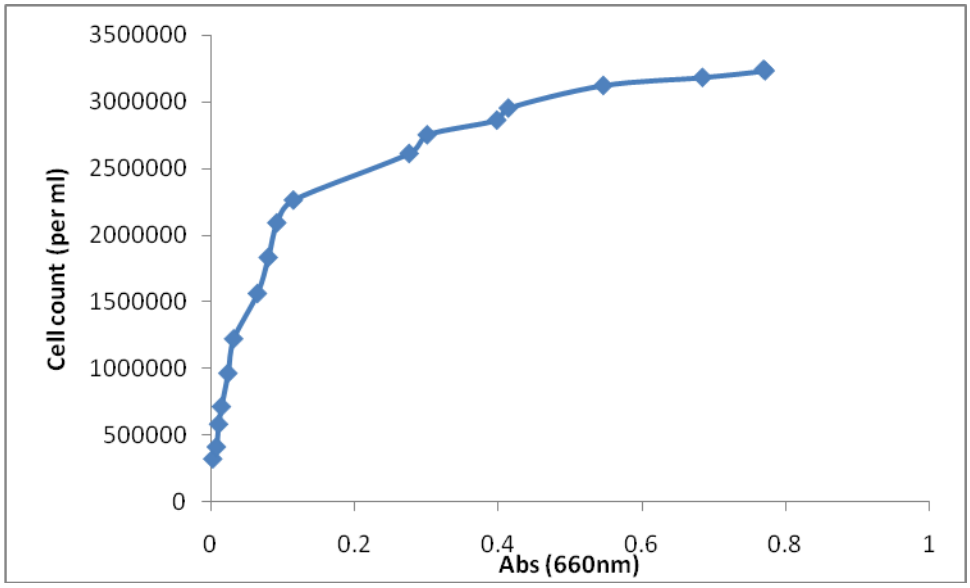

Figure 20 - Cell count of *P.carterae* temperature treatment in F2 media

VITA

TIAMIYU GANIYAT ADETOLA

Candidate for the Degree of

Master of Science

Thesis: EFFECT OF NITROGEN, IRON AND TEMPERATURE ON YIELD AND COMPOSITION OF MICROALGAE

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Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: EFFECT OF NITROGEN, IRON AND TEMPERATURE ON YIELD AND COMPOSITION OF MICROALGAE

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Major Field: Plant and Soil Sciences

Scope and Method of Study: The scope of the study was to assess the effect of nutrients (nitrogen and iron) and temperature on the yield and fatty acid composition of three microalgae as well as develop a growth rate function in response to nitrogen, iron and temperature. Three micraoalgae *Nannochloropsis salina*, *Chlorella sp*., and *Pleurochysis carterae* were cultured in different culture media F/2, K and Hoagland while inducing different level of treatment (low and high) from the required nutrient composition. The study was conducted in the laboratory at the agronomy research station at Oklahoma State University, photoperiod of 16 hours light and 8 hours light was used. Absorbance using the photospectometer, cell count with both microscope and automated cell counter, specific growth rate was calculated, lipid extraction was carried out using the modified Bligh and Dyer method and fatty acid were analyzed with the Gas chromatography. Factorial treatment combination using the SAS program was used to determine the effect of different culture media at different levels of nutrients and temperature treatment on cell growth and fatty acid composition.

Findings and Conclusions**:** Results showed that *Nannochloropsis sp.* and *Chlorella sp.* had highest growth rate when cultured in low nitrogen concentration (37.5g/L) in the F2 media, while *Pleurochysis carterae* did not respond well to nitrogen stress. *Nannochloropsis salina. Chlorella sp., Pleurochysis carterae* highest growth rate were at high iron treatment (6.2g/L) in K, H and F2 media respectively. *Nannochloropsis salina,*and *Pleurochysis carterae* response to temperature had its highest growth rate at high temperature 32 ºC in K and F2 media respectively while *Chlorella sp* at 25 ºC (control). The results show that fatty acid composition of microalgae required for biofuel production is best when cultured at control or recommended nutrients composition, and temperature of 25 ºC. Modifying nutrient concentration and temperature would increase yield of microalgae but the overall fatty acid composition might not be ideal for biofuel production.

ADVISER'S APPROVAL: Dr. Vijaya Gopal Kakani