OPTIMIZED PRODUCTION of OMEGA-3 AND OMEGA-6 FATTY ACIDS FROM *Chlorococcum novae-angliae*

by

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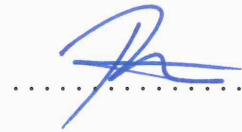

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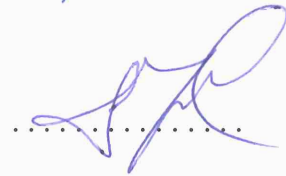

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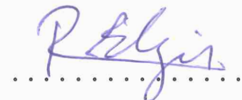

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ABSTRACT

OPTIMIZED PRODUCTION of OMEGA-3 AND OMEGA-6 FATTY ACIDS FROM *Chlorococcum novae-angliae*

Essential fatty acids (ω-3 and ω-6) are key examples of highly demanded nutraceutical supplements as they are substantially required for the development of nervous system and brain in children, and to maintain a healthy cardiovascular system in adults. Major sources of essential fatty acids in nature are fungi, plant, fish, and microalgae. Among them, microalgae feedstocks have gained significant attention recently as they grow much faster and provide higher biomass productivity compared to plant species or fungi. Moreover, microalgae are primary producers of essential fatty acids. Although there are many alternatives for ω-3 and ω-6 fatty acids in the international market which are mainly fish oil-based, pollution of water resources, accumulation of heavy metals, organic contaminants, dioxin in fish bodies, undesirable odor of fish oils, and reduced fish stocks due to overfishing became major concerns for sustainability of these resources. Therefore, microalgae based ω-3 and ω-6 fatty acids are more desirable in the market as they appeal more to vegans and vegetarians due to their plant-based nature. In this study, *Chlorococcum novae-angliae,* a green microalgae that natively synthesize high amounts of ω-3 and ω-6 fatty acids has been exposed to various growth conditions (light, nitrogen, salinity, and temperature stress) to optimize their growth conditions for maximal production of ω-3 and ω-6 fatty acids as a potential candidate for achieving large-scale production goals. For this reason, lipid content and fatty acid profiles of *Chlorococcum novae-angliae* were investigated in order to find out biosynthesis levels of ω-3 and ω-6 fatty acids.

ÖZET

OMEGA-3 ve OMEGA-6 YAĞ ASİTLERİNİN *Chlorococcum novae-angliae* TÜRÜ YEŞİL ALG'DE ÜRETİMİNİN OPTİMİZASYONU

Temel yağ asitleri, nutrasötik takviyelerin örneklerindendir ve çocuklarda sinir sistemi ve beyin gelişimi, yetişkinlerde kardiyovasküler sağlık için yadsınamayacak derecede ihtiyaç duyulduklarından dolayı çok popülerdirler. Temel yağ asitlerinin doğadaki temel kaynakları mantarlar, bitkiler, balıklar ve mikroyosunlardır. Bunların arasında, mikroyosunlar bitkilere ve mantarlara oranla daha hızlı büyüdüklerinden ve daha yüksek biokütle ürettiklerinden ziyadesiyle ehemmiyet kazanmıştır. Üstelik, mikroyosunlar temel yağ asitlerinin doğadaki birincil üreticileridir. Uluslararası pazarda, ω-3 ve ω-6 yağ asitlerinin pek çok alternatifi olmasına rağmen (ki bunlar genellikle balık yağı temellidirler), su kaynaklarının kirlenmesi, organik kirleticilerin, ağır metallerin, dioksinlerin balıklarda birikmesi, balık yağlarının istenmeyen kokusu, balık stoklarının azalması balık kaynaklı ω-3 ve ω-6 yağ asitlerine karşı endişeyle bakılmasına neden olmuştur. Dolayısıyla, mikroyosun kaynaklı ω-3 ve ω-6 yağ asitleri veganlara ve vejetaryanlara hitap etmeleri ve bitkisel temelli doğaları nedeniyle markette daha çok talep edilmektedirler. Bu çalışmada, *Chlorococcum novae-angliae,* ω-3 ve ω-6 yağ asitlerini yüksek oranlarda doğal olarak sentezleyen bir yeşil mikroyosun, çeşitli büyüme koşullarına maruz bırakılarak (ışık, azot, tuz ve sıcaklık stresi), büyük ölçekte üretim yapma hedeflerine ulaşması amacıyla büyüme koşullarının optimize edilmesi için araştırılmıştır. Bu sebeple, *Chlorococcum novae-angliae*'nin yağ içeriği ve yağ asidi profilleri ω-3 ve ω-6 yağ asitlerinin biyosentezin seviyelerini anlamak amacıyla araştırıldı.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Unit
α	Alpha	
β	Beta	
γ	Gamma	
ω	Omega	
µl	Microliter	

Abbreviation	Explanation
Acetyl-CoA	Acetyl-Coenzyme A
ALA	Alpha-linolenic Acid
AA	Arachidonic Acid
CCAP	Culture Collection of Algae and Protozoa
CO_2	Carbon Dioxide
DHA	Docosahexaenoic Acid
DH-GLA	Dihomo-gamma-linolenic Acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic Acid
FAEEs	Fatty Acid Ethyl Esters
FAME	Fatty Acid Methyl Esterification
FAS	Fatty Acid Synthase
FTIR	Fourier-transform Infrared Spectroscopy
G	Gram
GLA	Gamma-linoleic Acid (γ-linoleic acid)
GC-FID	Gas Chromatography-Flame Ionization Detector
GC-MS	Gas Chromatography-Mass Spectrometry
HDL	High Density Lipoprotein
KAS	Keto-acyl Synthase
Kg	Kilogram
LA	Linoleic Acid
LC-PUFA	Long Chain Polyunsaturated Fatty Acids
MTBE	Methyl-tert-butyl-eter
MUFA	Monounsaturated Fatty Acids

ORP	Open Raceway Pond
PBR	Photo Bioreactor
PCB	Polychlorinated biphenyls
PTFE	Polytetrafluoraethylene
PUFA	Polyunsaturated Fatty Acids
ROS	Reactive Oxygen Species
Rpm	Revolutions per minute
RUBISCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SAG	Sammlung Algenkulturen Göttingen
SC-PUFA	Short Chain Polyunsaturated Fatty Acids
SD	Standard Deviation
SDA	Stearidonic acid
SFA	Saturated Fatty Acid
VLDL	Very Low-Density Lipoprotein

1. INTRODUCTION

Algal biomass is considered as a promising raw material for well-balanced diet as we explore its fortifier effects on human health. Although consumption of algae as a food traces to 2000 years back, it has been largely started to be consumed as food supplement since early 1950s (Kay and Barton, 1991). Aside from being used as a single cell protein due its high nutritional value, different forms of algae-food combinations have started to emerge in the markets such as tablets, capsules, and liquids attracting consumers (Ward and Singh, 2005). Algae infused products like chocolates, snacks, yoghurts, soft drinks have also gained remarkable attention in recent years (Martins et al., 2013). In addition, usage of algae as space food for the astronauts due to its high protein, fatty acid, vitamin, and antioxidant content is one of the hottest topics in algae food industry (Matos, 2017). Particularly, microalgae biomass is considered as a key source for omega-3 (ω-3) and omega-6 (ω-6) fatty acids compared to fish oils as they are the primary producers of these fatty acids in nature (Topuz, 2016). Both ω-3 and ω-6 fatty acids have strong impacts on cardiovascular health, visual function, memory and learning and cognitive maintenance and infant brain development. Yet, daily intake of ω-3 and ω-6 fatty acids is not at sufficient levels due to low nutritional of foods and inadequate diet habits of individuals (Martins et al., 2013). Therefore, there is a growing demand for algae based superfood products or food supplements including high levels of ω-3 and ω-6 fatty acids. Although fish oil is convenient in the market as ω-3 and ω-6 fatty acids source, some concerns have occurred in recent years (Lenihan-geels et al., 2013). Underlying reasons of those concerns are undesirable odors of fish oils and burping, accumulation of heavy metals and organic contaminants in fish bodies, and reduced fish stocks due to overfishing (Tonon et al., 2002). There is also a growing interest from vegan and vegetarian individuals who are not willing to use fish oils as food supplement for omega fatty acids. Thus, algal species, microalgae in particular, seem to be a promising candidate to produce ω-3 and ω-6 fatty acids. Microalgae are sustainable sources for the rapid production of ω-3 and ω-6 fatty acids since they grow faster compared to higher plants and do not require arable land and excessive fertilizers (Chatterjee et al., 2017). Extraction of essential fatty acids is easier as microalgae has simpler fatty acid profiles compared to fish, fungi, and other oil-bearing plants. Meanwhile, microalgae produce ω-3 and ω-6 fatty acids naturally, and their production capacities need to be enhanced in order to reach efficient and economical large-scale production. To achieve this goal, several biotechnological studies have been conducted to boost microalgal capabilities in order to achieve optimized production of ω-3 and ω-6 fatty acids (Nichols et al., 2010). One of these attempts is to expose microalgae to stress by changing culture conditions in order to promote lipid

accumulation (Qiao et al., 2016). According to previous studies, shifts in temperature and light, salinity, and nitrogen deplete conditions have remarkable effects on microalgal lipid production (Gacheva and Gigova, 2014; Pal et al., 2011). In this study, green microalgae species, *Chloroccoccum novae-angliae* strain SAG 5.85 was exposed to four stress parameters (light, nitrogen, salinity, and temperature) in order to maximize its ω-3 and ω-6 fatty acids production. Total lipid content and fatty acid profiles were investigated in order to understand biosynthesis level of ω-3 and ω-6 fatty acids. Therefore, total lipids were obtained from the harvested biomass by conventional solvent extraction method (Breuer et al., 2013) and quantified by gravimetric analyses. The lipids extracted from algal biomass were esterified to be analyzed through a GC-FID (Gas Chromatography-Flame Ionization Detector) in order to identify and quantify fatty acid composition. In addition to most commonly sought ω-3 fatty acids, i.e. eicosapentaenoic acid (EPA, C20:5n3) and docosahexaenoic acid (DHA, C22:6n3), the production of other essential ω-3 and ω-6 fatty acids such as α-linolenic acid (ALA, C18:3n3) and linoleic acid (LA, C18:2n6c), and other key ω-6 fatty acids such as gamma-linoleic acid (GLA, C18:3n6), dihomo-gamma-linolenic acid (DGLA, C20:3n6) and arachidonic acid (AA, C20:4n6) were determined.

2. LITERATURE REVIEW

Daily life habits of modern societies are quite far from pre-industrial times in terms of working hours, nourishment, and sleep durations (Shridhar et al., 2015) After the industrial revolution, business world has become more competitive and this environment forced people to find practical and rapid ways of keeping daily routines as they race against time. Therefore, consumption of unhealthy fast-food, processed foods, and snacks have become widespread and turned into one of the majors threats to human health as they include fried oil, inadequate nutritional content, and chemical additives such as stabilizers and shelf life extenders (Nerli et al., 2015). Beside insufficient nourishment, irregular sleep, and stress are other explicit factors which lead to heart diseases, psychological and physiological problems such as depression, insulin resistance, obesity, diabetes, high blood pressure, and atherosclerosis (Doughman et al., 2007).

Another critical issue that needs to be addressed urgently is the food security. Food security is one of the most pressing issues for all countries regardless of their economic development status. Huge numbers of people have been suffering from acute malnourishment all around the world, particularly in Eastern and Southern Africa (Achadi et al., 2016). Increased population and limited food and water resources are the main problems for a considerable amount of people (Baroni et al., 2007). Particularly, under-developed and developing countries experience severe famines and water shortages leading to many problems such as reduced agricultural activities with low crop yields or epidemic diseases (Schmidhuber and Tubiello, 2007). While developing countries suffer from food insecurity due to military conflicts, adverse impacts of climate change and water shortages, developed countries mostly deal with inadequate nutrition since processed foods and fast foods containing low nutritional value are introduced into markets (Simopoulos, 2016)

It is obvious that a well-balanced diet plays key role in human health in order to maintain a strong immune system (resistance to illnesses), protect nervous system, and overall development of the body (Röthlin, 1993). As our comprehension has changed about the importance of diet on human health, most studies have focused on improving diet qualities and elevating nutritional values in food. As a result, there is a growing market in that regard offering variety of healthy food products with a wider range. In recent years, many products have been marketed in order to provide vital requirements for adequate and balanced nutrition. For instance, dubbed as “super foods”, which have exceptional doses of vitamins and minerals, have become desirable by consumers. Also, functional foods enriched with

long chain polyunsaturated fatty acids (LC-PUFAs) including cereals, beverages, cheeses, yogurts, eggs, milk, spreads, and dressings have gained attention from public and medical communities depending on their intensifier health benefits (Martins et al., 2013). Particularly, EPA, DHA and ALA as dietary supplements have become popular since they are substantially required for brain and nervous system development, cardiovascular health, visual function, memory and learning and cognitive maintenance and infant development (Dyall, 2015). They also have fortifier impacts on the prevention of hypertension, premenstrual tension, hypertonia, cancer, and hyperlipidemia (Nicolai et al., 2004). They also play key biological roles in the maintenance of membrane fluidity, acylation of proteins, lipid peroxidation, gene interactions, vascular changes (Liu et al., 2014; Robertson et al., 2013). Also, concentrations of very low-density lipoprotein (VLDL) and high density lipoprotein (HDL) in human blood plasma are associated with ω-3 fatty acids as these fatty acids affect TAG production (Liu et al., 2014). Some critical impacts of ω-3 fatty acids are demonstrated in Figure 2.1.

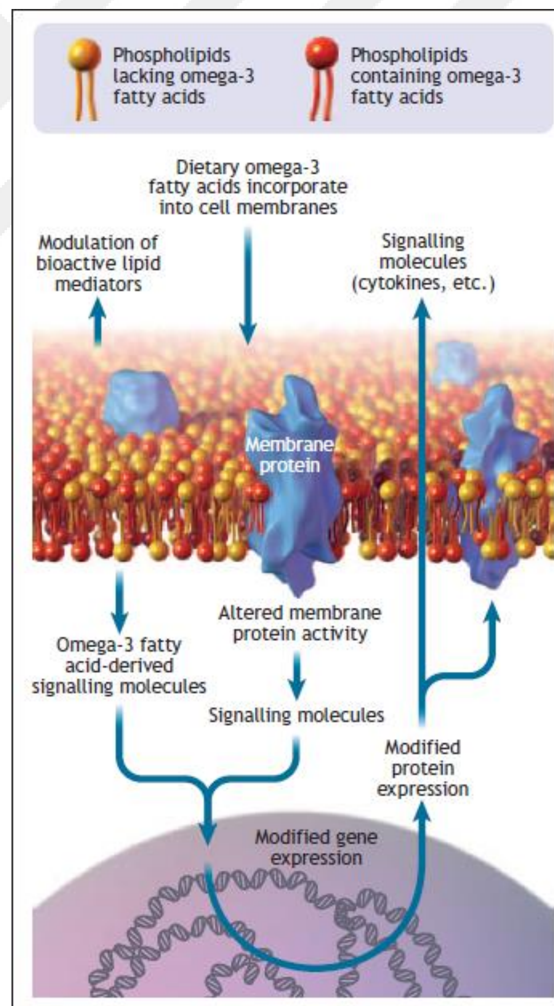


Figure 2.1. Phospholipid bilayer of cell membrane in cooperation with ω-3 fatty acids and their biological impacts (Adopted from Surette, 2008).

Specifically, DHA has significant impacts on impulses generated between the brain cells (Swanson et al., 2012). It also affects visual functions intensively since it is an essential structural fatty acid for retina membranes and takes part in signaling mechanisms in the eye such as photo transduction (Shindou et al., 2017). The other important ω-3 fatty acid, EPA, is vital for circulation system in mammals as it hinders platelet aggregation (Park and Harris, 2002). Moreover, presence of required EPA dose throughout the human body facilitates regulation of inflammation and strengthens immune system (Calder, 2013). Also, EPA-derived substances play vital roles in hindering heart diseases and suppressing chronic and inflammatory mechanisms (Swanson et al., 2012). In reducing inflammation and abolishing reactive oxygen species (ROS) at the cellular level, EPA and DHA also have a strong role in defense mechanism of the body (Winwood, 2013). Since the human body cannot generate the EPA, DHA and AA from ALA at sufficient levels, EPA and DHA should be taken with diet at proper ratios (Nichols et al., 2010). ALA is a key structural component, required for EPA and DHA biosynthesis and produced in very limited amounts in mammals (Saunders et al., 2013). ALA and AA are precursors for prostaglandins, critical mediators for immune system response such as inflammation, regulating defense mechanism of the body by patent vasodilators after injuries and infections (Araujo et al., 2019). Along with EPA, DHA, and ALA, stearidonic acid (SDA, C18:4n3), and docosapentaenoic acid (DPA, C22:5n3) are vital ω-3 fatty acids respectively which have been suggested as important necessities for human well-being (Adarme-vega et al., 2012). Also, ω- 6 fatty acids that consist of LA, GLA, DGLA, and AA are required at proper ratios with ω-3 fatty acids (Simopoulos, 2016). Certain vegetable oils are essential sources of ω-6 fatty acids. For example, LA is abundantly present in western diets as corn oil, sunflower oil, soybean oil, and safflower oil are commonly used (Saunders et al., 2013). While ω-6 fatty acids are synthesized from LA, ω-3 fatty acids are derived from ALA (Khozin-Goldberg et al., 2011a). Arachidonic acid and GLA are considered as important ω-6 fatty acids which are developed from LA (Abedi and Sahari, 2014). Arachidonic acid is also present at high levels in red meats whereas EPA and DHA are mostly obtained by consuming fatty fish and seafood such as salmon, mullet, and mackerel (Nichols et al., 2014). However, microalgae are natural primary producers of ω-3 and ω-6 fatty acids (Nicolai et al., 2004). By consuming microalgae directly and/or other small fish and zooplanktons feeding on microalgae through the food chain, most fish can store large amounts of EPA and DHA (Peltomaa et al., 2013; Nichols et al, 2010). Also, chloroplasts of green leafy vegetables such as purslane, spinach, and walnuts are also rich sources of ALA (Simopoulos, 2004). Gamma-linoleic acid is initial product of ω-6 fatty acid metabolism that will be further synthesized to DGLA after entrance of LA to human body. This fatty acid is very essential for ω-6 fatty acid metabolism in which dihomo-gamma-linolenic acid (DH-GLA) is converted to AA by the action of Δ5-desaturase enzyme (Fan and Chapkin, 1998). It was reported that some diseases such as cancer and diabetes have disruptive impact

on the conversion of LA to GLA (Simon et al., 2014). Therefore, some studies suggest that daily intake of GLA could be a possible solution for the circulation of ω-6 fatty acid metabolism. Arachidonic acid is the most commonly seen LC-PUFA among phospholipids that needs to be liberated by lipase enzymes to produce prostaglandins D, E, and F (Collinius, 2016). Also, this fatty acid mostly constitute brain membranes together with DHA and has remarkable impacts on inflammatory responses, resistance to allergenic, and cell signaling (Shanab et al., 2018). Moreover, central nervous system and immune system are considerably affected by the levels of AA. Arachidonic acid and its metabolites are required for skeletal muscles for creating pain (Tallima and El Ridi, 2018). Also, AA helps animals which lives under low temperatures in adapting to conditions as natural anti-freezer (Shanab et al., 2018).

In terms of consumption, recent findings propose that recommended daily intake level of DHA and EPA is accepted as 0.2 to 0.3 g/day for a healthy individual (Abyor et al., 2011). This level rises up 1.0 to 4.0 g/day for an individual with coronary heart disease (Candela et al., 2011). Unbalanced intake of LC-PUFAs might lead to cardiovascular diseases and trigger cardiac death and/or mental illnesses (Zárate et al, 2017). A proper ratio of LC-PUFA of the ω-3 and ω-6 groups is also vital for a healthy nourishment, and sufficient dietary intake has strong positive impacts on human health (Simopoulos, 2016). Especially, if ω-3 levels are far lower than ω-6, ω-6:ω-3 ratio will increase and deviate from the expected ratio of healthy diets. According to literature, ω-6:ω-3 ratio is nearly one throughout the human evolution, yet typical western diet ω-6:ω-3 ratio ratio is 15:1 to 16.7:1 today (Simopoulos, 2002). Excessive amounts of ω-6 leading to high ω-6:ω-3 ratio is considered as one of the most important causes of cardiovascular diseases, cancer, inflammatory and autoimmune problems (Simopoulos, 2015). Hence, it is indicated that ω-6: ω-3 ratio must be kept in proper levels to maintain normal functions of the human body.

In case of ω-3 deficiency, hair loss, hemorrhagic dermatitis, impaired wound healing, reduced growth in children, hemorrhagic folliculitis of the scalp, weak visual acuity and neuropathy could be seen (Simopoulos, 2002). Particularly, regular ω-3 and ω-6 supplementation during pregnancy have remarkable positive impacts on fetal development, pregnancy duration and visual acuity (Swanson et al., 2012). Visual functions are developed at birth and rapidly completed during first year of life. There are specific mediators carrying DHA and AA from the mother to fetus. For instance, infants fed with DHA containing breast milk show better neurodevelopment compared to their peers fed with fatty acids-free formula milk (Tonon et al., 2002). In addition, some studies show that ω-3 and ω-6 supplementation reduces risk of early preterm delivery in high risk pregnancies (Coletta et al., 2010).

Consequently, growing market demand for ω-3 and ω-6 fatty acids has increased the number of overfished stocks and unrestrained hunting, despite monitoring global fish hunting by strict regulations and tight control. On the other hand, fish oil cannot be recommended as pure sources of EPA and DHA because of the probable accumulation of chemical contaminants, heavy metals (i.e. copper, mercury), and organic pollutants (i.e. PCB, and dioxin) in water bodies due to industrial and agricultural discharges, and other anthropogenic activities leading to water pollution (Akan et al, 2012). Also, oxidative stability of fish oil is indigent depending on type of fish compared to microalgae-based products. Particularly, PUFA content of cooked fish is a contradictive topic in terms of susceptibility of PUFAs to oxidation. Also, high temperature and heat exposure might deteriorate fatty acids in cooked fish (Rymer et al., 2010). Although, microalgae oil is vulnerable to oxidative stress, this situation can be eradicated by short reaction times at reduced temperatures (Frankel et al., 2002).

It should also be noted that, fish-based ω-3 and ω-6 fatty acids are not preferred by vegan and/or vegetarian people. Recent reports claim that vegetarians are not willing to consume animal-based products which suggest microalgae-based ω-3 and ω-6 fatty acids seem to be a viable alternative approach for fulfilling the consumer needs in a much wider range (Adarme-Vega et al., 2014). However, as mentioned earlier, supplying the essential nutrients and meeting the demands of increased population and their diverse needs have become a significant problem because of non-arable lands, insufficient water resources, and environmental pollution. To cope with increased human consumption trends, creation of alternative resources such as improving production capacities of plants and qualities of land-based crops by genetic manipulations are considered. Alas, commercial use of genetically modified crops may not be the best solution due to ongoing social and scientific debates (Martins et al., 2013; Robertson et al., 2013).

To address these shortcomings, several biotechnology companies have started to utilize microalgae feedstock in large scale operations to produce EPA and DHA as they are primary producers of ω-3 and ω-6 fatty acids (Xie et al., 2015). As reliable sources of bioactive compounds, microalgae have great potential to generate significant amounts of EPA and DHA, and considered as essential sources of LC-PUFAs. As fish and other living creatures in marine environments are exposed to numerous contaminants depending on the extent of pollution of surface waters, microalgae-based ω-3 and ω-6 fatty acids have gained significant attention in recent years because they are not contaminated by heavy metals densely (Nichols et al.,2010).

Compared to land crops, microalgal cells have much faster growth rates and higher biomass productivities (Bucy et al., 2012). In addition, microalgae production systems offer environmental advantages as there is no need for arable land space for cultivation and freshwater resources if marine or brackish species are preferred (Hu et al., 2008). Also, various phototrophic microalgae can acclimate themselves to heterotrophic conditions when sufficient sunlight is not available (Winwood, 2013). Yet, phototrophic microalgae confer the most promising outcomes for sustainability benefits as they can be cultivated with renewable inputs such as sunlight, atmospheric carbon dioxide, and recycled waste streams bearing nitrogen and phosphorus. As shown in Figure 2.2, microalgae are great raw material which could be used for energy, animal and human nutrition, and medical products.

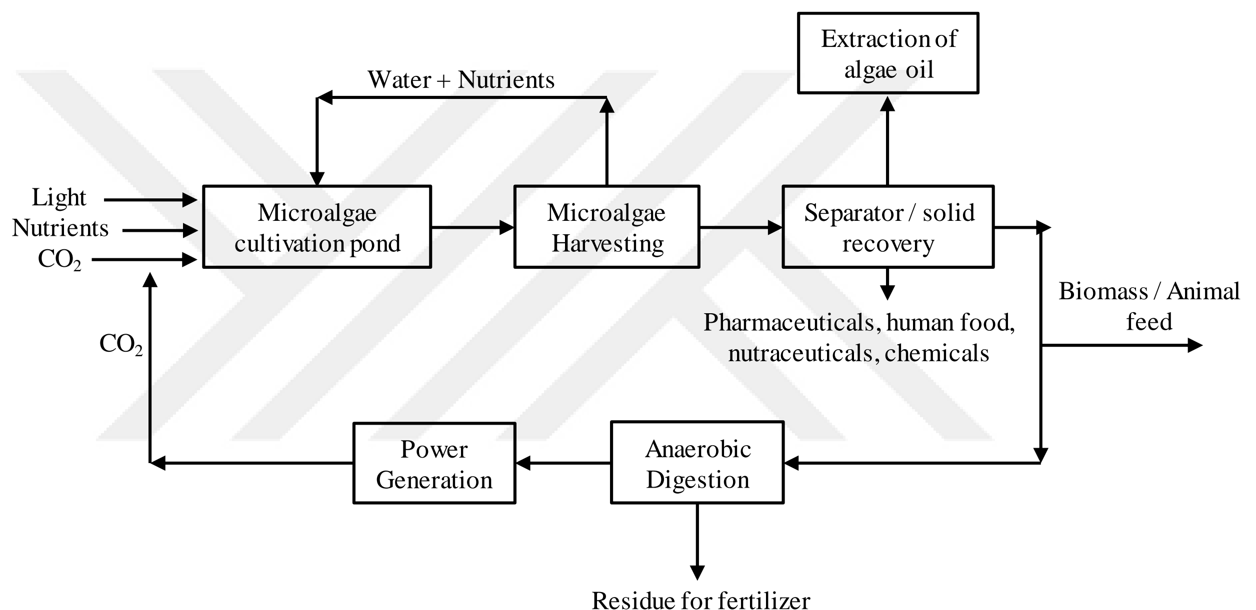


Figure 2.2. Schematic diagram for microalgae biotechnology (Modified from Harun et al, 2010).

2.1. Polyunsaturated Fatty Acids (PUFAs)

Fatty acids are important structural components that play crucial roles in metabolism such as storage and transport of energy, and regulation of gene functions (Robertson et al., 2013). Also, they are essential for all membrane structures (Abedi and Sahari, 2014). Fatty acids can be present throughout the cell as both free or part of complex lipids (Rustan and Drevon, 2001). While fatty acids integrated lipids confer thermal and mechanical insulation, PUFAs are vital for different mechanisms such as generation of eicosanoids, interaction with enzymes, maintenance of membrane fluidity, lipid peroxidation, acylation of proteins, and gene interactions (Liu et al., 2014; Rustan and Drevon, 2001). Eicosanoids that emerged from AA, DHA, and EPA have crucial functions in cell signaling and inflammatory processes (Mozaffarian and Wu, 2011). Moreover, they have intensive

effects on defensive responses to several diseases such as coronary artery, diabetes, cardiac arrhythmias, atherogenesis, and hyperlipidemia (Surette, 2008). It was reported that supplements of LC-PUFA in daily diet prevent major depression and Alzheimer's disease since they have considerable fortifier effects on cognitive functions (Collinius, 2016).

Fatty acids can simply be defined as long carbon chains which carry a methyl group and a carboxyl group at both ends (Wiktorowska-owczarek et al., 2015). The closest carbon atom to carboxyl group is depicted with α. Then, the next carbon is designated by β symbol. Similarly, the closest carbon to double bond around methyl end is named with ω (Rustan and Drevon, 2001). Figure 2.3 shows the general concept of nomenclature used in fatty acids terminology. In ω-3 fatty acids, position of first double bound is generally between third and fourth carbon with reference to ω carbon. In ω-6 fatty acids, the first double bond is placed between sixth and seventh carbon (Adopted from Rustan and Drevon, 2001).

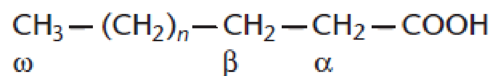


Figure 2.3. Nomenclature of fatty acids (Adopted from Rustan and Drevon, 2001).

Polyunsaturated fatty acids undergo further processing after their production in fatty acid metabolism such as addition of carbon atoms and desaturation. Also, PUFAs are metabolized with a specific pathway which is called β-oxidation in mitochondria or peroxisomes (Rustan and Drevon, 2001). This process is basically curtailing fatty acids into acetyl-coenzyme A (Acetyl-CoA) subunits. Then, those forms of fatty acids can be converted to ketone bodies or transferred into tricarboxylic acid cycle end up with the generation of CO_2 and H_2O (Bajaj et al., 2008). Basically, PUFAs are categorized into two groups regarding their chain lengths. For instance, LA and ALA are short chain polyunsaturated fatty acids (SC-PUFAs) whereas EPA, DHA, and AA are classified as long chain LC-PUFAs. Linoleic acid and ALA are two essential fatty acids which needs to be taken regularly by daily diets as humans cannot *de novo* synthesize these essential fatty acids due to lack of Δ12 and Δ15 desaturase enzymes which create double bonds in fatty acid backbones (Russo, 2009). Therefore, daily diet should include rich appropriate content of fatty acids (Collinius, 2016). Linoleic acid and ALA are important precursors for LC-PUFAs such as AA, EPA, and DHA. Linoleic acid and ALA can be converted to EPA and DHA by elongation and desaturation steps. Biosynthesis of ω-3 and ω-6 fatty acids derived from LA and ALA are illustrated in Figure 2.4. The biosynthesis of long chain PUFAs from LA and ALA is completed by β-oxidation step in peroxisomes (Robertson et al., 2013). Human enzymes have the capability to synthesize LC-PUFAs from LA and ALA, but they are not

sufficient to supply required amounts Wiktorowska-owczarek et al., 2015). Particularly, AA and DHA, main phospholipids of brain, are scarcely produced in human body (Shanab et al., 2018). Also, this capacity is highly vulnerable to aging and some diseases (Khozin-Goldberg et al., 2011a). Hence, recent studies propose that AA and DHA should also be taken into account as essential fatty acids (Singh, 2005; Le et al., 2009).

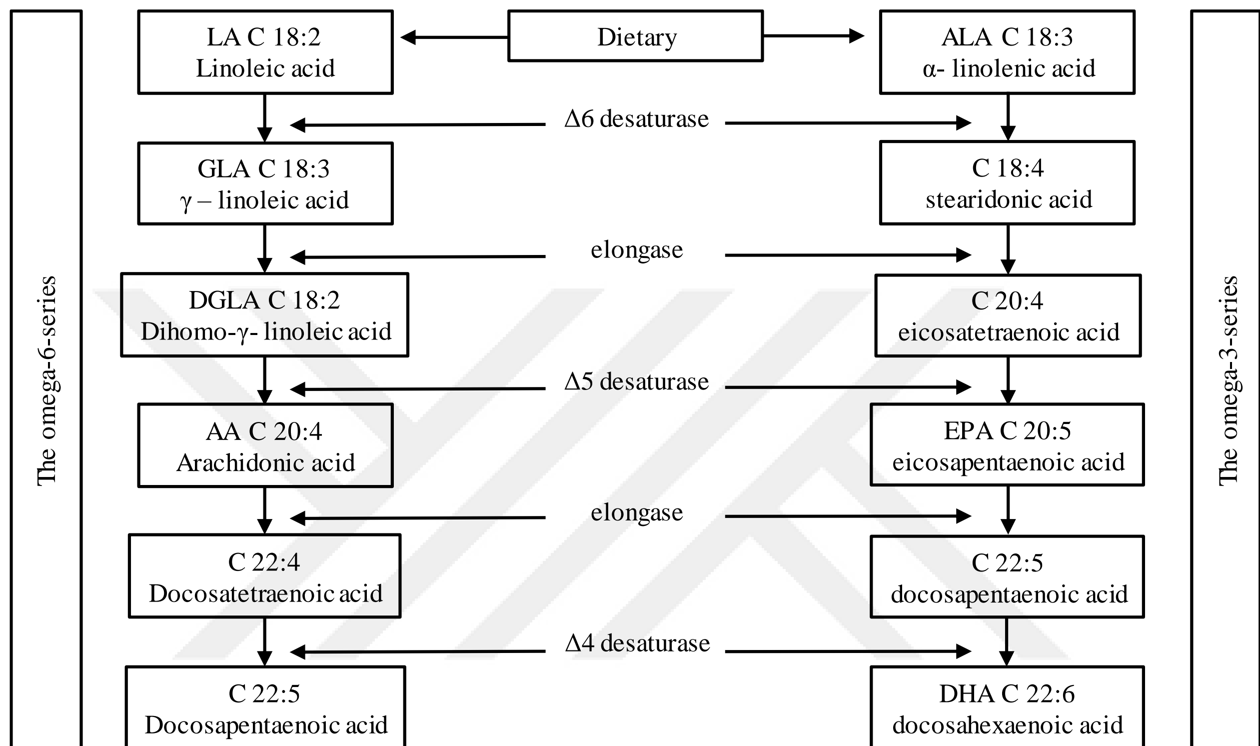


Figure 2.4. Synthesis of ω-3 and ω-6 fatty acids (Modified from Wiktorowska-owczarek et al., 2015).

2.2. Biosynthesis of Lipids and LC-PUFAs in Microalgae

Algae are excellent organisms which are capable of living in extreme conditions, thus, they can be easily found in any habitat (Mohan et al., 2014). If the structure of food chain is considered from down to up, algae constitute more than half of the primary production (Raven and Johnston, 1991). They sequester CO_2 in the atmosphere more than terrestrial plants (Bhola et al., 2014). Also, they are considered as promising biofuel sources since they can generate biofuels much more sustainably than oily plants such as canola, soybean, corn, coconut, and palm oil. (Voloshin et al., 2016). Compared to these plants, microalgae require less area to meet fuel needs. Table 2.1. shows oil yields and required area of plants and microalgae in order to satisfy energy demands in the United States of America.

Table 2.1. Comparison of some sources of biodiesel (Modified from Christi, 2007).

Crop	Oil Yield (L/ha)	Land area needed (M ha)[a]	Percent of existing US cropping area[a]
Corn	50-77	1540	846
Soybean	25-75	594	326
Canola	28-32	223	122
Jatropha	20	140	77
Coconut	23	99	54
Oil palm	25-33	45	24
Microalgae[b]	20-35	2	1.1
Microalgae[c]	31-68	4,5	2.5

[a] for meeting 50% of all transport fuel needs of the United States
[b] 70% oil (by wt) in biomass
[c] 30% oil (by wt) in biomass

If biosynthesis pathways of fatty acids and triacylglycerols (TAGs) are compared, many genes and enzymes isolated from algae and plants are found to be analogous (Yu et al., 2011, Fan et al., 2011). Although the lipid biosynthesis pathways are almost same among algae and higher plants, lipid synthesis in algae are much less studied compared to plants (Mohan et al., 2014). During CO_2 fixation, photophosphorylation reactions occur in thylakoid membranes which are the primary places for *de novo* synthesis of fatty acids. Calvin cycle reactions take place in stroma where carbohydrate production is carried out. Many structural and functional components for the survival of algae are produced through Calvin cycle such as TAGs depending on the species and culture conditions (Huang et al., 2010). Basically, lipid biosynthesis pathway could be summarized in four steps. Firstly, algae capture CO_2 by photophosphorylation reactions in thylakoid and fix CO_2 to carbohydrates in stroma. Also, during photosynthesis algae have the ability of producing their own carbon sources which is known as photosynthates. Those sugars produced in stroma and photosynthates are converted to acetyl-CoA (energy rich compound) which deposits energy for further lipid biosynthesis (Mohan et al., 2014). While glucose is converted to pyruvate by glycolysis in cytosol, photosynthates are activated by acetyl CoA synthetase in stroma and serve as main source of acetyl-CoA (Khozin-Goldberg et al., 2011). The conversion of acetyl CoA to malonyl-CoA is the start point of *de novo* fatty acid biosynthesis (Wiktorowska-owczarek et al., 2015). Carbon dioxide catalyzed by acetyl CoA carboxylase transforms acetyl CoA into malonyl CoA in which energy is consumed through this reaction. Through this cascade reactions, seven molecules of malonyl CoA are produced. Following, seven molecules of malonyl CoA and one molecule of acetyl CoA react with NADPH result in production of palmitate (Mohan et al., 2014). Combination of acetyl CoA and malonyl CoA is catalyzed by KAS III to form C4:0-ACP (Gong and Miao, 2019). In detail, seven molecules of malonyl CoA go into repetitive cycles where each cycle adds two carbon to fatty acyl chain. This

four cycle repetitive reaction chain is catalyzed by fatty acid synthase (FAS) which is a multienzyme complex (Mohan et al., 2014) After that, synthesis of palmitic acid (C16:0) occurs that will be utilized in the production of higher fatty acids. Palmitic acid is extended resulting in formation of stearate (C18:0). Stearate is further desaturated by the action of Δ9 desaturation enzyme to form oleic acid (Robertson et al.,2013). First, C18 fatty acids, particularly ω-9 fatty acid, oleic acid (OA, C18:1n9), are desaturated by Δ12 desaturase enzyme resulting in production of LA in endoplasmic reticulum (Nicolai et al., 2004). Then, LA can be converted to ALA by the function of Δ15 desaturase enzyme or directly transferred to general ω-6 biosynthesis pathway (Gill and Valivety, 1997). Those LA and ALA are catalyzed by Δ6 desaturases followed by Δ6 desaturase/elongase and Δ5 desaturase enzymes resulting in EPA and DHA. Also, other alternative routes might be used during biosynthesis of PUFAs. These pathways are generally initiated by Δ9 elongation and following that Δ8 and Δ5 desaturations occur. All pathways utilized in the production of PUFAs depend on microalgae species, but marginally differ from each other. For instance, some haptophytes such as *Isochrysis galbana*, *Pavlova salina*, and *Emilinia huxleyi* produce PUFAs via alternative routes initiated by Δ9 elongation. On the other hand, PUFAs are generated by general ω-3 and ω-6 pathways in *Phaeodactylum tricornutum* (Khozin-Goldberg et al., 2011b). Basically, these processes consist of desaturation and elongations steps catalyzed by specific enzymes such as fatty acid desaturases (Liang et al., 2013). Fatty acid desaturases are key enzymes which introduce *cis* double bonds at specific positions through the chain (Khozin-Goldberg et al., 2011a). As stated before, the biosynthesis of PUFAs can be carried out by different metabolic pathways in microalgae. Those production ways vary in microalgae species, but C20 intermediates are shared in all pathways (Khozin-Goldberg et al., 2011a).

2.3. Crucial Parameters Affecting Microalgal Metabolism

Microalgae show outstanding flexibility to dynamic environmental conditions with highly adaptive capacities. They produce various biosynthetic compounds or substances with unique properties as a result of their defense and adaptive mechanisms to survive (Guschina and Harwood, 2006). Some of these compounds have great potential to be utilized as precursors or raw materials for value added bioproducts in various sectors and industries. Essential abiotic factors such as light intensity, temperature, pH, salinity, and nutrient limitation (especially phosphorus and nitrogen) have considerable impacts on biochemical pathways in microalgae, affecting growth and lipid production (Pal et al., 2011). It should be noted that, the effects of these parameters are species specific and optimal conditions for one specific microalgae species may not always be favorable for other microalgae species (Robertson et al., 2013). Therefore, it is very essential to understand how these parameters play role on metabolic pathways in different microalgae species.

2.3.1. Light Stress

Light is the most vital parameter for photosynthesis which significantly affects metabolic activities of microalgae (Singh and Singh, 2015). Quantity of energy utilized in microalgal cell depends on light intensity that they absorb. Therefore, microalgal biomass growth is strongly bounded to the light regime. For example, it was shown that *Chromochloris zofingiensis* (formerly *Chlorella zofingiensis*) increases its C/N (carbon/nitrogen) ratio in open outdoor enclosed tubular PBR systems on sunny days (Ma et al., 1997). In a study by Carvalho and Malcata, it was reported that *P. lutheri* increased lipid accumulation and cell density under high light intensity (Carvalho and Malcata, 2005). Another study by Seyfabadi and coworkers showed that increment in light intensity suppresses production of chlorophyll a, monounsaturated and polyunsaturated fatty acids in *C. vulgaris* (Seyfabadi et al., 2011). High light intensity is a favorable condition for microalgal growth and production of pigments as it increases photosynthetic activity, however, it might not be favorable for PUFA production. In a study carried out by Orcutt and Patterson, it was shown that increased light intensity caused reduced PUFA production in *Nitzschia closterium (Cylindrotheca fusiformis)* (Orcutt and Patterson, 1974). However, Brown and colleagues showed that production of PUFAs reached maximum rate for *Thalassiosira pseudonana* during exponential phase under high light intensity (Brown et al., 1996). Another study conducted by Solovchenko and colleagues stated that *Parietochloris incise* (green microalgae) decreased growth rate and production of carotenoids to chlorophyll ration under low light intensity (Solovchenko et al., 2008). Findings for effects of light intensity on microalgal growth and fatty acid composition are very contradictive in literature (Juneja et al., 2013).

2.3.2. Salinity Stress

Another important stress parameter is related to salinity levels. It was proposed that salt stress could be applied instead of light stress as research shows that high salt levels in the growth media promotes production of lipid based pigments, specifically carotenoids. However, increased levels of salt in media reduces microalgal growth (Mishra and Jha, 2009). Another study showed that growth of *H. pluvialis* was reduced under salt stress, but there is a significant increment in carotenoid levels (Tam et al., 2012) It could be inferred that salt stress decreases photosynthetic efficiency by obstacle light utilization (Romanenko et al., 2017). Also, salt content of growth media creates osmotic stress for microalgae resulting in accumulation of low molecular mass compounds and osmolytes. Moreover, membrane permeability might change as a consequence of lipid catabolism (Alyabyev et. al., 2011). Some studies suggested that microalgal lipid content increases under salt stress. One of

those studies measured salt tolerance of three microalgae species [i.e. *Chloroccocum* sp. (freshwater), *Microcystis* sp. (freshwater), and *Chaetoceros* sp. (marine)] by observing their growth and lipid contents (Asulabh et al., 2012). Microalgae cells were exposed to three different salt concentration (0,013 mM, 0,014 mM, 0,034 mM for freshwater algae and 0,6 mM, 3 mM, and 6 mM for marine algae, respectively). While growth of microalgae cells did not differ much, lipid contents were higher compared to controls on the 5th and 6th days. Also, some studies showed that salt stress could increase degree of unsaturation leading to increased ω-3 levels and lipid content (Rismani and Shariati, 2017).

2.3.3. Temperature Stress

Shifts in temperature influence cellular structural components like cell membranes and inner organelles by changing composition of lipids and proteins. Also, those alterations in temperature have strong impacts of enzymatic activities as a result of oscillations in reaction kinetics (Juneja et al., 2013). The changes in cell permeability, enzymatic processes, and regulatory aspects affect essential mechanisms such as photosynthesis and respiration. All these cellular changes at molecular and structural levels are correlated with the inductions or reductions in cell growth. For instance, low temperature results in declined reaction rates, membrane flexibility, and electron transfer whereas high temperatures lead denaturation of proteins, reduced ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) activity, disrupted cell membrane and permeability, and disfunction of photosynthetic systems (Gacheva and Gigova, 2014). It was shown that decreasing temperature has led to increased EPA and DHA levels in various microalgae species. Shifting temperature 25 °C to 15 °C during cultivation of *P. tricornutum* resulted in higher EPA and DHA content (Qiao et al., 2016). Also, *Nannochloropsis oculata* and *Isochrysis galbana* showed optimum productivity for EPA and DHA between 14 °C to 20 °C, respectively (Aussant et al., 2018). In another study conducted by Lynch and Thompson, it was shown that decreasing temperature 30 °C to 12 °C during cultivation of *Dunaliella salina* increased fatty acid unsaturation rate (Lynch and Thompson, 1982).

2.3.4. Nitrogen Stress

Another major stress parameter is nutrient limitation, specifically nitrogen deprivation in culture media. Nitrogen is a vital element for all living organisms since it is found in DNA and RNA and amino acid structures (Kim et al., 2016; Juneja 2013). Nitrogen also plays an important role in microalgae cells since accessory pigments such as chlorophylls a and b, vital for photosynthetic efficiency and non-photochemical active pigments (such as carotenoids), contain nitrogen. In the

absence of nitrogen, while protein rich compounds such as chlorophylls a and b are consumed to maintain cell growth and serve as nitrogen pools for vital proteins, lipids and carbohydrates are increased for long term energy storage as a response to nitrogen stress (Li et al., 2008). Building on this biochemical phenomenon, nitrogen stress is applied to increase the accumulation of lipids in microalgae. In experimental conditions, there are two ways of creating nitrogen stress (Figure 2.5.). One of them is preparing nitrogen deplete medium which is deprived of nitrogen. The other one is to grow microalgae under nitrogen limited medium in which concentration of nitrogen is insufficient for microalgal growth (Figure 2.5.). While microalgal cells growing in nitrogen deprived media show reduced growth and low levels of chlorophylls a and b, they are able to increase fatty acid contents.

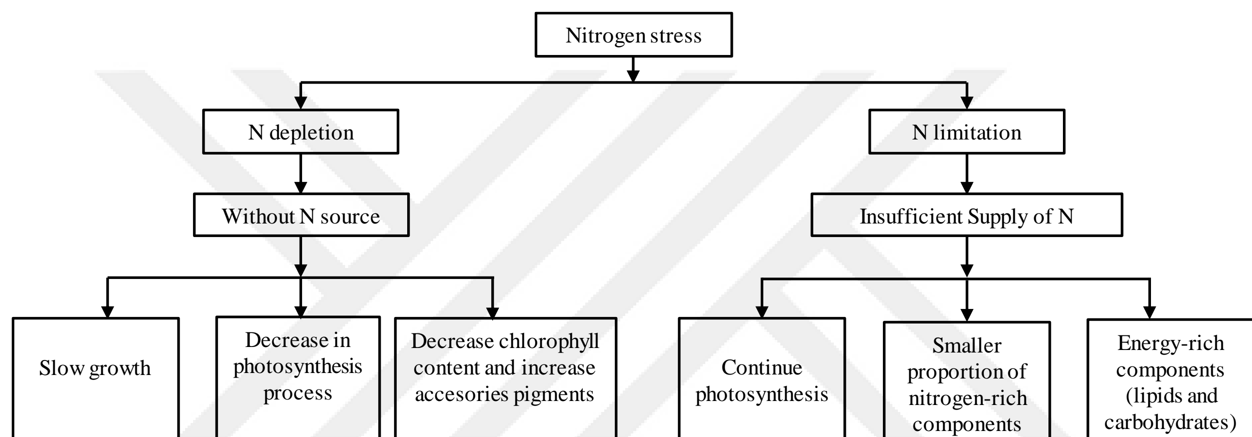


Figure 2.5. Types of nitrogen stress affecting lipid production (Modified from Benavente-Valdés et al., 2016)

For instance, Li et al. showed that *Ettlia oleoabundans* (formerly *Neochloris oleoabundans*) stopped cell division after nitrogen was completely consumed, however, resulting biomass was rich in lipids and storage oils (Li et al., 2008). Another study found that nitrogen deplete conditions triggered monounsaturated triacylglycerols, in contrast, nitrogen limited conditions resulted in elevated PUFA contents in *E. oleoabundans* (Bona et al., 2014). Responses of green algae to nitrogen starvation differ from each other (Rodolfi et al., 2009). For instance, lipid content of *C. pyrenoidosa* was increased under nitrogen deplete conditions whereas *Tetraselmis suecica* and *Dunaliella spp.* slightly decreased lipid production under same conditions (Borowitzka 1988).

2.3.5. Phosphorus Stress

Another crucial parameter affecting microalgal metabolic pathways is phosphorus. It is considered as limiting nutrient in natural waters and aquatic systems. This element is significantly vital for phytoplankton growth since it is utilized in microalgae cells for energy and nucleic acid

metabolism (Brett et al., 2000). Also, phosphorus enters mostly phospholipid membranes and other cell compartments as a key component. Phosphorus have considerable impact on enzymatic activities, transport processes, photosynthesis, and inheritance of genetic material (Juneja et al., 2013). Microalgal growth is significantly affected by phosphorus concentration whereas photo active pigments are not tightly depended on phosphorus concentrations (Kozłowska-Szerenos and Zieliński, 2000). Some studies declare that phosphorus deprived media conditions promote lipid accumulation in microalgal cultures as a result of alteration in biochemical pathways in response to stress, but it did not change PUFA levels significantly (Blair et al., 2014). It was proposed that increased lipid accumulation might be result of consumption of 24 NADPH generated from electron transport chain (Hu et al., 2008). Another study conducted by Liang and colleagues highlighted that concentration of phosphorus in growing media have crucial impact on lipid production in green microalgae (Liang et al., 2013). They found that phosphorus deplete conditions increased lipid productivity rate in *Chlorella sp*. Also, Hamouda and Abou-El-Souod reported that low phosphorus concentration increased lipid content of Scenedesmus obliquus (green microalgae), however, this level of phosphorus created stress on microalgae resulting in impaired lipid productivity (Hamouda and Abou-El-Souod, 2018).

2.4. Essential Steps of Microalgal Lipid Studies

Even though it depends on the final product, there are some important steps that need to be applied in microalgae production processes. Basically, these steps constitute the main framework of most microalgal biotechnology research. For the main objective of this particular study, selection of appropriate microalgae strain is one of the most important step as biochemical compositions of microalgae species and their productivity highly vary under stress conditions (Larkum et al, 2012). Table 2.2 shows how lipid content vary in different microalgae species. Under certain stress conditions, accumulation of lipids while limitation on biomass productivity is very common behavior in microalgae. The lipids produced in algae (phospholipids, betaine lipids, glycolipids, TAGs) might differ from each other based on culture stages, culture conditions and the species (Sharma et al, 2012). For lipid studies, it is important to know lipid contents and cell wall compositions of microalgae species prior to exposure to experimental conditions.

Table 2.2. Lipid contents of certain microalgae species (% dry biomass)(Modified from Chisti, 2007).

Microalgae	**Lipid Content (% dry weight)**
Schizochytrium sp.	50-77
Botryococcus braunii	25-75
Chlorella sp.	28-32
Crypthecodinium cohnii	20
Duneliella primolecta	23
Isochrysis sp.	25-33
Nannochloris sp.	20-35
Nannochloropsis sp.	31-68
Neochloris oleoabundans	35-54
Nitzschia sp.	45-57
Tetraselmis sueica	15-23
Chlorococcum sp.	6-28

All methods and treatment processes are shaped regarding on selected species throughout the study. Following this step, microalgae cells are cultivated in open ponds or closed photobioreactors (PBRs) to produce sufficient amount of biomass under controlled conditions. Cultivation process is significantly important for algal growth and effective production because growth parameters of microalgae species could be controlled at this stage. Cultivation process mainly depends on CO_2, light, pH, temperature, dissolved O_2, and nutrients. In the presence of right amounts of nutrients, light and CO_2 levels, microalgae cells could grow rapidly and double under 24 hours.

After cultivation, microalgal biomass are harvested and directly used or dewatered by certain techniques such as flocculation, flotation, gravity-assisted settling, centrifugation, filtration, and direct drying (Sharma, 2013). Following harvesting, several cell disruption methods could be applied to enhance lipid recovery from the cells such as laser treatment, microwave, water bath, ultrasonic, and blender (Mcmillan et al., 2013). Disruption of the cell wall is necessary prior to lipid extraction. Lipids can be also extracted by using organic solvents, supercritical fluid extraction (SFE), conventional Soxhlet extraction, or accelerated solvent systems from the biomass (Mcmillan et al., 2013). Extracted lipids are quantified gravimetrically, trans esterified into fatty acid methyl (or ethyl) esters (FAMEs or FAEEs respectively), identified and quantified by analytical methods such as gas chromatography (GC) or high-performance liquid chromatography (HPLC).

2.5. Cultivation and Harvesting

There are certain types of cultivation systems which have been designed specific for production capacity (lab-, pilot-, or demo-scale) and type of microalgae species, but mainly categorized into two types as open ponds and closed PBRs. Open ponds are quite convenient in algal biotechnology to produce biofuels or valuable bioactive compounds. Shapes and forms of PBRs are also variable with respect to final product, and level of sterility required. Four types of open ponds are mostly utilized in microalgae production systems, such as open raceway ponds (ORPs), shallow big ponds, circular pond tanks, and closed ponds (Harun et al., 2010). All those types are highly suitable for large-scale production, despite some disadvantages. For instance, contamination risks are one of the most important problems in open pond systems as controlling the contamination in open to air systems are harder than closed PBRs (Janssen et al., 2003). Also, there are some crucial parameters which affect efficiency of pond systems such as their locations, climatic conditions, and light availability (Harun et al., 2010). However, open pond systems are preferred since they could be easily operated and cheaper to manufacture. Moreover, they require low operational costs and energy (Narala et al., 2016). Some examples of cultivation systems utilized in for different bioproducts from different algae species are shown in Table 2.3.

Table 2.3. Microalgal species with high relevance for biotechnological applications (Modified from Pulz and Gross, 2004).

Species/group	**Product**	**Reactor type**
Spiriluna platensis/Cyanobacteria	Phycocyanin, biomass	Open ponds
Chlorella vulgaris/Chlorophyta	Biomass	Open ponds, glass-tube PBR
Duneliella salina/Chlorophyta	Caretenoids	Open ponds
Haematococcus pluvialis/ Chlorophyta	Caretenoids	Open ponds, PBR
Odentella aurita/Bacillariophyta	Fatty acids	Open ponds
Porphyridium cruentum/Rhodophyta	Polysaccharides	Tubular PBR
Isochrysis galbana/Chlorophyta	Fatty acids	Open ponds
Phaedactylum tricornutum/Bacillariophyta	Lipids, fatty acids	Open ponds

Apart from these, closed PBRs are better alternatives to control contamination compared to open pond systems. Contrary to open ponds, PBRs also provide better quality control on operational parameters (Christi, 2007). Growth conditions, pH, temperature, dissolved CO_2, and culture sterility are adequately controlled in PBRs (Janssen et al., 2003). Better control of the culturing environment provides enhanced biomass productivity. Similar to pond systems, there are different types of PBRs,

basically classified into two main groups as tubular and flat-plate types (Harun et al., 2010). Tubular PBRs are made of glass- or plastic-based, vary in their geometry and tubing configurations specific to each systems with different flexibilities (Molina et al., 2001). They can be vertical, horizontal, and helical configurations to provide large surface areas for illumination. Flat-plate PBRs are intensively utilized as they consume less power compared to others (Ugwu et al., 2008). High photosynthetic capacity and less oxygen generation are other essential reasons of why closed PBRs have been mostly preferred. There are certain types of flat-plate PBRs which are constructed to achieve maximum light penetration and higher cell density yields. Basically, they differ from each other by construction materials such as glass or plastic types. Also, V-shaped and inclined type PBRs are also utilized in production systems. Despite these advantages, compared to pond systems, closed PBRs are more expensive, especially for the initial capital stages of the cultivation process (Narala et al., 2016).

Choosing suitable PBRs need to be evaluated regarding to physiologies of microalgae being cultivated (Pulz and Gross, 2004). After choosing appropriate cultivation system, selected microalgae species are exposed to controlled growth parameters such as light, temperature, salinity, dissolved O_2/CO_2, pH, and nutrients depending on the type of species cultivated (Norsker et al., 2011). Cultivated biomass is dewatered by an appropriate technique without damage given to biomass. This process is applied to gather up all microalgae cells from the cultivation media. Filtration, flocculation, and centrifugation are commonly utilized techniques for dewatering microalgae biomass (Sharma, 2013). An optimal dewatering method should be chosen considering physiology of microalgae, maximum biomass yield, and energy requirements with respect to economic feasibility (Fasaei et al., 2018). As initial step of dewatering, flocculation provides facilities further progress (Harun et al., 2010). Basically, microalgae cells cannot be observed in self-aggregation since their membranes carry negative charge (Knuckey et al., 2006). Flocculation process allows them to coagulate and to be separated from the media since flocculants (cationic chemicals such as metal salts) ease aggregation of microalgae cells by countering negative surface charge (Al Hattab et al., 2015). Those chemicals are commonly multivalent salts such as aluminum sulfate, ferric chloride, ferric sulfate, cationic polymers and chitosan (Grima et al., 2003). Flocculation is a convenient process applied in many studies, but some of the synthetic flocculants used in the process can be toxic to environment (Sharma, 2013).

Another technique utilized in algal dewatering process is filtration. This process basically separate microalgae cells from the culture media by an appropriate filtering device based on the dimensions of algal cells (Grima et al., 2003). Types of filtration techniques differ from each other regarding on filter size and propellant power (pressure and vacuum). Those techniques are dead-end

filtration, microfiltration, ultrafiltration, pressure filtration, vacuum filtration, and tangential flow filtration (Grima et al., 2003, Harun et al., 2010). Filtration process is more recommended method compared to flocculation since it does not carry environmental threat, alas, it is not cost effective to certain extent. Apart from those two techniques, centrifugation is fast and easily applicable technique. Centrifugation is one of the most feasible method to harvest microalgae biomass compared to other methods in terms of cell viability and harvesting efficiency. Advantages and disadvantages of some of the dewatering techniques commonly utilized in algal biotechnology are presented in Table 2.4.

Table 2.4. Comparison of harvesting and dewatering technologies (Modified from Fasaei et al., 2018).

Technology	**Advantages**	**Disadvantages**
Centrifugation	• Continuous • Efficient for large scale processing • High recovery	• High capital cost
Pressure filtration	• Low energy demand • High recovery	• Discontinuous • Clogging and fouling
Vacuum filtration	• Continuous	• Relative high harvesting cost • Clogging and fouling
Membrane filtration	• Efficient for small scale processing • High recovery	• Fouling • High capital cost
Sedimentation	• Easy to apply • Low energy demand	• Slow rates • Large operational area • Low recovery • Suitable for large size algae
Chemical flocculation	• Low energy demand • Low equipment cost	• Difficult recovery of flocculants
Drum drying	• Mature technology	• High energy demand
Spray drying	• Suitable for high value product	• High energy demand
Solar drying	• Low cost	• Slow rate • Risk of contamination and biomass loss

Principle of centrifugation is based on separation of microalgae cells and culture media through centripetal acceleration which allows to recover biomass easily by removing the supernatant (Harun et al., 2010). However, this technique also has some drawbacks. For instance, cell disruptions would be seen if speed of centrifugation was not adjusted appropriately (Sharma et al., 2013). Also, its energy consumption is very intensive resulting in higher operational costs (Grima et al., 2003).

Nevertheless, centrifugation is still considered as the most reliable and efficient technique compared to other harvesting methods (Harun et al., 2010).

2.6. Cell Disruption Methods

Cell disruption is a crucial step in the process of lipid extraction by allowing solvents to reach intracellular lipids. This step should not be skipped over since it has considerable impact on lipid recovery, quality, and operational costs (Byreddy et al., 2015). The most commonly used disruption methods in literature are expeller pressing, bead beating, microwave assistance, lyophilization, ultrasonication, osmotic shock, grinding with liquid nitrogen, shake mill, autoclaving, ferromagnetic layer treatment, antibiotics treatment, enzyme solution treatment and salt treatments (Benavente-Valdés et al., 2016; Byreddy et al., 2015; Mohan et al., 2014). One study suggests that combination of lyophilization and ultrasonication increases lipid yield significantly (Onay et al., 2016). In a study by Dvoretsky and colleagues reported that combination of antibiotic solution and microwave radiation gave the highest lipid recovery from *C. vulgaris* biomass (Dvoretsky et al., 2016). Another study proposes that microwave assisted lipid extraction is the most applicable method as it is very practical and efficient (Lee et al., 2010). Also, integrated application of bead beating and ultrasonication could be suggested for enhanced lipid recovery and product quality (Prabakaran and Ravindran, 2011). Also, another study carried out by Byreddy and coworkers showed that lipid recovery from *Schizochytrium* sp. S31 and *Thraustochytrium* sp. AMCQS5-5 were enhanced remarkably by exposure of algal biomass to osmotic shock (Byreddy et al., 2015). Nevertheless, bead beating, also utilized in this study, has been considered as one of the most efficient techniques in recent years since it provides excellent cell wall disruption and applicable for large scale productions (Günerken et al., 2015; Baldev et al., 2015). Bead beating is basically defined as mechanical disruption of microalgae cell walls by grinding with quartz or metal beads in bead beating tubes or vessels (Passos and Uggetti, 2015). The crucial factors affecting bead beading process are bead sizes, bead compositions, speed, time, and sample characteristics (Miranda et al., 2012). The main advantages of this technique are the requirement of less power and its ease of application to algal biomass compared to other methods (Passos and Uggetti, 2015, Lee et al., 2012). One study showed that bead beating of *Botryococcus braunii* provided two-fold higher lipid yield over control compared to other cell disruption techniques such as ultrasound and high pressure homogenization (Lee et al., 1998). Also, in a study by Lee and colleagues showed that high level of cell disruption of *Botryococcus sp., C. vulgaris*, and *Scenedesmus sp.* was observed at 2800 rpm by exposing algal biomass through 5 minutes Lee et al. (2010). In our study, bead beating and ultrasonication methods were combined for cell disruption.

2.7. Lipid Extraction from Microalgae

Although, there are several lipid extraction techniques in literature, still some challenges exist to move toward large-scale production of lipid-based products since those techniques could not be well standardized or achieve satisfactory levels (Li et al., 2014; Baldev et al., 2015). The solvents used in lipids extraction and their health impacts, costs, efficiencies, and whether they are labor-intensive or not are vital points which dramatically affect the fate of the study as this step is related to quality and quantity of lipid products (Ranjith Kumar et al., 2015; Yang et al., 2014). Lipid extraction efficiencies and fatty acid methyl esters yields mostly depend on cell wall compositions of microalgae, temperature, type of organic solvents, biomass to solvent ratio, and significantly affected by cell disruption techniques (Dvoretsky et al., 2016; Lee et al., 2010; Baldev et al., 2015).

Study by Dvoretsky et al., concluded that ethanol/petroleum ether (2:1) as solvent gave the highest lipid yield (Dvoretsky et al., 2016). Also, they stated that dry biomass to solvent ratio could be ranged between 1:100 and 1:200. Another considerable finding revealed from this study was that the temperature for lipid extraction should be kept between 45-50°C. Another study carried out by Li and coworkers found that using mixture of polar and non-polar solvents in Soxhlet extraction was more efficient than single non-polar solvent used to extract lipids (Li et al., 2014). However, they expressed that Soxhlet extraction method resulted degradation of LC-PUFAs. Another important finding from their study that total lipid extracted from *Tetraselmis sp.* M8 was higher when using (Dichloromethane/methanol) compared to direct saponification (Ethanol/KOH), (Propane: Hexane), (Chloroform/Methanol) supercritical CO_2 extractions. Moreover, they reported that direct saponification resulted lower fatty acid and lipid yields. Matyash and colleagues proposed that using MTBE (metyl-tert-butyl-eter) in lipid extraction provided more purified lipid recovery and higher lipid yield compared to Folch and Bligh&Dyer methods (Matyash et al., 2008). Study carried out by Jones and colleagues stated that excellent lipid recoveries were obtained by using 2-ethoxyethanol (2-EE) as solvent (Jones et al., 2012). However, Bligh&Dyer method has become one of the most applied lipid extraction techniques since it was widely utilized in several fields such as molecular biology, biophysics and biomedical researches including lipid biochemistry and lipidomics studies (Sündermann et al., 2016). This method and its different versions are very common and applicable in lipid studies as it was shown in Figure 2.6.

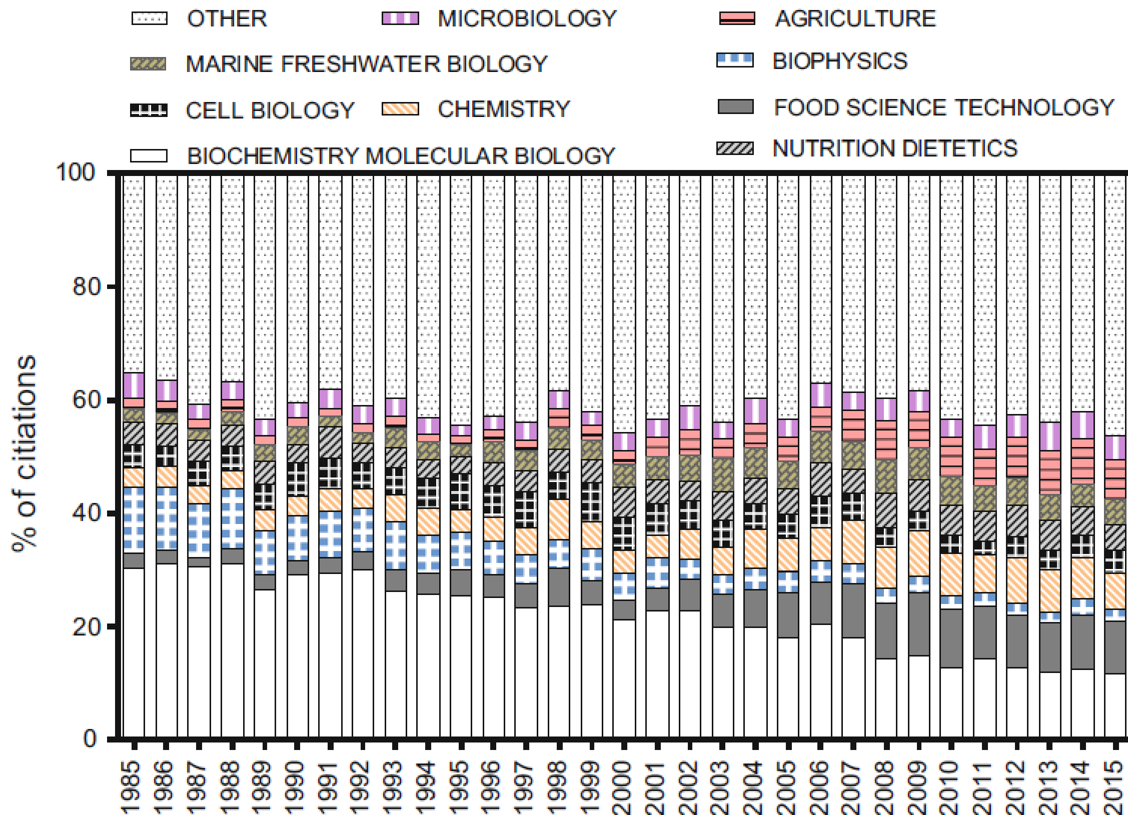


Figure 2.6. Distribution of citations per research area according to categorization of Web of Science and year in percent (Adopted from Sündermann et al., 2016).

The study by Sheng et al., revealed that highest lipid recoveries from *Synechocystis* PCC 6803 (shown in Figure 2.7) were obtained by Bligh&Dyer and Folch methods and defined these methods as “gold standards” (Sheng et al. 2011). They proposed that chloroform/methanol based methods could integrate into cell membranes easier due to higher polarity compared to MTBE, ethanol, isopropanol, butanol, hexane, and acetic ester. Superiority of Bligh&Dyer method in FAME yield compared to other extraction methods was demonstrated in Figure 2.7. Also, another study made by D'Oca and coworkers. reported that highest lipid recovery from *Chlorella pyrenoidosa* was obtained by chloroform:methanol (2:1 v/v) extraction compared to other solvents such as methanol, chloroform, ethanol and hexane (D' Oca et al., 2011). In this study, a modified version of Bligh&Dyer method was used and strengthen by the combination bead milling and ultrasonication. Water bath was also used for fatty acid methyl esterification, thus, it was expected to see that all lipids were liberated from membranes and compartment of organelles by temperature exposure. This method has been adopted from Breuer et al., (2013).

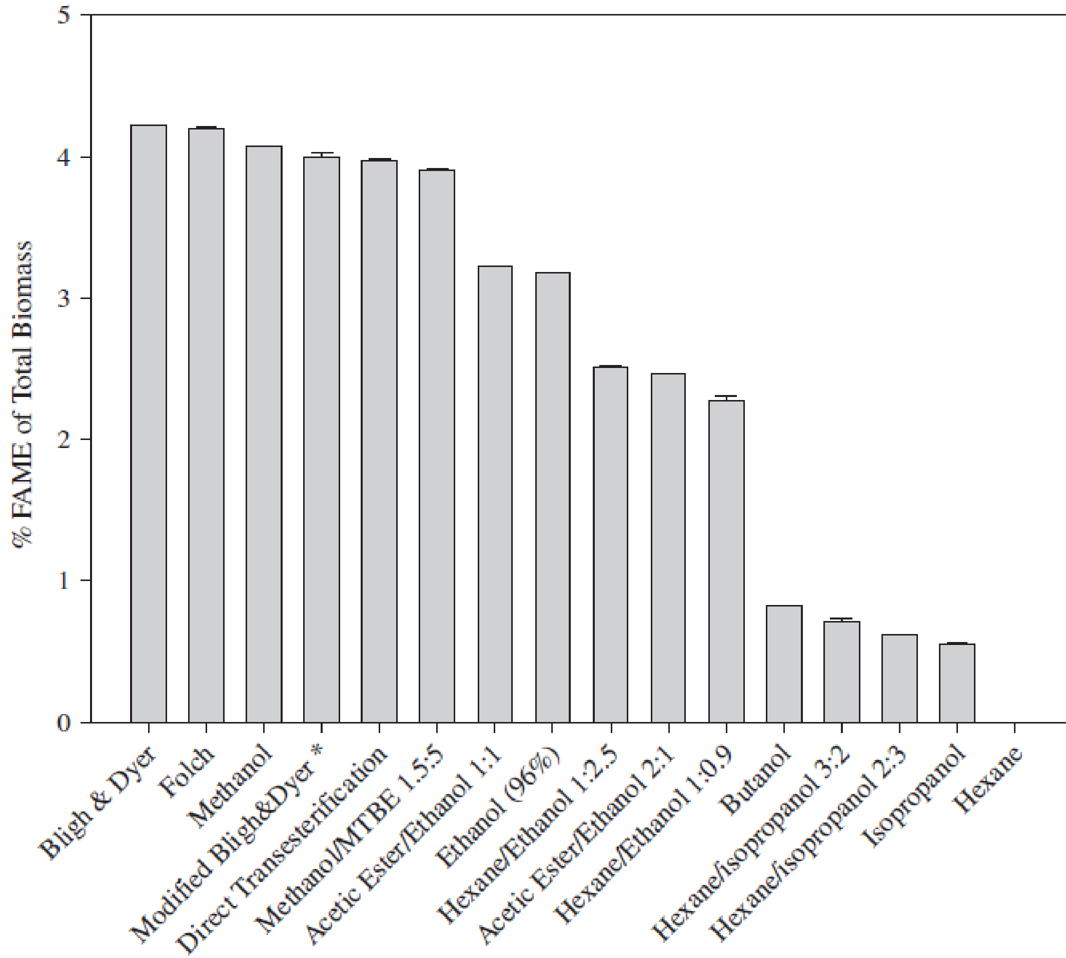


Figure 2.7. Percent FAME of total biomass after total lipid was extracted using different solvents/solvent combinations from *Synechocystis* PCC 6803. All ratios are v/v. (Adopted from (Sheng et al., 2011).

Bligh&Dyer method is an old, yet simple and rapid method which can completely extract all lipids in a simple process (Sündermann et al., 2016). The method described below is original version of (Bligh and Dyer, 1959) method. Originally, this method was based on homogenization of target tissue with chloroform/methanol (1:2, v/v) mixture by using a blender. One volume of chloroform is added to resulting homogenate and blended for 30 seconds. Following, one volume of water is added and vortexed for another 30 seconds. The mixture is filtered through filter paper under vacuum by using Büchner funnel. Then, the filtrate is transferred into a graduated cylinder and kept for the complete separation. Volume of the chloroform layer containing lipids is recorded. Alcoholic layer was removed by a rotary evaporator. This method is still one of the most commonly utilized techniques due its simplicity (Iverson et al., 2001). Since endogenous water present in the tissue provides better extraction of neutral and polar lipids, drying the biomass is not necessary. Over the years, Bligh and Dyer method has been modified in order to increase its lipid extraction efficiency (Ranjith Kumar et al., 2015). Different versions of this method have been used mainly by changing

the methanol, chloroform and water ratios, solvent volumes, incubation time or acid addition (Weerheim et al., 2002, Jensen, 2008, Hajra, 1974). The modified version used in this study is described in "Materials and Methods" section.

2.8. Transesterification Methods

Fatty acid methyl (or ethyl) esterification step is an essential step for the identification and quantification of fatty acids in any lipid sample. Fatty acid methyl esters are formed through a thermochemical reaction catalyzed by acid or alkali reagent in which fatty acids present in mono-, di-, and triglycerides react with an alcohol (methanol or ethanol) and turn into FAME (or FAAE if ethanol is used) and glycerol (Passos and Uggetti, 2015). Typical esterification reaction is illustrated in Figure 2.8. Fatty acid methyl esters from algae biomass are commonly obtained by using *in situ* transesterification and two-step transesterification methods. *In situ* transesterification directly convert algal biomass into FAMEs without lipid extraction step (Laurens, 2015). This method could be applied to small size of samples (i.e. 4-7 mg) for the bench scale productions (Laurens et al., 2012). Two-step transesterification techniques utilize conventional solvent based systems where lipids are extracted from algal biomass using organic solvents and proceeded by transesterification (Silas et al., 2015).

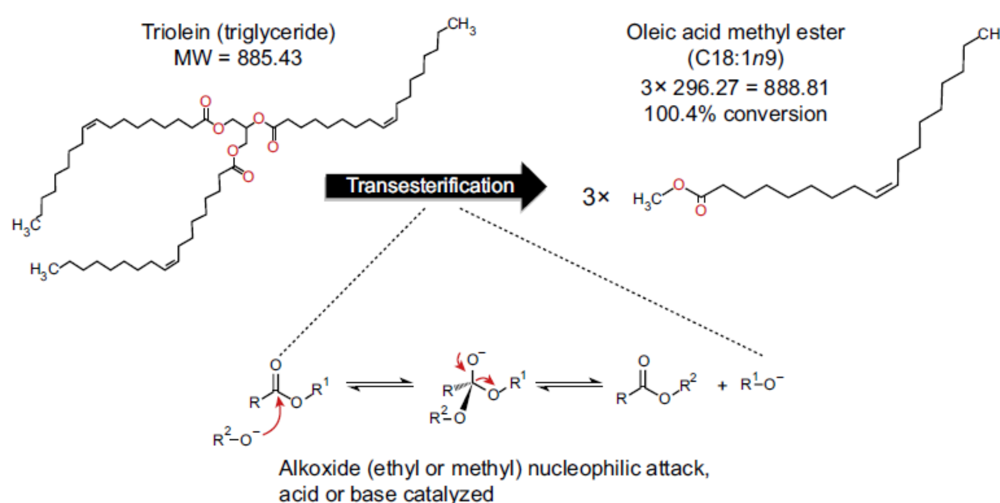


Figure 2.8. Conversion of triolein triglyceride onto oleic acid methyl ester (Adopted from Laurens, 2015).

There are certain variables affecting FAME yields such as moisture content, solvent type, catalyst type/concentration, temperature, pressure, stirring, time of reaction and sample size (Ehimen et al., 2010; Passos and Uggetti, 2015). The study by Nagle and Lemke revealed that using acid

catalyst instead of alkaline is more accurate since alkaline catalyst could lead to saponification in samples with high free fatty acid (FFA) content (Nagle and Lemke, 1990), and suggest usage of methanol containing 0,6 N hydrochloric acid for 6 minutes at 70 °C to obtain optimized biodiesel from microalgae. In a study by Miao and Wu, conversion of microalgal lipids to biodiesel was the highest by applying 100% acid catalyst for 4 h at 30 °C (methanol to biomass ratio:56:1 as molar) among tested conditions (Miao and Wu, 2006) . Another study made by D'Oca and colleagues concluded that esterification by boron trifluoride (BF_3)/methanol after Soxhlet extraction (methanol as solvent) gave better results compared to *in situ* transesterification (D'Oca et al., 2011). Thao and coworkers reported that sulfuric acid (H_2SO_4) mediated transesterification provided high FAME content and biodiesel yield (Thao et al., 2013). In our study, two-step H_2SO_4 catalyzed transesterification method was utilized. Samples were incubated in water bath for 3 h with methanol containing 5% H_2SO_4.

3. MATERIALS AND METHODS

3.1. Model Organism and Maintenance of Culture

Chlorococcum novae-angliae strain SAG 5.85 is a unicellular, terrestrial microalga which belongs to Chlorococcaceae family under Chlorophyta division (green algae). This strain was obtained from The SAG Culture Collection of Algae (SAG, Germany) and maintained in modified Bold's 3N (MB3N) culture medium. Components and their concentrations for MB3N culture medium are provided in Table 3.1 and Table 3.2 below.

Table 3.1. Modified Bold's 3N Components for one liter cultivation medium.

Components	Final Concentration
$NaNO_3$	5 ml/L (8,82 mM)
$CaCl_2.2H_2O$	0,7 ml/L (0,17 mM)
$MgSO_4.7H_2O$	1 ml/L (0,3 mM)
K_2HPO_4	2,5ml/L (0,43 mM)
KH_2PO_4	2,5ml/L (0,43 mM)
NaCl	0,84 ml/L (0,43 mM)
P-IV Metal Solution	6 ml/L
Soil Water	40 ml/L
Vitamin B_{12}	1 ml/L
Biotin	1 ml/L
Thiamine	300 µl/L

Table 3.2. Components for P-IV Metal Solution.

Components	Amount	Final concentration
$Na_2EDTA.2H_2O$	0,75 g/L	2 mM
$FeCl_3.6H_2O$	0,097 g/L	0,36 mM
$MnCl_2.4H_2O$	0,041 g/L	0,21 mM
$ZnCl_2$	0,005 g/L	0,037 mM
$CoCl_2.6H_2O$	0,002 g/L	0,0084 mM
$Na_2MoO_4.2H_2O$	0,004 g/L	0,017 mM

Maintenance of the cultures was sustained in sterile air-filter (0.22 µm pore size) capped tissue culture flasks containing 20 ml MB3N broth and also agar plates. Both flasks and plates (Figure 3.1) were

kept in a plant growth chamber (Model GC 401, Nüve, Ankara, Turkey) operating under 25 ºC±1, illuminated with cool white fluorescent bulbs providing 1800 lux light intensity for 14:10 hr (light:dark) cycles and passaged every seven to eight days. Prior to experimental culture batches, maintenance cultures were inoculated into 0,5 L glass gas wash bottles in order to obtain the seed cultures.

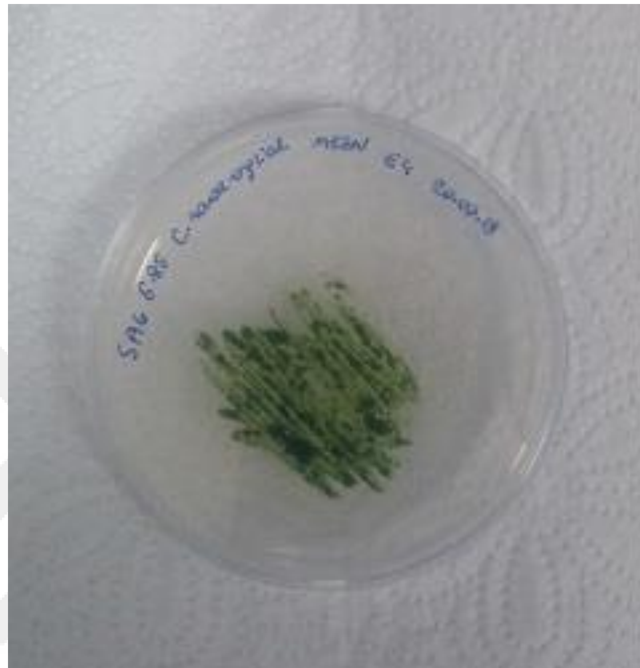
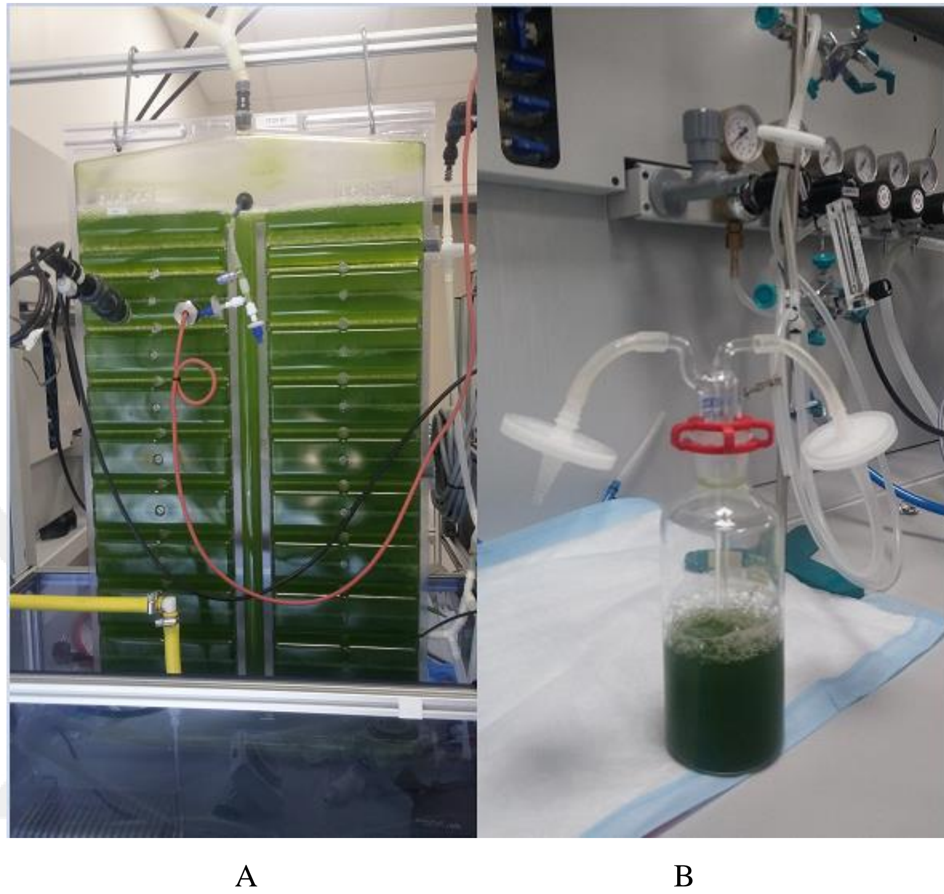


Figure 3.1. *Chlorococcum novae-angliae* strain SAG 5.85 on MB3N agar plate.

3.2. Seed Culturing in Batch Reactors

Seed cultures were prepared axenically in 0,5 L gas wash bottle reactors (Figure 3.2B) operated in batch mode. The reactors were supplied with 0,22 µm filtered air at 0,5 L min^{-1} and provided with 16243 lux light intensity for 14:10 h (light:dark) cycles. Levels of pH of the reactors were kept between 6.5-8. Homogenization within the reactors were achieved by air diffusers and no additional mixing was provided. Working volume for the reactors were 340 mL MB3N medium inoculated with 10 mL maintenance cultures as described in previous section. The seed cultures were grown until they reach mid-exponential growth phase (i.e. 4 to 5 days), and transferred to experimental reactor, i.e. 6 L flat panel photobioreactor (PBR) system (Model 6L FPA, Subitec Gmbh, Stuttgard, Germany) operating under batch reactor conditions as shown in Figure 3.2A. Cell concentrations were measured daily by optical density measurements (taken at 680 nm wavelength) using a spectrophotometer (DR 3900, Hach Lange, Manchester, United Kingdom), and cell counts were determined every other day

by using a hemocytometer (Neubauer, Isolab, Wertheim, Germany) using a light microscope (BM-250, Boeco, Hamburg, Germany).



A B

Figure 3.2. 6 L Photobioreactor (A) and gas wash bottle (B) systems used for experimental and seed cultures respectively.

3.3. Experimental Reactor System

Seed cultures achieving mid-exponential phase were harvested at 4500 rpm for five minutes at ±4℃ using a centrifuge (Model U-320R, Boeco, Hamburg, Germany) and inoculated into 6 L PBR for experimental conditions at 3X10^6 cells/mL concentration. Few drops of polypropylene glycol (Sigma, Germany) as anti-foam agent were added in order to prevent excessive foaming in the reactors. Culture pH was adjusted by pure, filtered CO_2 flow to keep pH values ranging between 7,5-8,0. Temperature was kept at 25 ºC±1 for all experimental conditions (Table 3.3) except temperature stress condition. Temperature was decreased from 25 ºC to 17 ℃ by considering that it would trigger lipid accumulation and PUFA production. There are some studies that maximize PUFA production and lipid content by decreasing temperature more than 10 ℃, however, temperature was dropped just 8 ℃ in this study as there is no temperature stress experiment on *C.novae-angliae*. Also, temperature

could be decreased maximum 8 °C in PBR used in this study. For the nitrogen stress, concentration of $NaNO_3$ was reduced to 1,47 mM from 8,82 mM. Since MB3N's medium contains high levels of nitrogen (Table 3.1), $NaNO_3$ concentration was lowered to be able to create nitrogen stress. Light intensity was decreased from 11100 lux to 5500 lux in order to trigger PUFAs production as some studies showed that the light intensities ranged between 5000-7000 lux maximized growth rate, lipid accumulation, and PUFA production. It was thought that 5500 lux light intensity would lipid content and PUFA production by creating stress on this species which was grown under 11100 lux light intensity. Also, concentration of NaCl was increased 2X fold to generate salinity stress. Although there are some studies present in literature which folded NaCl concentration to more than two times, it was thought that these concentrations might show inhibitor effect on *C. novae-angliae* as this species is terrestrial green microalgae.

Table 3.3. Experimental matrix for tested stress factors.

Parameter	**Control**	**Light**	**Nitrogen**	**Salinity**	**Temperature**
Light Intensity	11100 lux	**5500 lux**	11100 lux	11100 lux	11100 lux
Aeration rate	100 L/min	100 L/min	100 L/min	100 L/min	100 L/min
Temperature	25 ºC±1	25 ºC±1	25 ºC±1	25 ºC±1	**17 ºC±1**
$NaNO_3$ conc.	8,82 mM	8,82 mM	**1,47 mM**	8,82 mM	8,82 mM
NaCl conc.	0,43 mM	0,43 mM	0,43 mM	**0,86 mM**	0,43 mM

3.4. Harvesting of Biomass and Determination of Dry Cell Weight/Growth Rate

Algal biomass reaching early stationary phase on Day 7, was harvested by centrifugation at 4500 rpm for 5 min at ±4°C temperature. Out of the total 6 L biomass, 3 L was separated to obtain wet biomass and stored at -20ºC for further processing. Remaining 3L biomass was harvested and lyophilized (Hypercool, Gyrozen, Frankfurt, Germany) to obtain dried biomass, stored at -80 ºC prior to lipid and fatty acid analyses. Dry cell weight measurements for wet cultures were carried out according to protocol given below:

Aluminum tray with filter paper on glass petri dish was kept in furnace (Daihan Scientific, Wonju, South Korea) at 450°C for 2 hours. Dried filters were kept in desiccator until usage. Following, weight of empty filter papers was recorded. From each experimental condition, 10 ml of algal culture was taken and filtered through 0,45 µm, 47 mm diameter glass fiber filter paper (Merckmillipore, Darmstadt, Germany) under vacuum pressure. Filter paper with biomass kept on paper towel for half an hour for air drying. Later, filter paper with biomass were transferred to furnace

and kept at 105°C overnight and the difference between the empty filter paper was recorded to determine g/L dry cell weight (DCW) for each experimental condition.

Dry weight measurements for lyophilized biomass were measured as below:

1) Prior to harvesting, tare weights of falcon tubes used for centrifugation were recorded.
2) Biomass were harvested using falcon tubes and lyophilized.
3) Falcon tubes with lyophilized biomass were weighed and recorded.
4) Tare weights of falcon tubes were subtracted from the final recorded weight.

Growth rate for each reactor calculated according to equation 3.1 given below:

$$\text{Specific growth rate } (\mu) = \ln(X_1 - X_0)/N_1 - N_2 \quad (3.1)$$

where

X_1: Cell number at the end of exponential phase

X_0: Cell number at the end of exponential phase

N_1: Number of days at the end of exponential phase

N_2: Number of days at the beginning of exponential phase.

3.5. Disruption and Homogenization of Algal Cells

For disruption of cell walls and homogenization of algal cells prior to lipid extraction, bead beating method was used. Mainly, 100 mg of lyophilized algae samples were mixed with 0,3 g of 0,1 mm and 0,1 g of 0,5 mm diameter glass beads and homogenized using a bead beater (Minilys, Bertin Technologies, France) for 60 seconds for 8 times with 60 seconds of cooling intervals on ice. Prior to bead beating process, glass beads were prepared by keeping them in 1 N HNO_3 acid overnight to remove any contaminant. Following day, glass beads were washed five times with deionized water (DIW) and kept in 105 ºC overnight stored in glass petri dishes to remove humidity. Beads and bead beating tubes were autoclaved and kept sterile till usage.

3.6. Extraction and Quantification of Total Lipids

As mentioned in earlier sections, a modified version of Bligh & Dyer method was carried out to extract total lipids from algae samples. This modified method was adapted from (Breuer et al., 2013; Bligh and Dyer, 1959). 100 mg of lyophilized algae biomass from each experimental condition was weighed and homogenized using bead beating as described in section 3.5. Before the extraction, nonadecanoic acid (C19:0) (Sigma Aldrich) at 50 mg/L concentration was prepared to be used as

internal standard (IS) for fatty acid quantification. Nonadecanoic acid was dissolved in chloroform:methanol 4:5 (v/v), and 1 ml of chloroform:methanol containing nonadecanoic acid was added to each beat beating tube along with the algae samples. Then, bead beating was carried out 8 times at 2500 rpm for each tube. After each bead beating, 120 sec intervals were taken to cool the tubes on ice. Following, the extracts were transferred into clean 50 mL glass centrifuge tubes with Teflon insert screw caps. It was important to take all the beads from bead beater tubes into centrifuge tubes to make proper cell disruption. Bead beating tubes were washed at least three times, by adding 1 mL of chloroform:methanol, and glass tubes were briefly vortexed and put into sonication bath (Sonorex Super RK 102 H, Bandelin, Germany) for 10 min. A mixture of 2,5 mL of DIW water containing 50 mM Tris and 1 M NaCl was added to transfer lipids towards chloroform phase. The pH was adjusted to 7 using 1 N HCl solution. Again, tubes were vortexed and sonicated for 10 min. The tubes were centrifuged for 5 min at 1200X*g*. Chloroform phase (bottom) were transferred into clean glass tubes by using glass Pasteur pipettes. Tare weights of the tubes were recorded before adding chloroform. Any interference from top phase was avoided while picking bottom phase. Extraction steps were repeated by adding chloroform to recover lipids completely. Chloroform was evaporated from the sample under N_2 gas stream. After evaporation of chloroform weight of tube containing lipid extracts were measured. Initial record (tare weight of tube) was subtracted from final measurement which provided the amount of extracted lipids.

Percentage of lipid in dry algae biomass calculated as Equation 3.2 provided below:

$$\text{Lipid Content } (\%) = (M_L/M_S) \times 100 \quad (3.2)$$

where

M_L: Lipid mass(g)

M_S: Sample mass(g)

Dried extracts were stored at -20 °C prior to fatty acid methyl esterification (FAME) step described in next section.

3.7. Preparation of Fatty Acid Methyl Esters (FAMEs) by Transesterification of Lipids

For the transesterification of fatty acids, 50 mL of methanol containing 5% (v/v) sulfuric acid was prepared. Next, 3 mL of methanol containing sulfuric acid solution was added into sample tubes containing dried lipids and closed tightly. The samples were incubated in a water bath (Maxturdy-30, Daihan Scientific, Wonju, South Korea) at 70 °C for 3 h and vortexed in every 30 minutes to avoid boiling. Later, samples were cooled and added with 3 mL of DIW water and 3 mL of hexane. Following, the tubes were vortexed and kept in test tube rotator (Wisemix RT-10, Witeg, Wertheim,

Germany) at 15 rpm for 15 min. The samples were then centrifuged at 1200X*g* for 5 min and upper 2 mL of hexane phases were collected and transferred into clean glass tubes. A washing step was done using 2 mL DIW added to each collected hexane. Finally, the tubes were vortexed and centrifuged at 1200X*g* for 5 min (Breuer et al., 2013) and transferred to GC vials stored at -20ºC until analytical analyses.

3.8. Analytical Measurements of FAMEs

Identification and quantitation of FAMEs were conducted on a GC system (7820A, Agilent, USA) equipped with a flame ionization detector (FID). Fatty acid methyl ester samples extracted from all experimental and control samples was analyzed for composition profiling by injecting 5 µl of sample into HP-88 column (Agilent) with dimensions of 100 m length x 0,25 mm diameter x 0,2 µm film thickness in splitless mode. The temperature program run on GC was as follows: initial 140°C hold for 5min; followed by ramp at 4°C/min rate to 240°C with a 15 min hold. Column flow was set at 1 ml min and nitrogen was used as carrier gas set at 280°C. Run time for each sample was appx. 45 min. Identification and quantification of individual FAMEs were done using a standard fatty acids mix (Supelco 37 FAME Mix, Sigma Aldrich) injected at 1 µl using same method. By comparing peak area of reference standard with peak area of target substances; Concentrations of fatty acids in our lipid extracts were calculated. Peak area for each sample was given as Table A1 presented in Appendix A.

3.9. Statistical Analyses

All data obtained from GC analyses for fatty acid composition of *Chlorococcum novae-angliae* expressed as means of triplicates for each parameter and statistically analyzed with two-tailed paired t-test in MS Excel (version 16.42 Microsoft, Redmond, Washington, USA). Results were considered statistically significant at $P < 0.05$.

4. RESULTS AND DISCUSSIONS

In this chapter, *Chloroccocum novae-angliae* cell growth, total lipid content, and fatty acid compositions were discussed with respect to four stress parameters, i.e. light, nitrogen, salinity and temperature.

4.1. Growth Rates and Lipid Content Under Stress Conditions

Cell growth rates of *Chlorococcum novae-angliae* for each experimental condition was evaluated according to cell count, optical density, dry cell weight, and PBR productivity. Cell count numbers, optical density, and pH fluctuations are illustrated in Figure 4.1. Total lipid content for each experiment was measured gravimetrically and calculated on the basis of dry cell weight. Average total lipid contents obtained from experimental conditions ranged between approximately 14-22%. These values were expected as the total lipid of the closest sister species of *Chlorococcum novae-angliae,* i.e. *Chlorococcum sp.*, were reported to be between 6-28% of its dry cell weight (Table 2.2) (Hasan et al.,2016; Mahapatra and Ramachandra 2013). As presented in Table 4.1, total lipid contents of all tested experimental conditions were lower than the control group. Decreases in total lipid contents compared to control group were not statistically significant except light stress. These differences are discussed in detail below with respect to each experimental condition.

Table 4.1. Comparison of dry cell weight, total lipid content, reactor productivity and growth rate of *Chlorococcum novae-angliae* under experimental conditions.

Reactor	**Dry cell weight (g/L)**	**Total lipid (% dry weight)**	**Reactor productivity (g/L)**	**Growth rate (cells.d^{-1})**
Control	0,48±0,34*	21,77±3,48	0,33±0,17	0,61
Light stress	0,14±0,13	16,33±0,71	0,15±0,01	0,23
Nitrogen stress	0,37±0,01	13,93±7,04	0,35±0,04	0,45
Salinity stress	0,28±0,06	17,40±2,90	0,28±0,04	1,32
Temperature stress	-	-	0,0041	-

*Results are the expressed as one standard deviation (n=3).

4.1.1. Effects of Light Stress

First of all, light intensity is a crucial factor that affects microalgae growth and lipid production considerably as it directly impacts photosynthetic activity and CO_2 uptake by changing the levels of photosynthetic pigments (Khan et al., 2018). Since high light intensity create oxidative stress on PUFA production, reduced light intensity was preferred for this study. A study by Daliry et al., showed that cultivation of *C. vulgaris* under 5000-7000 lux maximized lipid accumulation and growth rates (Daliry et al.,2011). Another study conducted by Khan and colleagues provided that highest biomass and carbohydrate yields were obtained by using red LED light source at 5000 lux (corresponding to appx. 90 μ mol $m^{-2}s^{-1}$) during the cultivation of *Microcystis aeruginosa* (Khan et al., 2016). A Study by Ifeanyi et al., reported that highest optical density was obtained for Aphanocapsa algal population under 5000 lux on 9th day of cultivation (Ifeanyi et al., 2011). Also, Krzemińska and colleagues found that *Scenedesmus obliquus* and *B. braunii* maximized their growth rates under continuous illumination (Krzemińska et al. , 2014). Another study conducted by Ota et al., found that growth rate of *Chlorococcum littorale* was the highest under 100 μ mol $m^{-2}s^{-1}$ (Ota et al., 2015). Rehman and Anal determined optimal light intensity as 4340 lux for *Chloroccoccum* sp. TISTR 8583 (Rehman and Anal, 2019). Therefore, it was assumed that *Chloroccoccum novae-angliae* would increase growth rate and lipid content under continuous 5500 lux light intensity. In this study, the cell numbers under light stress reached appx. $4X10^6$ cells/ml on the 6th day of cultivation (Figure 4.1A). When cell growth rates were compared among each experimental condition, low light stress, i.e. 5500 lux, resulted in the lowest cell growth rate of 0,23 cells.d^{-1} (Table 4.1), yet, total lipid content obtained from light-stress reactor as 16,33±0,71 (% dry cell weight) was not significantly less than other experimental stress conditions except the control group (Table 4.1).

Dry cell weight (0,14±0,13 g/L) and reactor productivity (0,15±0,01) under light-stress were also the lowest compared to other experimental conditions (Table 4.1). Therefore, it was clearly inferred that cell growth was suppressed under low light intensity in *Chlorococcum novae-angliae*. These findings were contradictive to other studies which investigate impacts of light on microalgal growth and lipid content. Therefore, further research needs to be carried out in order to optimize light intensity for proper growth and lipid production of *C. novae-angliae*. Based on the reported values for the control group, 11000 lux light intensity was the optimal for the cells as they showed highest growth rate, lipid yield, and dry cell weight (Table 4.1). However, it should be noted that, only white visible lights were used and the impacts of different wavelengths, such as red light which is favored for enhancing photosynthetic activity, carbohydrate production, and cell growth in literature was not utilized in this study. Besides, effects of non-continuous illumination on *C. novae-agliae* and light

saturation intensity point which maximizes growth rate of microalgae should be carried out for *C. novae-agliae*.

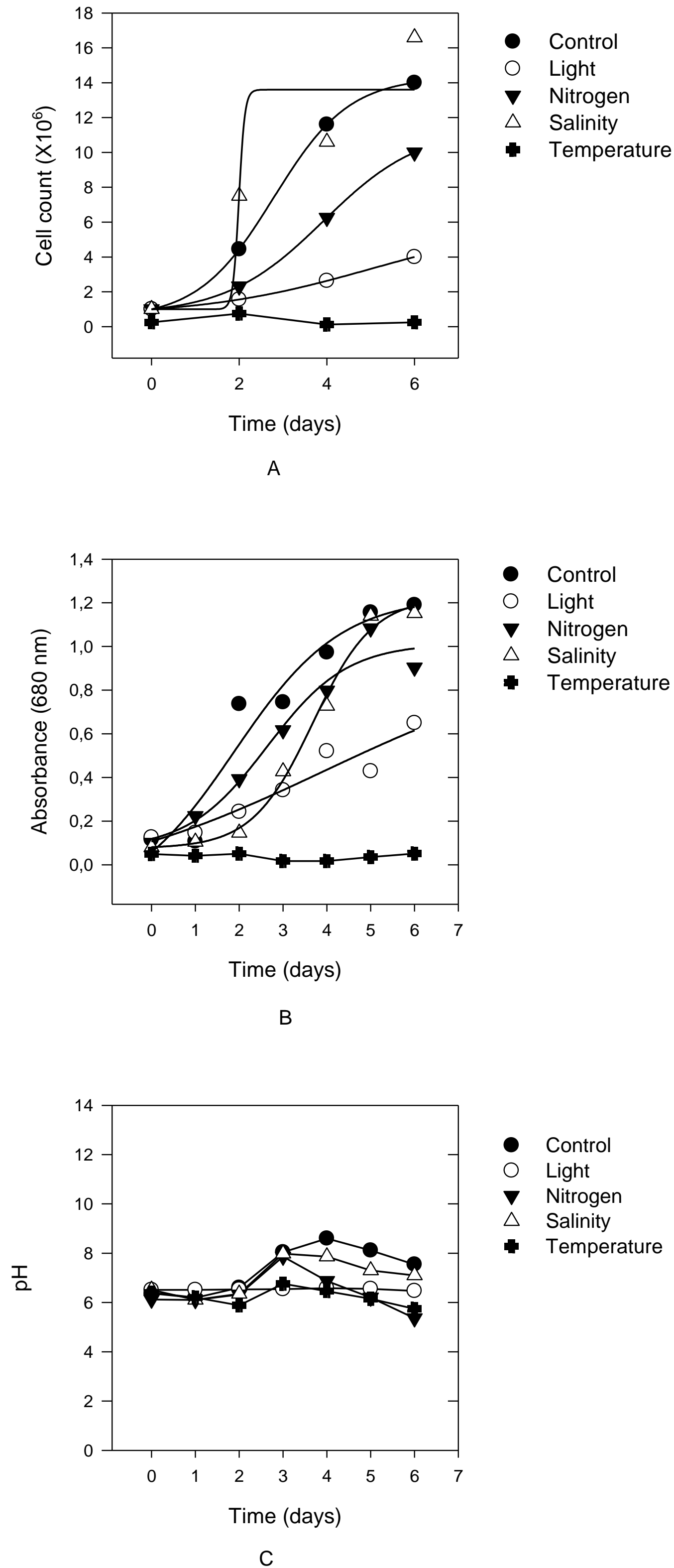


Figure 4.1. Cell count (A), optical density (B), and pH Measurements (C) of *C. novae-agliae* under tested conditions.

4.1.2. Effects of Nitrogen Stress

Nitrogen is one of the most important limiting nutrients that influence microalgal growth and lipid accumulation. In this study, a nitrogen-stress of 1,47 mM, corresponding to one sixth of nitrogen concentration of original MB3N medium was used to trigger lipid production in *C. novae-agliae* cells since there are numerous studies in literature suggesting nitrogen limitation enhances lipid production in microalgae. For example, Rehman and Anal reported that lipid content of *Chlorococcum sp*. TISTR 8583 was increased by 29,59% under nitrogen limitation compared to the control while cell dry weights were lowered (Rehman and Anal, 2019). Li and co-workers found that *Neochloris oleoabundans* cells growing in soil extract medium which contains 3 mM $NaNO_3$ had the highest lipid content (Li et al., 2008). Also, they obtained the highest biomass concentration (3,2 g/L) with exposure 10 mM $NaNO_3$. Therefore, they stated that $NaNO_3$ is one of the most effective nitrogen sources for the optimal growth and lipid productivity of *N. oleoabundans* compared to other nitrogen sources such as KNO_3 and urea. They explained this situation with the early running out of external nitrogen at low cell density that led to more photosynthetic activity since the cells had been exposed to more light penetration resulting in higher lipid yields. Also, Bona et al., stated that highest biomass yield was observed under nitrogen replete conditions, i.e. 0,01M $NaNO_3$, however, they obtained higher lipid content from *Neochloris oleoabundans* with nitrogen limited conditions ($2{,}9x10^{-4}$ M) (Bona et al., 2014). Tornabene et al., obtained better lipid yield (54% dry weight) by growing *Neochloris oleoabundans* in 0,5 mM $NaNO_3$ in media (Tornabene et al., 1983). A Study by Kiran and colleagues reported that lipid yield of *Chlorella sp*. was maximized with BG-11 medium containing 5mM $NaNO_3$ which means one third of nitrogen of original medium (Kiran et al., 2016). Shen et al., investigated impacts of nitrogen sources and concentrations on *Scenedesmus dimorphus* and *Chlorella protothecoides* (Shen et al., 2009). While *S. dimorphus* produced highest lipid and biomass with high concentrations of urea, low KNO_3 concentrations led to higher lipid production in *C. protothecoides* cells. Chen and colleguaes observed effects of urea and nitrate on lipid production in green microalgae *Dunaliella tertiolecta* by using Nile Red Staining (Chen et al., 2011). They underlined that increased concentrations of urea inhibited cell growth while high levels of nitrate in media did not show inhibitory effects on microalgal growth. Also, low concentration of nitrate caused increased cell size and high lipid yield according to their study (Chen et al., 2011). Another study conducted by Dean and colleguaes confirmed that cell sizes of *C. reinhardtii and Scenedesmus subspicatus* were increased under nitrogen starvation while cell growths were limited (Dean et al., 2010). They determined lipid to amide ratio by Fourier Transform Infrared Micro-spectroscopy (FTIR) and found out that lipid toamid ratio were increased under nitrogen deficiency. Illman et al., reported that all *Chlorella* strains used in study, i.e. *C. vulgaris* Beijerink CCAP 211/11B, *Chlorella*

emersonii Shihira and Kraus CCAP 211/11N, and *Chlorella protothecoides* Kruger CCAP 211/8D, reduced dry weights under nitrogen starvation compared to the control while they increased lipid productivity significantly (Illman et al., 2000). Despite the increases in lipid accumulation, many studies also mention that nitrogen limited conditions might lead to growth inhibition (Shen et al., 2009). With particular interest to *Chlorococcum* species, a study conducted by Li et al., examined impacts of the type of nitrogen sources and concentrations on growth rates and lipid productivity of *Chlorococcum ellipsoideum* UTEX 972, *Chlorococcum nivale* UTEX LB2225, *Chlorococcum tetranse* UTEX 2227, and *Scenedesmus deserticola* (Li et al., 2013). They exposed those species to 5,8 mM $NaNO_3$ (nitrogen limitation), 17,6 mM $NaNO_3$ (nitrogen-replete), 2,9 mM urea (nitrogen limitation), and 8,8 mM (nitrogen-replete) conditions. They observed that all microalgae species exposed to nitrate deficiency decreased growth rates. The maximum biomass yield was obtained with *C. ellipsoideum* under urea-nitrogen deficiency compared to other experimental conditions. Also, this species increased lipid content under urea-nitrogen deficiency on the 16th day of cultivation. It was underlined that all species enhanced lipid productivity under nitrate-nitrogen deficiency compared to nitrate-nitrogen replete conditions. Another remarkable outcome from this study was the importance of cultivation time on lipid productivity because all species gave the highest lipid yields on the 16th day of cultivation (Li et al., 2013). It could be inferred that effects of type of nitrogen source and its concentrations and cultivation time on microalgal growth and lipid production vary in different species. Moreover, Li and colleagues mentioned that there are three types of responses to nitrogen deficiency such as Type A, Type B, and Type C (Li et al., 2013) The microalgae cells which enhance lipid productivity, but show decreases in biomass are categorized into Type A, the most common behavior under nitrogen stress. In Type B, algae cells decrease both biomass and lipid content under nitrogen stress. According to their study, *C. tetranse* which show reduction in both biomass and lipid contents independent from nitrogen sources were involved in Type B. However, there are no sufficient data for Type B pattern in literature as they mentioned. In Type C, both lipid content and biomass productivity are enhanced by nitrogen deficiency. Therefore, Type C microalgae cells are very suitable for large scale biofuel production.

In this study, both cell numbers and total lipid content were decreased under nitrogen stress compared to nitrogen-replete control group, thus, it could be inferred that *C. novae-angliae* followed Type B regime. In detail, algae cells exposed to nitrogen stress decreased their dry weights to 0,37±0,01 g/L (Table 4.1). Lipid accumulation, i.e. 13,93±7,04 %dry weight, lower than control group though the decrease was not statistically significant. Cell numbers under nitrogen-stress reached $1X10^7$ cells/ml on the 6th day of cultivation (Figure 4.1A). Growth rate of 0,45 $cells.d^{-1}$ was approximately two-fold higher than the cells under light nitrogen stress, yet lower than the control

(Table 4.1). Nevertheless, reactor productivity of 0,35±0,04 g/L was slightly higher compared to the that of the control group (Table 4.1). As there is no sufficient data present about effect of nitrogen on growth and lipid production of *C. novae-angliae* in literature, further investigations should be carried out to determine the best type of nitrogen source and its concentrations to maximize lipid content in *C. novae-angliae*. In this study, 1,47 mM $NaNO_3$ was used as nitrate-nitrogen source which might be insufficient for the enhanced lipid production as control cells grown in MB3N media had higher nitrogen concentration, i.e. 8,82 mM $NaNO_3$. This reduction in nitrogen concentration might have led to severe stress on microalgae cells and decreased lipid accumulation. In future studies, different type and concentrations of nitrogen should be tested on *C. novae-angliae* cells. Moreover, determination of cultivation time is another important parameter to maximize accumulated lipids. Harvested cells on the 6th day of cultivation on this study might be too early for cellular response to nitrogen stress in terms of accumulating lipids.

4.1.3. Effects of Salinity Stress

Salinity stress (by exposing *C. novae-angliae* cells to NaCl concentration of 0,86 mM) was the other stress condition tested in this study was with the assumption to reach increased levels lipid production. Salinity is a crucial abiotic factor which affects microalgal growth and biochemical composition of microalgae (Juneja et al., 2013b). Some studies showed that lipid production in microalgae could be induced by increased salinity levels (Fábregas et al., 1984; Zhila et al., 2011). In a study by Rismani and Shariati total lipid production was enhanced by 82% in *C. vulgaris* (Rismani and Shariati, 2018). In another study conducted by Asulabh and collugues , growth rates and lipid productivities of *Chloroccoccum sp*., *Microcystis sp*., and *Chaetoceros* were observed under salinity stress (Asulabh et al., 2012). They found that all growth rates were maximized on the 2nd and 3rd day, followed by dramatic decrease in following days. Duan and co-workers reported that lipid production was enhanced by adding different concentrations of NaCl at initial days of cultivation, however, cell growth inhibition was observed in following days in *C. vulgaris* (Duan et al., 2012). From these studies, it can be inferred that, similar to nitrogen stress, cultivation time is an important parameter for salinity stress as well. In another study conducted by Takagi and Yoshida reported that increased lipid content in *D. tertiolecta* was observed by changing NaCl concentration from 0,5 M to 1 M (Takagi and Yoshida, 2006). Interestingly, *B. braunii* as a freshwater algae increased lipid content, growth rate, and carbohydrate production under high salinity levels. Yet, its biomass concentration was maximized at lowest salinity level (Rao et al., 2007). Also, another study carried out by Vazquez-Duhalt and colleagues found that increased salinity levels led to decreases in protein content of *B. braunii* (Vazquez-duhalt et al., 1991), however, lipid productivity was increased. Ben-Amotz et al.,

observed that lipid productivity of *B. braunii* was increased by artificial media containing 0,5 M NaCl (Ben-Amotz et al., 1985).

In relationship to salt concentrations, pH is an essential parameter which influence metabolic processes of microalgae throughout the cultivation, since it affects availability of inorganic carbon sources by changing their solubility in culture growth media (Goldman, 1973). In a study by Alyabyev and colleagues, the impacts of changes in pH and NaCl concentrations in growing media on oxygen trade off rates and generation of heat were investigated (Alyabyev et al., 2011). They reported that pH was shifted to alkaline levels during cultivation of *C. vulgaris* and *Dunaliella maritima*, however, adding NaCl to media decreased pH levels and resulted in acidification. Also, it was mentioned that low concentrations of NaCl led to increase in pH for a while following a decrease compared to pH level of the control group (Alyabyev et al., 2011). In this study, experimental reactors were dosed with CO_2 when pH levels increased due to photosynthetic activity. Therefore, despite culture media contained increased levels of NaCl for the salinity stress, acidification did not occur. During the cultivation of *C. novae-angliae*, pH only shifted from 6,5 to 7,1 under salinity stress.

With respect to lipid contents, *C. novae-angliae* cells exposed to saline environment accumulated the highest lipid content, i.e. 17,4±2,9% dry cell weight, compared to other stress conditions, despite being lower than the control group (Table 4.1). Cell count numbers under salinity stress reached to $1,6X10^7$ cells/mL (Figure 4.1A). However, dry weights (0,28±0,05 g/L) of *C. novae-angliae* cells under salinity-stress were decreased compared to control (Table 4.1). Being exposed to increased salinity levels might have led to decreased photosynthetic activity since it creates additional osmotic stress on *C. novae-angliae* cells which are acclimated to terrestrial ecosystems. Meanwhile, the growth rate (1,32 $cell.d^{-1}$) under salinity stress was the highest compared to other experimental conditions. This trend was contrary to other studies such as Asulabh and colleagues mentioned that increased salinity could decrease growth rate of *Chlorococcum sp*., *Microcystis sp*., and *Chaetoceros sp*. (Asulabh et al., 2012). Yet, Juneja et al. underlined that maximal growth rates were observed in neutral pH (Juneja et al., 2013). As pH was also kept close to neutral pH levels in this study, higher growth rates are feasible. Although cell numbers under salinity stress conditions were spiked between 1th and 2th days, total lipid contents were not significantly higher than light reactor which had lowest growth rate and dry weight (Table 4.1).

Overall, fortifier impact of salinity on lipid productivity was observed compared to other stress conditions, albeit, total lipid content was still lower than the control reactor (Table 4.1). This result might be attributed to failure to determine appropriate concentration of NaCl to create intensive stress

on *C. novae-angliae*. Since there is no specific study for optimal NaCl concentration of *C. novae-angliae* in order to trigger lipid productivity, NaCl level in medium was only increased moderately in this study. As future investigation, *C. novae-angliae* cells could be exposed varying levels of NaCl concentrations in order to understand upper and lower limits of *C. novae-angliae* by observing growth rate and changes in biochemical composition.

4.1.4. Effects of Temperature Stress

Low cultivation temperatures, specifically 17 °C, which is lower than normal cultivation temperature of 25 °C, on microalgal growth and lipid accumulation. As temperature is one of the most important factors that have crucial impacts on algal membrane fluidity, permeability, and photosynthetic/respiratory actions, fatty acid compositions of microalgae are substantially affected by temperature shifts (Juneja et al., 2013; Sharma et al. 2012). In addition, enzymatic activities and membrane functions are very sensitive to thermal fluctuations. For instance, a very common trend in most microalgae and cyanobacteria species is that decreases in temperature cause increments in fatty acid unsaturation because membrane fluidity is lowered at low temperatures (Sharma et al., 2012). In fact, increases in degree of unsaturation under low temperatures are considered as adaptive responses to cold conditions. The main reason lies in the fact that, double bonds in unsaturated fatty acids ease membrane fluidity compared to saturated fatty acids which are tightly congested (Sharma et al., 2012). Whereas, increases in saturated fatty acids are mostly seen at high temperatures. Increased levels of unsaturated fatty acids in green microalgae to low temperatures has been widely known (Thompson, 1996). For example, impacts of low temperature on biochemical composition of *D. tertiolecta* have been broadly studied. In a study by Thompson, it was observed that decrease in cultivation temperature from 30 °C to 12 °C caused considerable increases in unsaturated fatty acids by 20% (G. A. Thompson, 1996). In another study completed by Sushchik et al., , it was reported that temperature shift from 25 °C to 30 °C decreased unsaturated fatty acid levels for both *C. vulgaris* and *B. braunii* (Sushchik et al., 2003). In another study carried out by Joh and colleagues, it was observed that lower temperatures ranged between 3 °C-7 °C resulted in higher unsaturated fatty acids by two-fold in *Chlorella ellipsoidea* (Joh et al., 1993). Modifications in fatty acid composition under low temperatures for eight species were examined in a study made by P. A. Thompson et al., (1992). They found that PUFAs levels in *Chaetoceros calcitrans, Chaetoceros gracilis, Chaetoceros simplex, Dunaliella tertiolecta, Isochrysis galbana, Pavlova lutheri, Phaeodactylum tricornutum, Thalassiosira pseudonana* were increased by shifting temperature 25 °C to 10 °C. Also, Aussant and coworkers investigated impacts of temperature on microalgal growth and lipid production for eight

microalgae species. They concluded that optimal temperature for the optimized production of EPA from *Dunaliella salina* ranged between 14 °C-20 °C (Aussant et al., 2018).

Despite several positive examples of lower temperature stress on lipid accumulation in literature, the growth of *C. novae-angliae* cells were inhibited suddenly on the very first day of cultivation in this study. Despite the inoculation of cultures with exact same cell numbers as other experimental stress conditions, optical density and cell counts were decreased in 30 minutes. The color of cultures in PBR turned into lighter yellowish-green indicating unhealthy photosynthetic response. The PBR was not immediately stopped with the expectation that *C. novae-angliae* cells would adopt to colder temperatures with time. However, cultures did not recover and seem healthy at the end of the seven days of cultivation. Biomass production was quite low and not sufficient for further characterization analyses. Both cell density and cell numbers were decreased dramatically compared to other parameters as it can be seen in Figure 4.1A and 4.1B respectively. Therefore, temperature stress condition has been excluded from the study with the conclusion that *C. novae-angliae* cells exposed to lower temperatures, i.e. 17 °C, compared to normal cultivation temperature of 25 °C negatively impacts culture growth.

4.2. Fatty Acid Composition of *Chlorococcum novae-angliae* Under Stress Conditions

As the main objective of this study, fatty acid composition of *Chlorococcum novae-angliae* was determined under stress conditions of light, nitrogen, and salinity. As explained in earlier, temperature stress conditions did not generate sufficient biomass for fatty acid analyses.

4.2.1. Saturated Fatty Acid Profile of *Chlorococcum novae-angliae*

Among all tested conditions including the control group, palmitic acid (C16:0) and stearic acid (C18:0) were found to be the most dominant ones with highest FA concentrations (Table 4.2). Similar to most other oleaginous green algae species, this result suggests that *Chlorococcum novae-angliae* could be a good candidate for biodiesel production, despite the fact that is biofuel potentiality is out of the scope of this study.

Table 4.2. Saturated Fatty Acid (SFA) Profiles of *Cloroccocum novae-angliae* under tested conditions.

Fatty Acids	Fatty acid Composition (ng/µl)			
	Control	**Light**	**Nitrogen**	**Salinity**
C6:0	1,62±2,8	2,20±3,12	0,47±0,82	1,62±1,23
C8:0	6,04±0,68	3,07±0,9	5,94±2,42	0,51±0,22
C10:0	0,64±0,55	0,33±0,3	0,2±0,02	0,75±0,22
C11:0	0,33±0,05	0,3±0,08	0,07±0,12	0,03±0,05
C12:0	0,74±0,31	0,45±0,18	1,28±0,23	0,17±0,07
C13:0	1,49±1,43	1,84±0,78	0,22±0,03	0,54±0,69
C14:0	38,83±3,46	27,03±9,37	46,45±6,58	7,92±4,64
C15:0	4,55±0,85	3,49±1,25	5,65±0,76	1,25±0,65
C16:0 (palmitic acid)	**344,1±20,57**	**270,45±81,12**	**492,46±61,26**	**87,65±45,92**
C17:0	17,44±3,35	10,33±4,64	19,87±19,87	3,88±2,09
C18:0 (stearic acid)	**47,28±3**	**28,87±10,01**	**78,45±8,11**	**21,06±10,54**
C20:0	13,21±1,97	7,53±1,89	17,52±2,55	3,44±1,63
C21:0	2,72±0,3	1,16±1,11	1,84±1,05	0,75±0,95
C22:0	5,32±1,24	3,53±2,64	6,51±0,81	6,25±5,84
C23:0	9,24±8,88	4,38±3,93	10,88±15,68	3,39±2,02
C24:0	9,24±4,52	1,15±0,44	12,68±19,68	9,02±10,6
Sum of SFAs	**497,81±27,40**	**366,11±103,92**	**700,50±96,3**	**148,23±77,3**

As can be seen in Table 4.2, total SFA concentration (366,11 ng/µl) was significantly decreased under low light intensity stress condition to compared to control by 26,4%. Expectedly, concentrations of both palmitic acid and stearic acid, which are important precursor SFAs for PUFA synthesis, were decreased by 21,4% and 38,9% compared to control respectively. Seyfabadi and colleagues reported that highest SFAs concentrations were obtained under 62,5 µmol photons $m^{-2}s^{-1}$ light intensity by 16:8 h light:dark cycle in *C. vulgaris* (Seyfabadi et al. 2011). They also mentioned that increased light duration led to elevated levels of SFAs. In this study, continuous illumination was used which might have caused photo-inhibition in *Cloroccocum novae-angliae*, as SFAs were higher in control reactor which had been illuminated with 11000 lux (app. 180 µmol µmol photons $m^{-2}s^{-1}$) and light stress reactors were illuminated with 5500 lux (app. 90 µmol µmol photons $m^{-2}s^{-1}$) continuously.

Under nitrogen stress, total concentration of SFAs were increased by 40,7% compared to the control group. Despite the decrease in total lipid content, nitrogen stress had significant impact on

enhancement of SFAs production, suggesting that free fatty acid (FFA) content was the highest for nitrogen stress conditions. Similar trends in increased levels of SFAs in *Neochloris oleoabundans* under nitrogen limitation had been reported by Tornabene et al., (1983). Hulatt et al., (2017) also observed increases in SFA concentrations from 30 $mg.g^{-1}$ to 122 $mg.g^{-1}$ in *Nannochloropsis* 211/78 (CCAP) under nitrogen limited conditions.

Lowest levels of SFAs were observed under salinity stress condition (Table 4.2). Total saturated fatty acid concentration was significantly decreased by 70,22% under salinity stress compared to control group. As mentioned earlier, this was unexpected and contradictory to literature as in a study by Xu and Beardall, shifting NaCl concentration from 0,4 M to 4 M has led to increased levels of SFAs in *Dunaliella* sp. (Xu and Beardall 1997). Also, Azachi et al., (2002) reported that higher ratio of stearic acid to palmitic acid was observed in *D. salina* cells growing in media containing 3,5 M NaCl. However, this ratio was quite lower compared to other stress parameters used in this study. Nevertheless, as mentioned earlier, *C. novae-angliae* is a terrestrial species and its tolerance to salt stress may not have been as strong as marine microalgae species such as *Dunaliella* as reported in these studies.

4.2.2. Monounsaturated Fatty Acid Profile of *Chlorococcum novae-angliae*

Apart from SFAs, monounsaturated fatty acid (MUFA) levels for each experimental stress condition were determined. Under low light intensity stress condition, oleic acid (C18:1n9c), an important ω-9 fatty acid, concentration was reported to be 26,60 ng/µl and was slightly decreased compared to the control group (Table 4.3). However, total concentration of MUFAs under light stress was reported as 393,61 ng/µl and increased by 14,4% compared to control group, despite the increase was not statistically significant.

Table 4.3. Monounsaturated fatty acid (MUFA) profiles of *Chlorococcum novae-angliae* under tested conditions.

Fatty Acids	**Fatty acid Composition (ng/µl)**			
	Control	**Light**	**Nitrogen**	**Salinity**
C14:1	28,53±6,68	18,29±13,39	10,42±2,12	0,97±0,55
C15:1	0,38±0,28	0,29±0,05	0,46±0,32	4,17±3,69
C16:1	11,04±1,32	8,32±2,81	27,2±5,24	3,08±1,74
C17:1	1,87±0,74	1,42±0,81	2,52±0,32	0,73±0,34
C18:1n9t	32,73±55,57	11,71±19,28	0,67±0,25	12,46±13,38
C18:1n9c (oleic acid)	**32,70±5,19**	**26,60±7,2**	**136,17±18,89**	**4,24±2,14**
C20:1	235,01±403,15	325,84±323,94	566,10±489,5	1,25±1,41
C24:1	1,67±1,89	1,15±0,71	4,25±1,96	4,56±2,01
Sum of MUFAs	**343,92±377,88**	**393,61±327,42**	**747,79±512,61**	**31,46±13,48**

As can be seen in Table 4.2, among all experimental conditions, highest oleic acid and total MUFA concentration levels were found under nitrogen limited stress conditions. Oleic acid concentration was significantly increased almost three fold compared to the control reactor and total MUFA concentrations under nitrogen stress reactor increased by 117% compared to the control. Meanwhile, lowest oleic acid and total MUFA concentrations were found to be under salinity stress conditions (Table 4.3). Total MUFAs content was found to be 31,36±13,48 ng/µl and decreased by 90,85 % compared to the control. Also, oleic acid concentration was reported as 4,24±2,14 ng/µl. It was observed that 87,03% reduction in concentration of oleic acid compared to the control under salinity stress. Ben-Amotz and collegues had reported that salt stress had crucial impact on synthesis of oleic acid in *B. braunii and I. galbana* (Ben-Amotz et al., 1985). They reported that increased concentration of NaCl in growing media resulted in elevated levels of oleic acid for these two species. However, salinity stress impaired oleic acid production in this study.

4.2.3. Polyunsaturated Fatty Acid Profile of *Chlorococcum novae-angliae*

In addition to essential omega fatty acids, all polyunsaturated fatty acid (PUFA) levels for each experimental stress condition were also determined. Per total PUFA content, highest concentrations were obtained in control group with 803,35 ng/µl (Table 4.4). Second highest levels of total PUFAs were observed in the cells exposed to nitrogen stress with 747,79 ng/µl, 7,74% lower than the control. Lowest levels of total PUFAs content, i.e. 263,88 ng/µl, were observed in the cells under salinity stress (Table 4.4). Xu and Beardall also observed a similar trend in *Dunaliella sp.* reported that shifting NaCl concentration from 0,4 M to 4 M decreased PUFAs levels (Xu and Beardall, 1997).

Table 4.4. Polyunsaturated fatty acid (PUFA) profiles of *Chlorococcum novae-angliae* under tested conditions.

Fatty Acids	Fatty acid Composition (ng/µl)			
	Control	**Light**	**Nitrogen**	**Salinity**
C18:2n6t	85,08±7,97	53,6±17,74	75,19±10,95	38,54±14,48
C18:2n6c (LA)	**222,50±63,13**	**152,54±70,18**	**311,25±48,58***	**38,33±18,28***
C18:3n6 (GLA)	**98,23±20,97**	**74,2±31,73**	**137,15±21,41***	**23,81±11,84***
C18:3n3 (ALA)	**381,09±334,90**	**191,08±329,27**	**164,92±285,01**	**134,18±60,67**
C20:2	1,09±0,38	0,61±0,5	0,72±0,24	3,05±2,56
C20:3n6 (DGLA)	**1,22±1,07**	**0,85±0,04**	**0,73±0,40**	**0,21±0,06**
C20:3n3/C22:1n9	4,46±1,41	7,57±7,24	3,35±0,36	1,91±1,75
C20:4n6 (AA)	**1,24±1,14**	**13,60±9,15**	**13,95±11,81**	**6,32±9,30**
C22:2	24,97±22,20	3,41±4,88	0,32±0,05	2,35±1,98
C20:5n3 (EPA)	**5,58±7,14**	**17,62±24,15**	**32,76±23,77**	**15±23,63**
C22:6n3 (DHA)	**2,87±2,90**	**3,12±4,29**	**0,76±0,35**	**0,21±0,25**
Sum of PUFAs	**803,35±272,857**	**522,59±402,67**	**741,10±222,7**	**263,88±120,51**

*Denotes the change is statistically significant with $P < 0,05$.

Total PUFAs was to be found 522,59±402,67, decreased by 34,94 compared to the control under low light intensity. Apart from that, there are certain studies present in literature suggesting that low light intensities lead to increased levels of PUFAs whereas PUFAs were decreased with low light intensity in our study. For instance, Sukenik and coworkers observed that PUFAs levels were increased by decreasing light intensity in *Nannochloropsis* sp (Sukenik et al., 1989) Although PUFAs were damaged by oxidative stress under high light intensities, some studies reported that PUFAs levels were increased by high light intensities.

4.3. Changes in Omega-3 and Omega 6-Fatty Acid Levels Under Stress Conditions

4.3.1. Omega 3-Fatty Acid Profile of *Chlorococcum novae-angliae*

Optimization of ω-3 and ω-6 fatty acid production in *Chlorococcum novae-angliae* exposed to various stress conditions was the main objective of this study. Average concentrations for each of ω-3 and ω-6 fatty acids were already presented in tabulated form in Table 4.4 above. As depicted more specifically in Figure 4.2, concentration of DHA (C22:6n3) among all experimental stress conditions increased only under light stress condition by 8,7% compared to the control despite the fact that this increase was not statistically significant. Peak area response of DHA in GC chromatograms were very small and almost undetectable (provided as Figure B2, B3, and B4 in Appendix B). In terms of detected DHA levels in *C. novae-angliae,* the results were lower than expected. For the only available study in literature, Lang et al., detected DHA levels in *Chlororococcum novae-angliae* to be 18,9%

of all total fatty acids which was the second highest level among SAG culture collection strains (Lang et al., 2011).

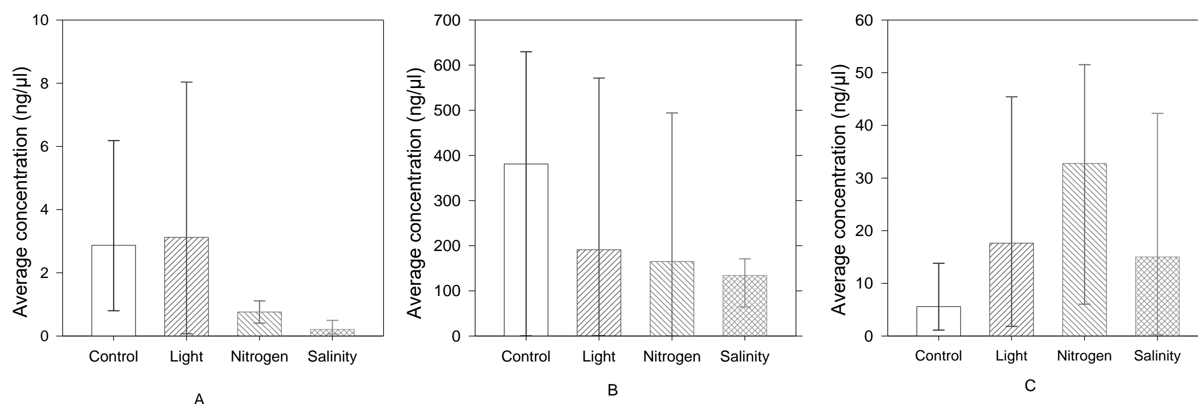


Figure 4.2. Average concentrations of ω-3 fatty acids in *Chlorococcum novae-angliae* exposed to stress conditions (A: DHA, B: ALA, C: EPA).

Both salinity and nitrogen stress conditions negatively affected the production of DHA levels in *C. novae-angliae* (Figure 4.2). This was rather the trend in literature as Rismani and Shariati reported that DHA was not detected in *D. salina* exposed to 0,5 M NaCl salinity stress (Rismani and Shariati 2017). Also, DHA was not detected in *Ankistrodesmus sp.*, *B.braunii*, *D. bardawil*, *D. salina*, *Nannochloris sp*., *Nitzschia sp*. under salt stress in a study by Ben-Amotz et al., (1985). Overall, salinity stress seems to have negative impact on production of DHA in green microalgae species.

The second investigated essential ω-3 fatty acids ALA (C18:3n3) which has a molecular structure making it harder to separate analytically in GC as it overlaps with eicosenoic acid (C20:1). Therefore, ALA peaks in the chromatograms were not fully and clearly separated from C20:1 even in standard (Appendix B, Figure B1). Therefore, it should be noted that reported concentrations of ALA for each experimental condition include summed concentration of ALA (C18:3n3) and eicosenoic acid (C20:1). That is being said, ALA was dramatically decreased compared to control reactor under all stress conditions (Figure 4.2B). Alpha-linoleic acid concentration was to be found as 191,08±329,27 ng/µl under low light intensity. Also, low nitrogen concentration in growing medium decreased ALA concentration, i.e. 164,92±285,01**,** by 56,72%. However, Bona and co-workers found that ALA content was increased on 4th and 7th of cultivation of *Neochloris oleoabundans* under nitrogen deplete conditions (Bona et al. 2014). Lowest concentration of ALA was observed under salinity stress, i.e. 134,18±60,67. Rismani and his colleague found that shifting NaCl concentration from 0,5 M to 1,5 M provided highest ALA content in *D. salina.* Also, they reported that growing *C. vulgaris* in media containing 200 mM NaCl resulted in higher ALA content

(Rismani and Shariati 2017). Inversely, ALA concentration under salinity stress was reduced compared to the control by 64,79%.

The third investigated essential ω-3 fatty acid, i.e. EPA (C20:5n3), concentrations increased under all experimental stress conditions (Figure 4.2C). The highest increase was observed for cells under nitrogen stress with 486,67% compared to the control group, though this change was not statistically significant due to high variability among replicates. Hulatt and collegues reported that EPA concentration was increased on 8th of cultivation compared to the control (Hulatt et al. 2017). Low light intensity provided increment in EPA content, i.e. 17,62±24,15, by 215,77%. This finding is supported by another study conducted by Van Wagenen et al. (2012). They reported that reduced light intensity provided elevated levels of EPA (Van Wagenen et al., 2012). Also, the cells under salinity stress increased EPA content, i.e. 15±23,63, by 168% compared to the control. Rismani and Shariati observed that high saline environments triggered accumulation of EPA in *D. salina*. Also, same study stated that *C. vulgaris* cells increased EPA levels compared to the control (Rismani and Shariati 2017). A similar analytical resolution problem was also experienced with EPA with C20:5n3 structure elutes at similar times with mycosanoic acid (C24:0) (Appendix B). As a result, EPA concentrations might be artificially increased, and results should be evaluated with caution.

4.3.2. Omega 6-Fatty Acid Profile of *Chlorococcum novae-angliae*

Among all tested experimental conditions, the most important essential ω-6 fatty acid, i.e. LA (C18:2n6c), concentrations increased significantly by 39,8% under nitrogen limited conditions compared to the control group (Figure 4.3A). Although there are some studies reporting that decreases in LA content by reduced nitrogen concentration in growing media, LA concentration was affected positively under nitrogen limited conditions. For instance, Breuer and colleagues reported that LA concentrations were decreased under nitrogen deplete conditions in *C. vulgaris, C. zofingiensis, D.tertiolecta, I. galbana, Nannochloropsis sp., N. oleoabundans* (Breuer et al. 2012). Also, Hulatt and co-workers showed that LA content was decreased on 8th day of cultivation under nitrogen limited conditions in *Nannochloropsis* 211/78 (CCAP) (Hulatt et al., 2017). Under low light intensity, LA concentration, i.e. 152,54±70,18 ng/µl, was decreased by 45,86% compared to the control. In a study by Wacker and colleagues, it was reported that LA concentration was higher under 40 µmol photons $m^{-2}s^{-1}$ compared to 300 µmol photons $m^{-2}s^{-1}$ in *Chromulina sp* (Wacker et al. 2016). However, other species, i.e. *Asterionella formosa*, *Chrytomonas ovata*, and *Cosmarium botrytis* increased their LA concentrations under 300 µmol photons $m^{-2}s^{-1}$ illumination (Wacker et al. 2016). Light intensity, i.e. 99 µmol photons $m^{-2}s^{-1}$, used for the stress might not be favorable for the cell to produce in this study.

The cells under salinity stress showed significant decrease, i.e. 38,33±18,28 ng/µl in LA content by 82,77%. Elevated level of NaCl might have led to reduced fatty acid unsaturation rate. Decreases in unsaturation level in microalgae under salt stress were mentioned in some studies (Zhila et al., 2011; Rao et al., 2007). Rao and colleagues observed that reported that increased levels of NaCl resulted in reduced LA content in *B. braunii* 572 cells (Rao et al., 2007). The peaks of LA were resolved well in all chromatograms. Therefore, it can be said that nitrogen stress triggered production of LA which is essential for generation of the other ω-6 fatty acids.

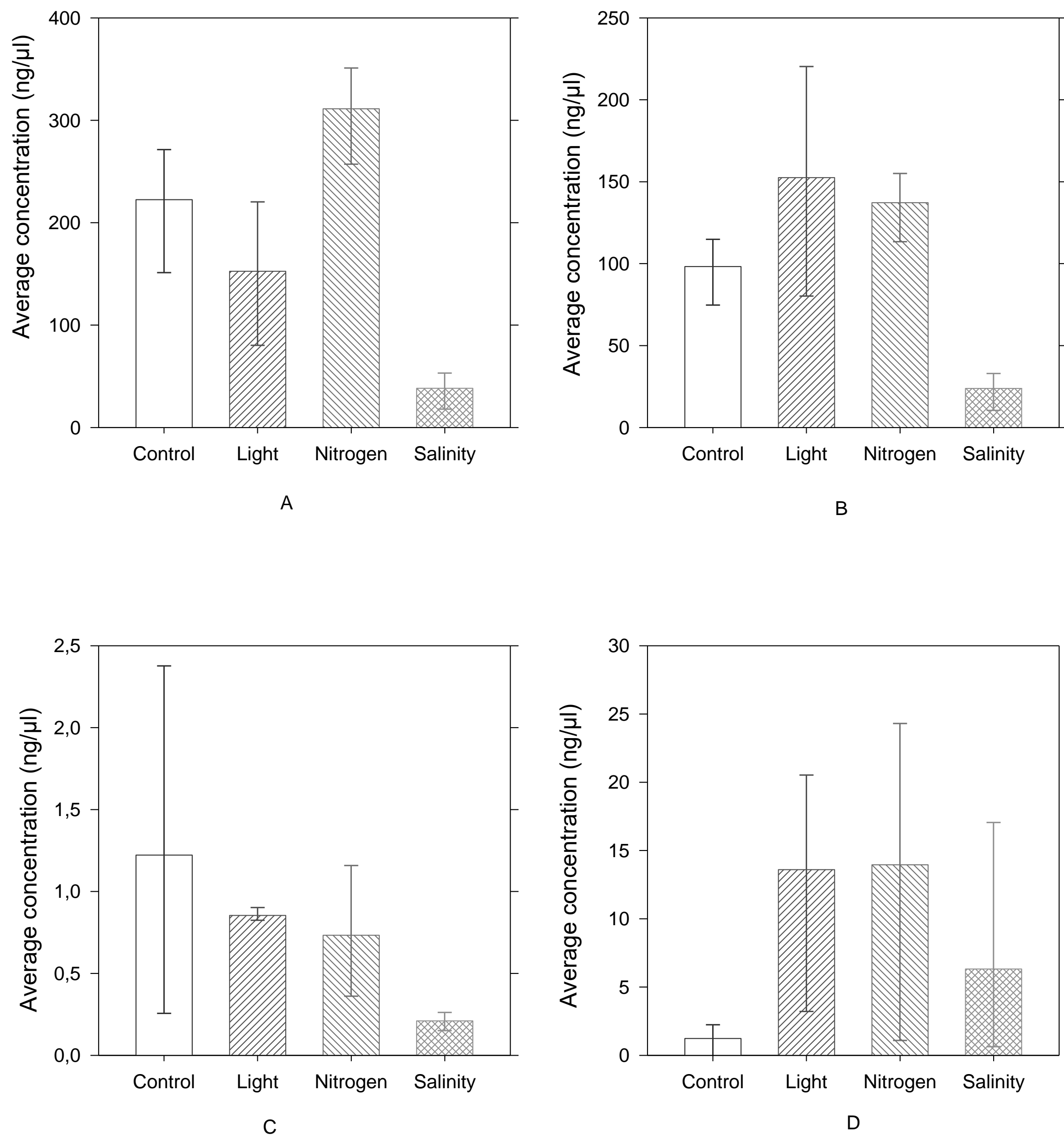


Figure 4.3. Average concentrations of ω-6 fatty acids in triplicates (A:LA, B: GLA, C: DGLA, D: AA).

Gamma-linoleic acid (C18:3n6) concentration, i.e. 137,15±21,41 ng/µl, was increased under nitrogen stress by 39,62% significantly, however, lowest concentration of GLA was found under salinity-stress, i.e. 23,81±11,84 ng/µl, decreased by 75 % compared to the control. Under light stress, GLA concentration was decreased 24,46% compared to the control. However, GLA could not be separated from C20:0 very well. This could be seen in Figure B8, B9 and B10. Therefore, increment in GLA concentration could be artificial.

Dihomo-gamma linoleic acid (C20:3n6) levels were found quiet low in all triplicates. Under light, nitrogen, and salinity stress, DGLA concentrations were found as 0,85±0,04 ng/µl, 0,73±0,40, and 0,21±0,06 ng/µl, respectively. Although, all stress factors decreased DGLA content compared to control. Salinity stress affected dramatically concentration of DGLA by decreasing 82,79% compared to the control.

Although Arachidonic acid could (C20:4n6) levels were increased for all stress parameters however; the data are not reliable since peaks of AA could not be separated from C20:3n3/C22:1n9. This situation could be seen in Figure B11, B12, and B13. Changes concentrations of omega 6 fatty acids were illustrated in Figure 4.3. Under light and nitrogen stress, AA concentrations were found 13,60±9,15 ng/µl and 13,95±11,81 ng/µl, respectively. This means 12-fold increase in AA concentrations compared to the control. Under salinity stress, AA concentration was increased by 409% and found to be 6,32±9,30 ng/µl. These increments are very artificial due to generation of unified peaks.

As an overall summary, changes in percent concentrations for ω-3 and ω-6 fatty acids compared to the control are shown in Table 4.5. With respect to ω-3 fatty acids, EPA concentrations were increased under all tested stress conditions reaching up to appx. 487% compared to control group and light stress only improved by appx. 9%. With respect to ω-6 fatty acids, LA concentrations were significantly increased under nitrogen stress by appx. 40% compared to control group. This was also the case of GLA concentration under nitrogen stress conditions (Table 4.5). Salinity stress condition caused significant decreases in LA and GLA concentrations by appx. 83% and 76% compared to the control group respectively. Dihomo-gamma linoleic acid concentrations were decreased whereas AA concentrations were increased under all tested experimental conditions, however, the changes were not statistically significant.

Table 4.5. Summary of the changes in percent fatty acid concentrations of ω-3 and ω-6 fatty acids under experimental stress conditions compared to the control group.

Types of fatty acids		**Stress Conditions**		
		Light	**Nitrogen**	**Salinity**
ω-3 fatty acids	DHA (C22:6n3)	8,7↑	73,6↓	92,7↓
	ALA (C18:3n3)	49,86↓	56,72↓	64,80↓
	EPA (C20:5n3)	215,57↑	486,67↑	168,63↑
ω-6 fatty acids	LA (C18:2n6c)	45,86↓	**39,89↑***	**82,77↓***
	GLA (C18:3n6)	24,46↓	**39,62↑***	**75,75↓***
	DGLA (C20:3n6)	30,11↓	40,4↓	82,79↓
	AA (C20:4n6)	1000,41↑	1029,19↑	411,84↑

*Denotes the change is statistically significant with $P < 0,05$.

As explained in materials and methods section, the experiments were carried out with technical replicates. As shown in most results, huge variations indicate that technical replicates did not work well as planned. Also, elution times for some of the key ω-3 and ω-6 fatty acids overlapped with other FAs which generated poor resolutions and combined results by summing up two FAs peak areas. Despite using a long and reliable FAME analysis column, analytical measurements were completed in a GC-FID, which could have yielded better resolutions if analyzed with GC-MS instrument. In a study by Aparicio-Ruiz and colleagues, this case has been reported that GC-FID provide poorer determination for volatile organic compounds, where GC-MS provided better selectivity and more precise results (Aparicio-Ruiz et. al, 2018). It should also be noted that, the extraction method used in our study (Breuer et al., 2013) recommended 5-10 mg of lyophilized algae in their protocol. However, in order not to miss any volatile fatty acids during extraction, 100 mg of lyophilized algae was used in this study. Higher biomass used was treated with same volume of solvents which might have caused low quality extraction as solvent volumes might have been insufficient to extract all lipids from the biomass. The other reason might be due to the fact that lipid extraction protocols may not be always proven to be best-performing among different types of microalgae species.

5. CONCLUSIONS

Polyunsaturated fatty acids cannot be synthesized in humans and other mammals, yet they have numerous positive health impacts on metabolic processes. Therefore, PUFAs should be taken with daily diets at required concentrations and proper ratios (Williams, C. M. 2000). Polyunsaturated fatty acids are linked to many vital functions in human body such as production of eicosanoids, enzyme-substrate relationships, membrane fluidity, acylation of proteins, gene interactions, and lipid peroxidation (Rustan and Drevon, 2001; Benatti et al.,2004). Omega-3 and omega-6 fatty acids also have vital functions in cell membranes as phospholipids and gene regulations. These fatty acids are the two major group of PUFAs. There are numerous clinical research present in literature which underlines the fortifier effects of ω-3 and ω-6 fatty acids in prevention of diseases such as atherosclerosis, arrhythmias, cancer, rheumatoid arthritis, asthma/allergy, and diabetes (Liu et al., 2014; Katan et al., 1994; Connor 2000; Burlingame et. al., 2009; Joint et. al., 2009). Supplementation with ω-3 and ω-6 fatty acids are also recommended by several clinic reports for maintenance of neurologic health as it was revealed that low levels of ω-3 and ω-6 fatty acids are associated with Schizophrenia and Alzheimer's and Parkinson's disease (Collinius, 2016).

For supplementation of ω-3 and ω-6 fatty acids, fish oils have been traditionally consumed. However, noticeable concerns have recently emerged as fish oils might contain various toxic contaminants in their body such as PCBs, heavy metals, organic impurities, and dioxins bioaccumulating from the aquatic environments where fish were caught or farm-raised. Since humans take part at the highest trophic level in the food chain, bioaccumulation of these contaminants poses serious threats to human health. Besides, microalgae are the actual primary producers of ω-3 and ω-6 fatty acids as zooplanktons and small fishes consuming microalgae accumulates these essential fatty acids in their flesh. With additional benefits of sustainable cultivation opportunities, large-scale production of ω-3 and ω-6 fatty acids from microalgae is preferred. Microalgae-based ω-3 and ω-6 fatty acids are also good alternatives for vegan and vegetarian people who do not prefer to consume animal based products.

With this motivation and rationale, the main objective of this study was to optimize culture conditions of *Chlororococcum novae-angliae* for the production of ω-3 and ω-6 fatty acids (particularly DHA, ALA, EPA, LA, GLA, DGLA and AA). For this purpose, four stress parameters, i.e. light, nitrogen, salinity, and temperature were selected to expose algae cells to promote of ω-3

and ω-6 fatty acids. As there were only two studies reported for *C. novae-angliae*, determination of correct stress conditions has been very difficult. *Chlororococcum novae-angliae* were cultivated several times in batch cultures to find out optimal conditions and at the beginning of early stationary phase for the control. Following, stress levels were selected based on literature survey, where studies are specifically related to lipid production from green microalgae.

For control group and other stress conditions, cultivation of *C. novae-angliae* was completed under 11100 lux light intensity which is considered optimal for the growth of most green microalgae as well as *C. novae-angliae*. For light stress conditions, light intensity was dropped from 11100 to 5500 in order to enhance lipid productivity and ω-3 and ω-6 fatty acids composition of *C. novae-angliae* as inferred from other literature studies. However, under low light intensity, lowest dry weight, growth rate, and reactor productivity were observed among all stress conditions. It was an obvious conclusion that growth and photosynthetic activity of *C. novae-angliae were* very sensitive to light intensity and lipid content was moderately decreased compared to the control. Meanwhile, total fatty acid composition and two main fractions (SFAs and PUFAs) were also decreased under light stress. Only MUFAs were increased by 14.4% where DHA level was increased by 8,7% under low compared to the control. Also, EPA, AA and GLA levels seemed to increase, despite the analytical resolutions were hard to interpret as these fatty acids eluted simultaneously with C24:0, C20:3n3/C22:1n9, C20:0 respectively. Dihomo-gamma linoleic acid and ALA were decreased under light stress. Also, oleic acid, palmitic acid, and stearic acid concentrations were negatively affected under low light intensity compared to control which confirmed decreased PUFA biosynthesis *de novo* from SFA.

Second tested experimental stress condition was achieved by exposing *C. novae-angliae to* low nitrogen levels. The growth media used for the control contained 8,82 mM of $NaNO_3$, whereas, $NaNO_3$ concentration was dropped to 1,47 mm in nitrogen stress reactor. Nitrogen stress caused cells to decrease their dry weights, lipid production, and growth rates. Although cell growth and overall productivities were negatively affected, highest SFAs, MUFAs, and PUFAs levels were observed under nitrogen stress suggesting the fraction of FFA within total lipoids were achieved by nitrogen stress. Also, highest concentration of palmitic acid, stearic acid, and oleic acid were found in the cells under nitrogen stress confirming higher *de novo* biosynthesis rates from SFAs. Eicosapentaenoic acid, LA, GLA and ARA were increased under nitrogen stress, however, the increments in EPA, GLA, and AA might have been over-predicted due to convergence of chromatogram peaks. Only ALA concentration was significantly increased by 39,9% compared to the control group. Nitrogen stress exposure of *C. novae-angliae* is a promising outcome revealed in this study as LA is the most essential

fatty acid for the production of ω-6 fatty acids. In further investigations, different concentrations of $NaNO_3$ could be tried in order to enhance growth rate and dry weight. Nevertheless, nitrogen stress was the most effective parameter that we used in this study in terms of overall ω-3 and ω-6 fatty acid composition.

Third tested experimental stress condition was salinity stress, by exposing *C. novae-angliae* to two-fold increased NaCl concentration of the original culture medium. The cells under salinity stress has shown highest growth rate and cell numbers were spiked on the initial days of cultivation. However, dry weight, reactor productivities, and lipid content were decreased compared to the control after six days of cultivation. Lowest levels of fatty acid concentrations were also obtained under salinity stress. The biggest decrease in concentration of palmitic acid, stearic acid, and oleic acid were observed under salinity stress. Only EPA and AA levels were increased, however, these data were also inconclusive due to analytical problems as explained in earlier sections. Concentrations of LA and GLA were decreased significantly by 82,77% and 72,75% respectively. Also, major reductions in concentrations of DHA, ALA, and DGLA were observed under salinity stress. These results confirmed that salinity stress should not be preferred to elevate ω-3 and ω-6 fatty acids levels in *C. novae-angliae*. Overall, salt stress created negative impacts on biomass yields and lipid content of *C. novae-angliae.* As a terrestrial microalgae species, *C. novae-angliae* might not have sufficient resistance to high levels of NaCl in growth media.

Last tested, yet failed stress condition was based on the assumption that exposing *C. novae-angliae* to lower temperatures would trigger lipid accumulation. Albeit, cell growth was dramatically inhibited on the very 1st day of cultivation. *Chlorococcum novae-angliae* cells in the reactor did not show adaptation to 17 °C. They were normally grown at 25 °C which seem optimal for the growth of for this microalgae microalgae. However, more temperature experiments need to be carry out in order to determine optimal growth condition for *C. novae-angliae*. As the decrease in temperature heavily affected microalgal growth and biomass production by the inhibition of photosynthetic enzymes, downstream analyses for lipid and FAME determination were not conducted.

It is important to highlight the fact that the effects of light, nitrogen, salinity and temperature shifts on metabolic processes and growth rates of microalgae are very specific to different species. Since this is the very first study which examines optimal growth conditions to enhance production of ω-3 and ω-6 fatty acids in *C. novae-angliae*, parameters that were selected to increase lipid content in other species might not be necessarily generate favorable results with respect to growth and lipid accumulation. Apart from the stress parameters, experimental errors might have led to fluctuated data

as harvesting step was considerably hard to achieve as microalgae cells adhere to flat panel PBR used in this study which resulted in biomass losses. Another experienced problem was the tubes used for lipid extraction analyses. During lipid extraction, 22 ml PTFE vials were used to prevent evaporation of chloroform and methanol mixture as Teflon™ caps are required to prevent cross-contamination. However, gravimetric calculation of lipids during extraction might not have been precise as the amount of harvested biomass (100 mg) used for extraction was quite lower than the tare weights of the tubes which were approximately 14 g.

In conclusion, *Chlorococcum novae-angliae* is a promising microalgae species producing high amounts of palmitic and stearic acid which are important for biodiesel production. Though not within the scope of this study, it indicates that *C. novae-angliae* can be considered for simultaneous biofuel, and ω-3 & ω-6 fatty acids production in large-scale biorefinery settings. Light and nitrogen stress experiments could be expanded more in depth for the further optimization processes. Particularly, nitrogen stress seems a promising approach in order to create positive impact on lipid content and fatty acid composition of *C. novae-angliae*. It should also be noted that analytical limitations negatively impacted the outcomes of this study. Specifically, there were more than one hundred peaks for each sample with certain peaks with high areas suggesting unidentified FAs synthesized at high concentrations. In further investigations, complete fatty acid analyses of *C. novae-angliae* could be analyzed by using a GC-MS instrument in order to understand content of lipid extracts more precisely and comprehensively. The other analytical problem was with elucidation of FAMEs through the column. The GC method that was followed in data analyses was not sufficient to separate certain ω-3 and ω-6 fatty acids peaks that were sought to be resolved more clearly. Nevertheless, this study is the first optimization of culture conditions of *Chlorococcum novae-angliae* for the synthesis of ω-3 and ω-6 fatty acids under various stress conditions which paves the way for further research to optimize lipid production and omega fatty acid production in *C. novae-angliae*.

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APPENDIX A: Identified and Unidentified Area of Fatty Acids for Replicates

Table A1. Peak area for each fatty acid in technical replicates.

Fatty acids	Peak area for parameters											
Saturated fatty acids	C1	C2	C3	L1	L2	L3	N1	N2	N3	S1	S2	S3
C6:0	0,00	0,00	0,00	0,42	0,28	5,16	0,00	0,00	1,26	1,12	2,66	0,54
C8:0	4,50	4,65	4,65	3,57	1,81	2,39	2,67	6,50	5,24	0,22	0,46	0,56
C10:0	1,93	3,16	3,16	0,58	0,53	0,18	0,55	0,60	0,51	1,36	2,29	2,48
C11:0	0,84	1,11	1,11	1,07	0,65	0,80	0,00	0,58	0,00	0,25	0,00	0,00
C12:0	3,34	1,51	1,51	1,87	0,87	1,29	3,98	4,58	3,19	0,32	0,72	0,53
C13:0	9,60	4,60	4,60	7,91	3,36	6,13	0,65	0,84	0,65	4,33	0,56	0,40
C14:0	125,28	106,41	106,41	108,28	53,97	103,92	168,62	164,23	128,85	10,31	40,97	27,43
C15:0	92,79	15,60	15,60	13,31	7,23	15,25	21,30	21,40	16,71	1,88	6,39	4,83
C16:0	19,11	1180,97	1180,97	1145,83	647,48	1097,95	1901,19	1932,90	1532,59	134,23	459,38	361,60
C17:0	35,09	30,36	30,36	23,44	14,75	26,55	37,65	35,48	28,45	2,81	9,87	7,17
C18:0	157,67	144,33	144,33	110,48	58,84	123,26	246,49	287,89	238,16	29,17	89,13	89,08
C20:0	39,65	31,28	31,28	26,93	13,90	23,28	50,28	46,72	37,63	4,05	11,88	10,49
C21:0	6,80	5,70	5,70	2,00	5,36	0,67	6,61	3,61	2,03	4,08	0,48	0,40
C22:0	11,72	7,43	7,43	6,03	3,01	12,45	11,60	11,45	14,19	4,66	6,37	24,73
C23:0	8,10	6,02	6,02	1,67	14,60	6,59	49,47	1,27	5,07	2,02	6,54	8,81
C24:0	8,70	20,85	20,85	2,57	1,03	12,59	51,25	1,26	5,07	0,19	46,34	13,96
Sum of SFAs	525,12	1563,97	1563,97	1455,95	827,65	1438,46	2552,31	2519,32	2019,59	200,99	684,03	553,01
% SFA	0,07	0,19	0,18	0,18	0,14	0,19	0,27	0,26	0,24	0,04	0,10	0,08
Monounsaturated fatty acids												
C14:1	92,79	71,57	71,57	83,39	9,34	87,69	35,34	40,23	26,54	1,31	4,90	3,34
C15:1	2,39	0,98	0,98	1,21	1,04	0,82	0,39	2,57	1,79	2,58	27,99	12,76
C16:1	43,08	37,85	37,85	35,75	17,60	34,78	82,41	115,22	85,35	4,55	16,59	10,92
C17:1	9,11	6,92	6,92	3,53	1,80	5,96	9,83	9,35	7,68	1,26	3,66	2,85
C18:1n9t	2,10	316,77	316,77	2,12	111,07	1,69	2,05	3,07	1,44	34,26	0,58	87,34
C18:1n9c	111,47	93,51	93,51	110,84	66,37	89,76	507,27	493,06	389,53	6,58	20,95	15,73
C20:1	1831,25	5,26	5,26	2079,98	2,00	1493,41	2336,43	2094,21	8,91	0,27	7,38	2,18
C24:1	0,00	5,52	5,52	2,67	0,64	1,75	9,15	3,34	6,45	8,31	3,32	8,65
Sum of MUFAs	2092,20	538,39	538,39	2319,48	209,84	1715,86	2982,86	2761,05	527,69	59,12	85,37	143,77
%MUFAs	0,26	0,06	0,06	0,29	0,03	0,22	0,31	0,29	0,06	0,01	0,01	0,02
Polyunsaturated fatty acids												
C18:2 n-6t	281,23	287,25	287,25	158,36	128,36	233,94	210,52	227,41	227,41	72,44	131,22	131,22
C18:2 n-6c	905,12	816,74	816,74	497,89	267,57	734,97	1170,61	857,47	857,47	59,82	146,19	146,19
C18:3 n-6	368,23	337,11	337,11	247,58	121,50	282,30	497,05	363,66	363,66	33,50	89,81	89,81
C18:3 n-3	1,00	1692,38	1692,38	3,91	1081,25	3,02	1,61	1629,52	1629,52	211,48	564,15	564,15
C20:2	3,71	2,21	2,21	2,59	1,15	0,48	2,13	2,06	2,06	6,67	1,59	1,59
C20:3 n-6	6,01	0,65	0,65	1,59	2,08	2,28	0,91	2,93	2,93	0,66	0,55	0,55
C20:3 n-3/C22:1 n-9	10,47	6,02	6,02	13,50	35,00	6,78	6,34	7,84	7,84	1,02	2,93	2,93
C20:4 n-6	3,34	0,00	0,00	43,12	43,20	2,24	55,54	2,49	2,49	1,45	38,96	38,96
C22:2	53,07	87,98	87,98	0,48	1,77	17,39	0,71	0,55	0,55	5,94	0,20	0,20

C20:5 n-3	2,49	4,07	4,07	87,38	4,05	2,30	91,05	115,20	115,20	3,58	61,20	61,20
C22:6 n-3	2,47	9,33	9,33	0,11	12,13	1,91	0,62	1,68	1,68	0,75	0,10	0,10
Sum PUFAs	1637,15	3243,74	3243,74	1056,53	1698,07	1287,60	2037,10	3210,81	3210,81	397,31	1036,91	1036,91
%PUFAs	0,21	0,39	0,38	0,13	0,28	0,17	0,21	0,33	0,38	0,08	0,14	0,14
Total Area	798529,00	835303,00	861567,00	806637,00	612335,00	774183,00	962284,00	965706,00	847653,00	472260,00	715604,00	734483,00
Total identified area	5490,99	5336,67	5060,82	4832,26	2735,55	4441,93	7562,26	7165,60	5755,59	657,53	1800,58	1733,69
Unidentified area	793038,01	829966,33	856506,18	801804,74	609599,45	769741,07	954721,74	958540,40	841897,41	471602,47	713803,42	732749,31
% Unidentified area	0,47	0,36	0,38	0,40	0,55	0,43	0,21	0,12	0,32	0,86	0,75	0,76
% identified area	0,53	0,64	0,62	0,60	0,45	0,57	0,79	0,88	0,68	0,14	0,25	0,24

APPENDIX B: GC Chromatograms for Reference Standard and Technical Replicates

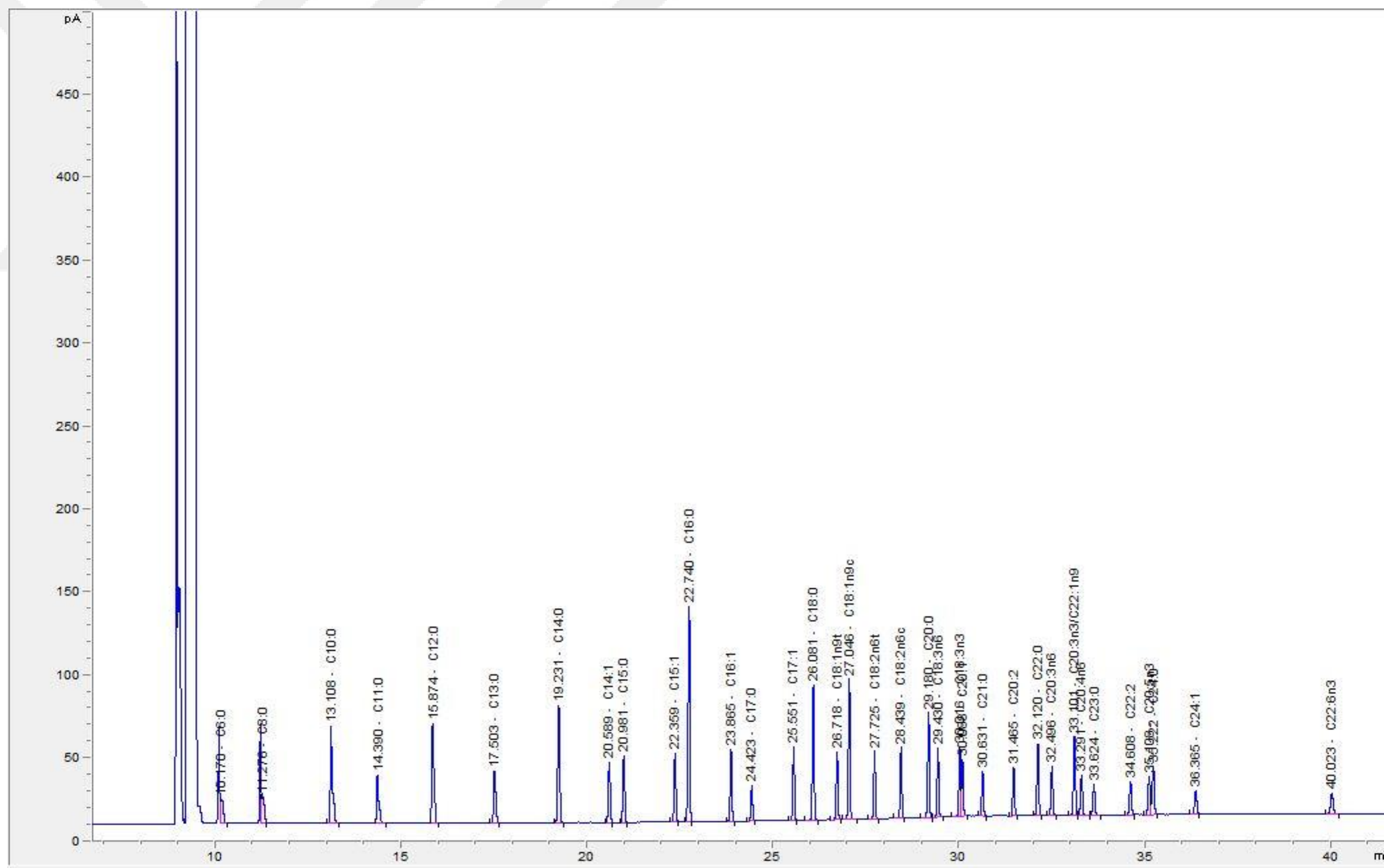

Figure B1. GC Chromatogram of reference standard.

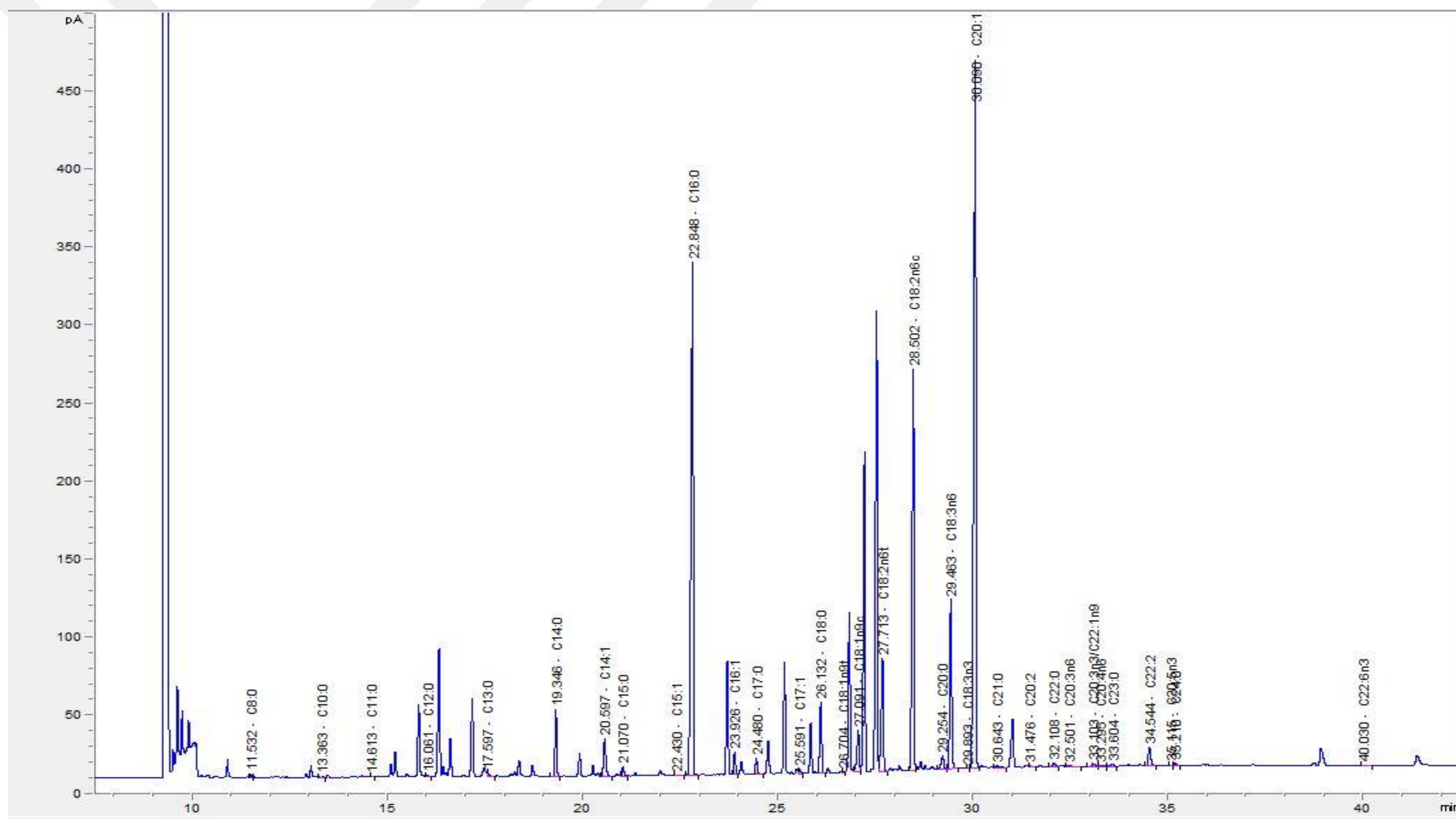

Figure B2. GC chromatogram of control for replicate sample-1.

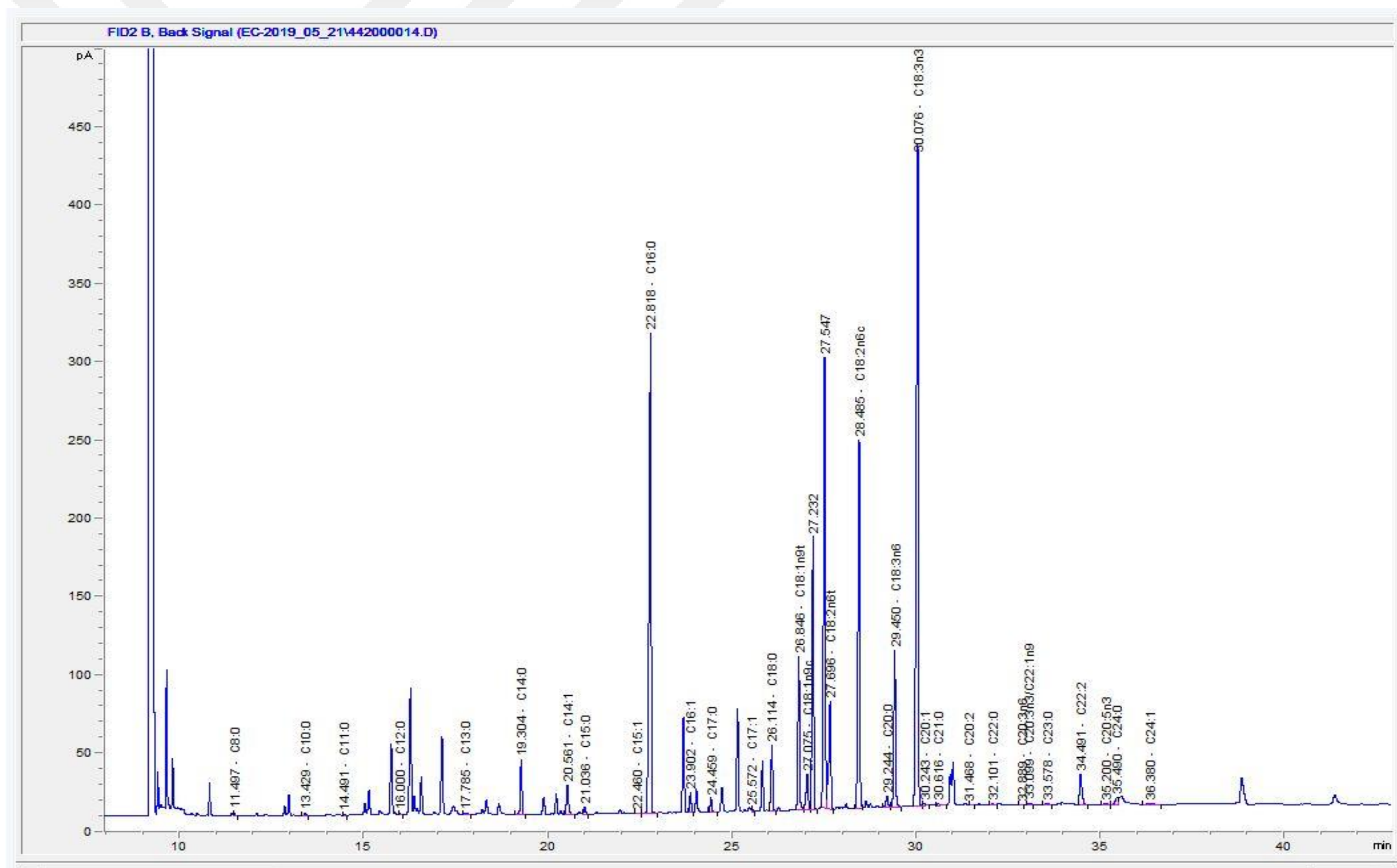

Figure B3. GC chromatogram of control for replicate sample-2.

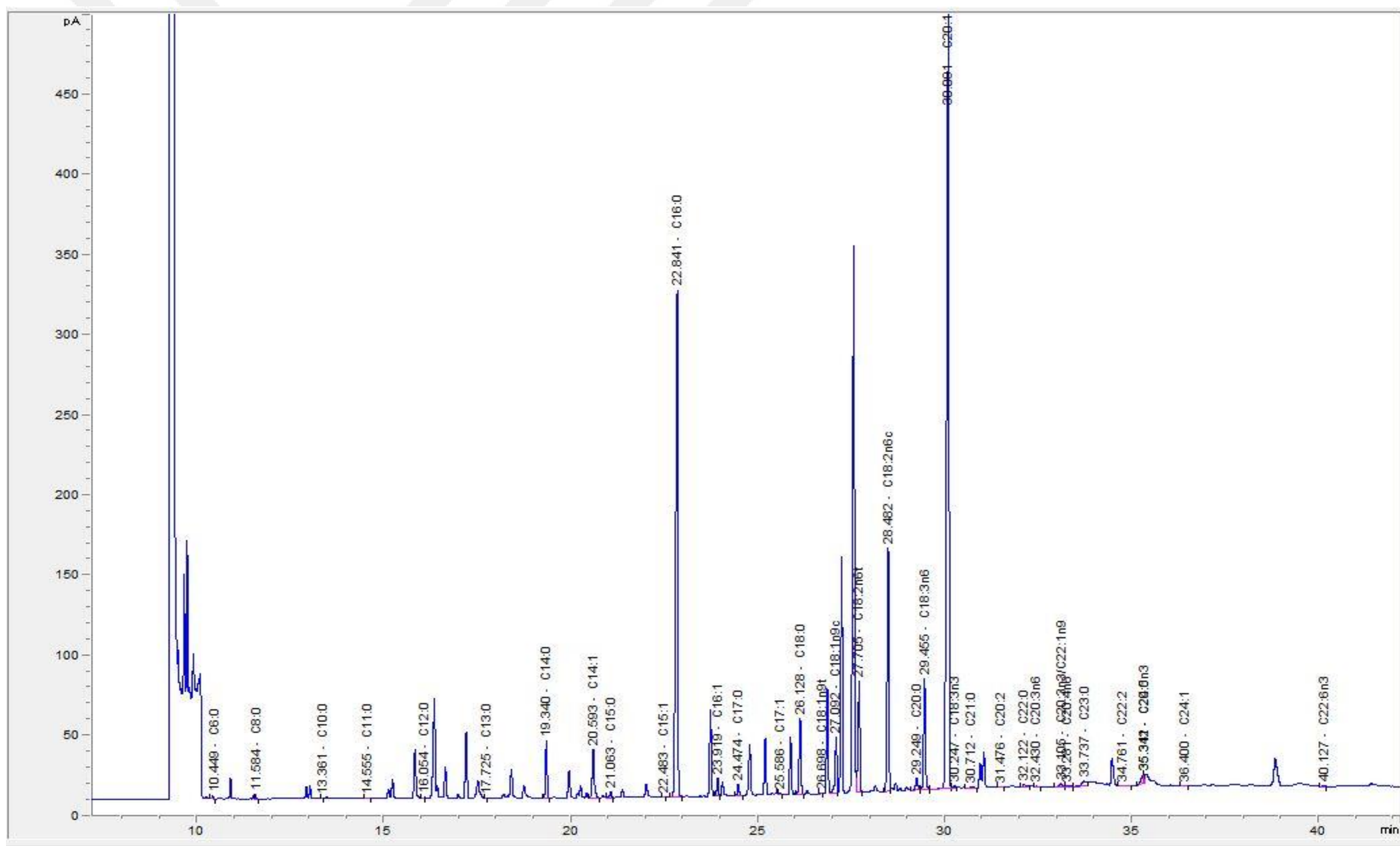

Figure B4. GC chromatogram of control for replicate sample-3.

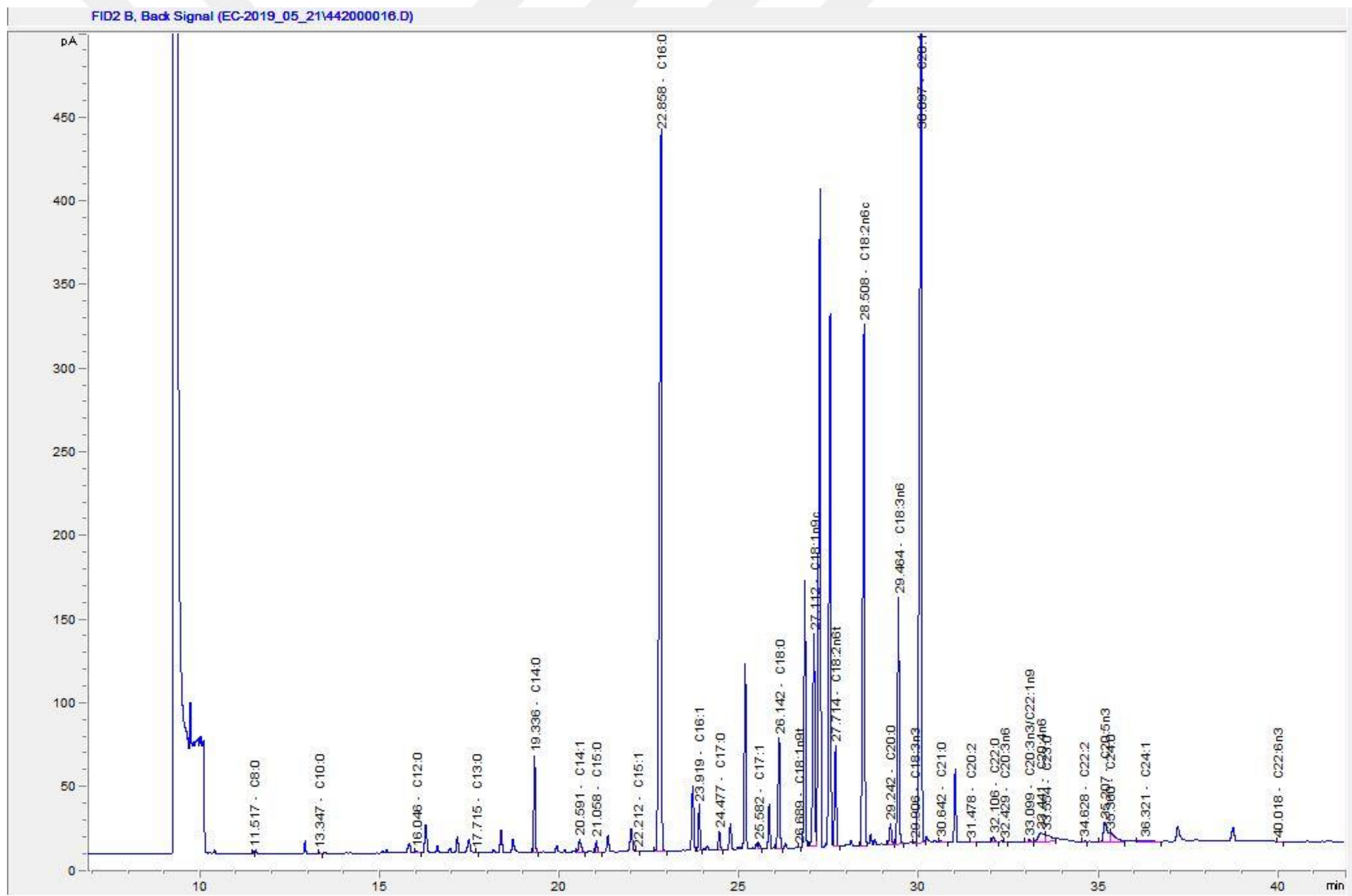

Figure B5. GC chromatogram of nitrogen stress condition for replicate sample-1.

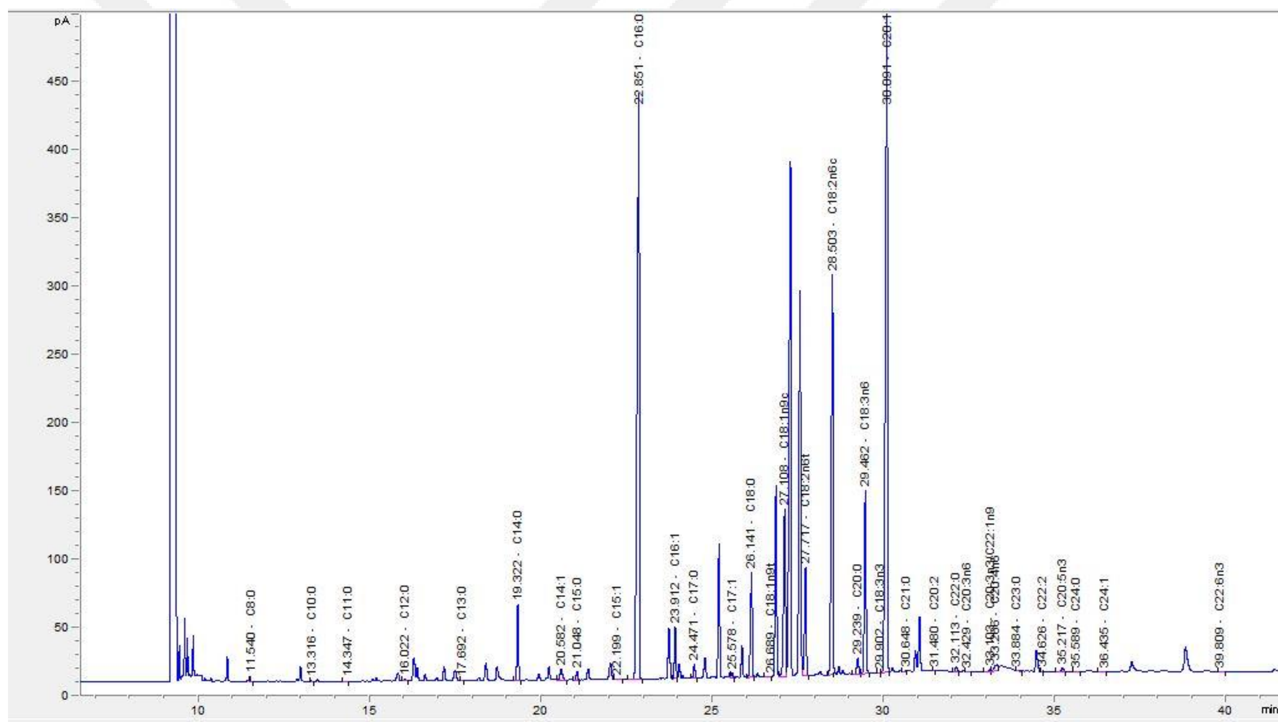

Figure B6. GC chromatogram of nitrogen stress condition for replicate sample-2.

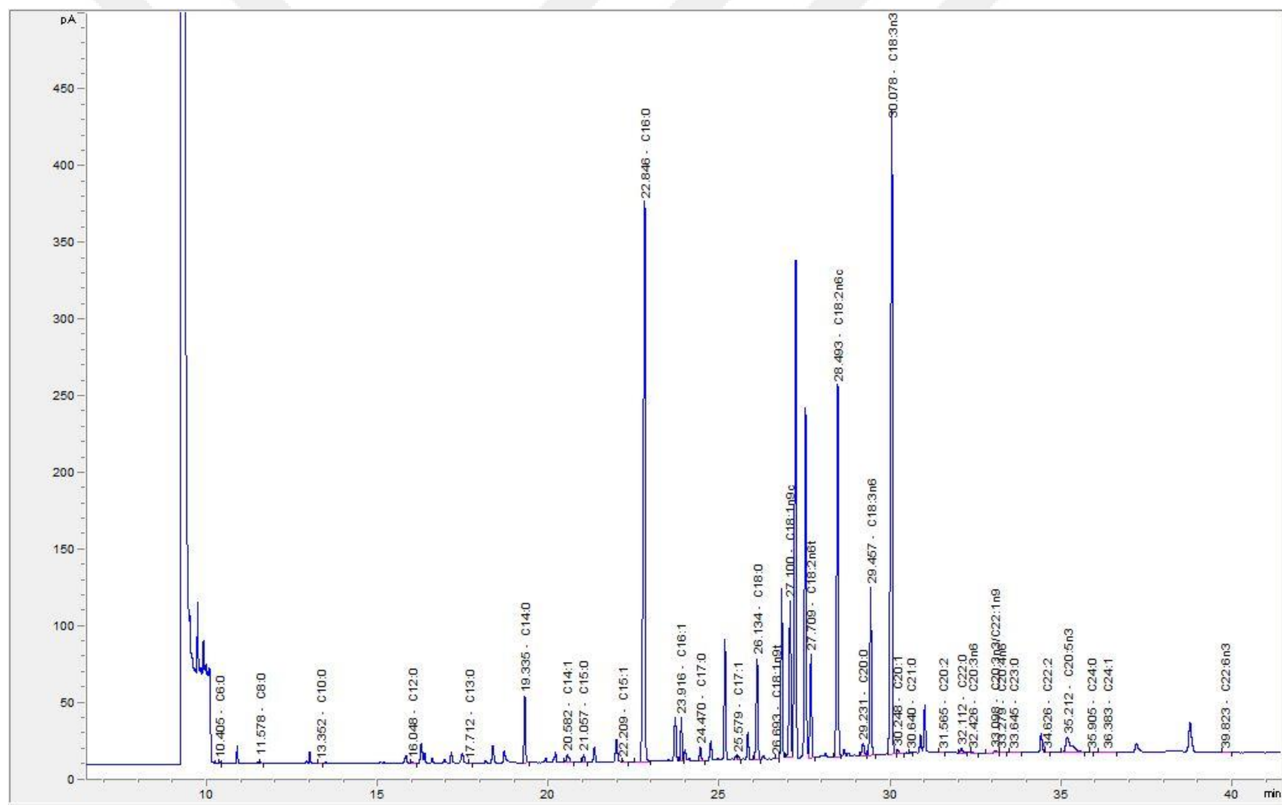

Figure B7. GC chromatogram of nitrogen stress condition for replicate sample-3.

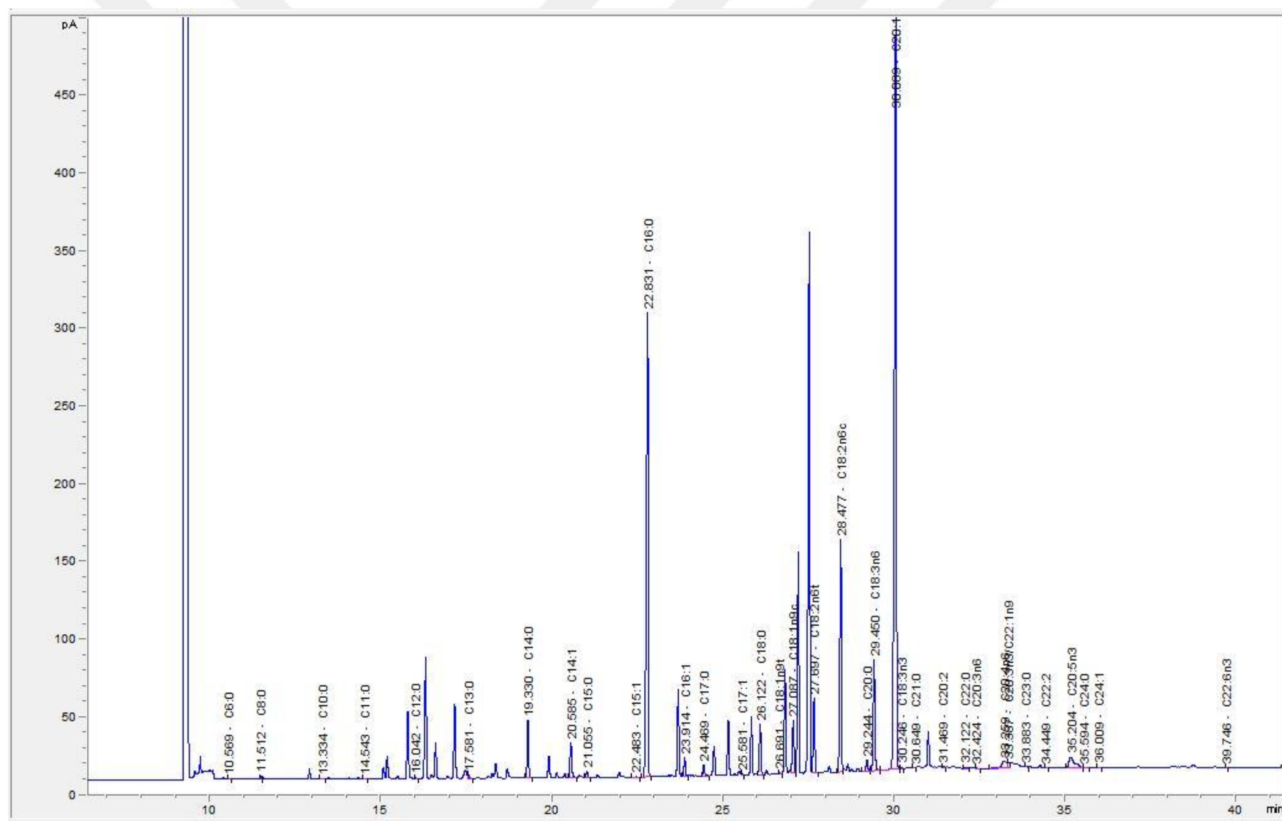

Figure B8. GC chromatogram of light stress condition for replicate sample-1.

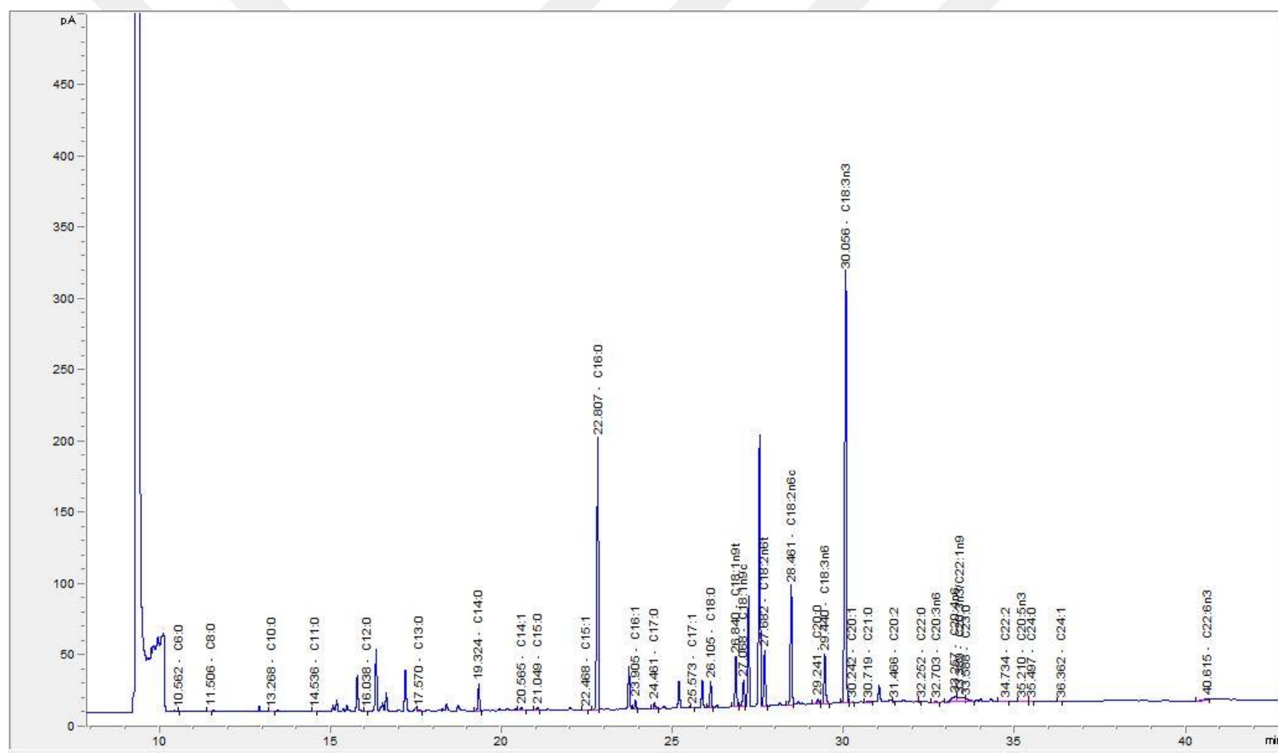

Figure B9. GC chromatogram of light stress condition for replicate sample-2.

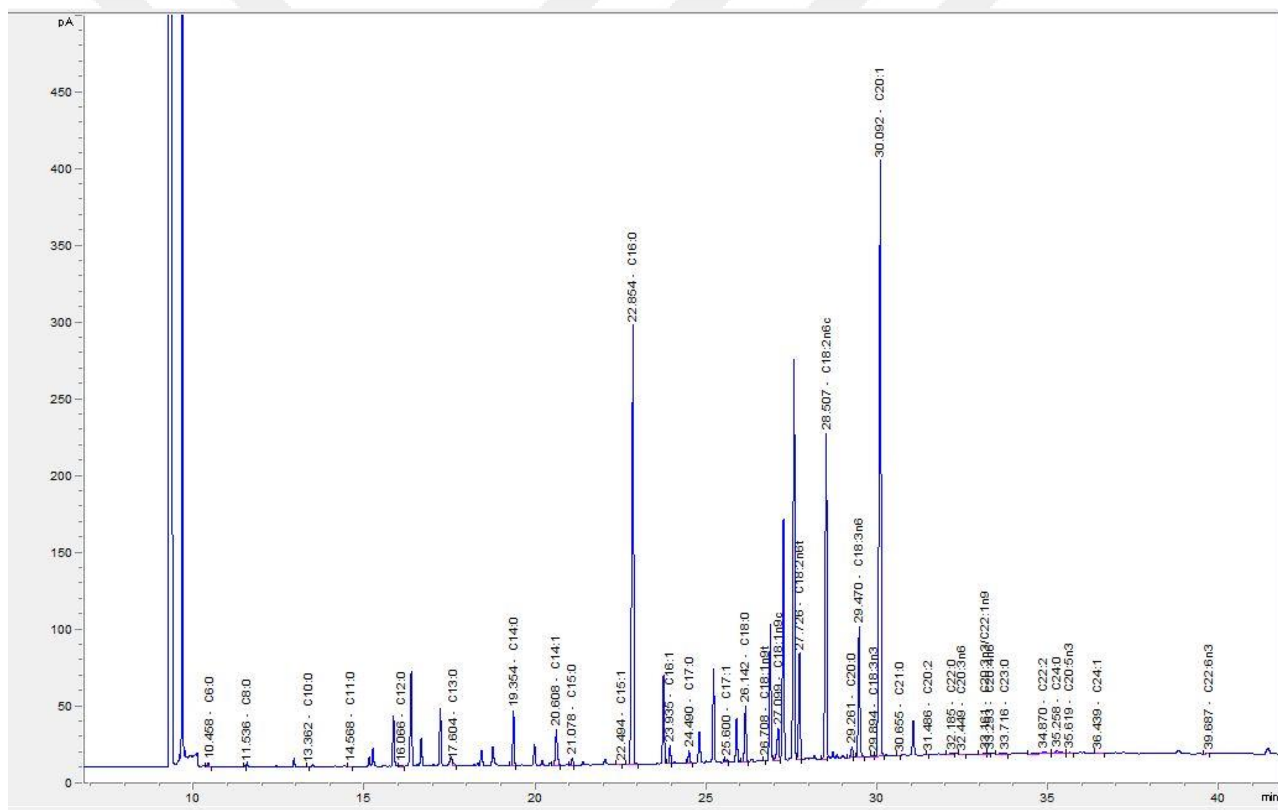

Figure B10. GC chromatogram of light stress condition for replicate sample-3.

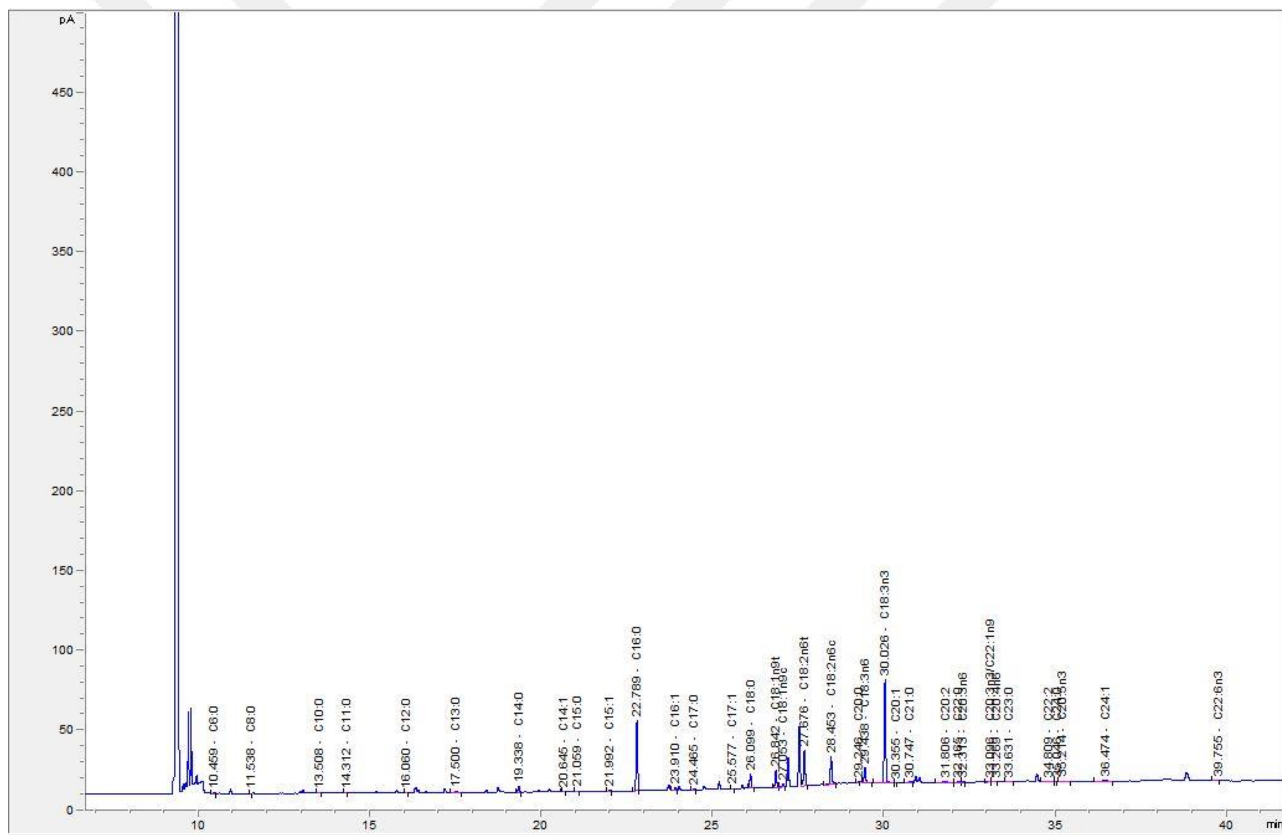

Figure B11. GC chromatogram of salinity stress condition for replicate sample-1.

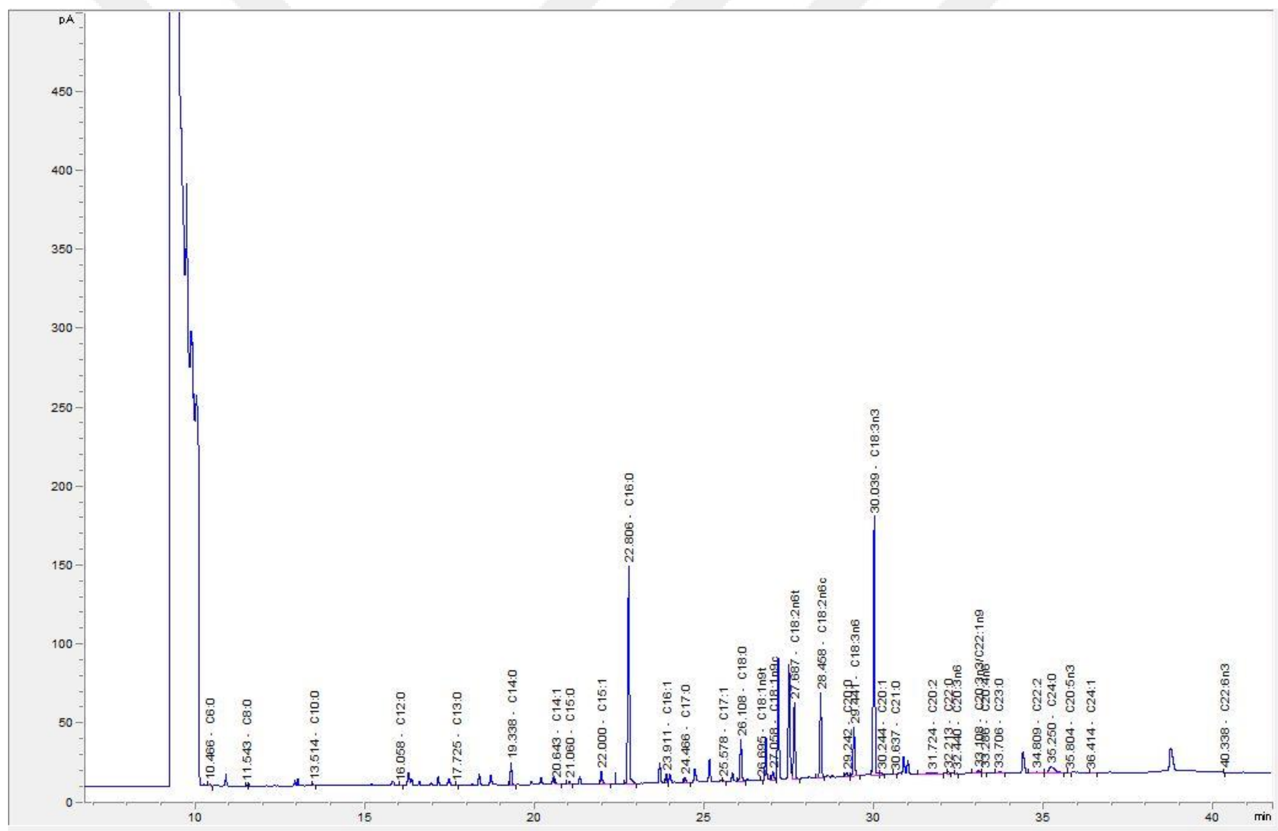

Figure B12. GC chromatogram of salinity stress condition for replicate sample-2.

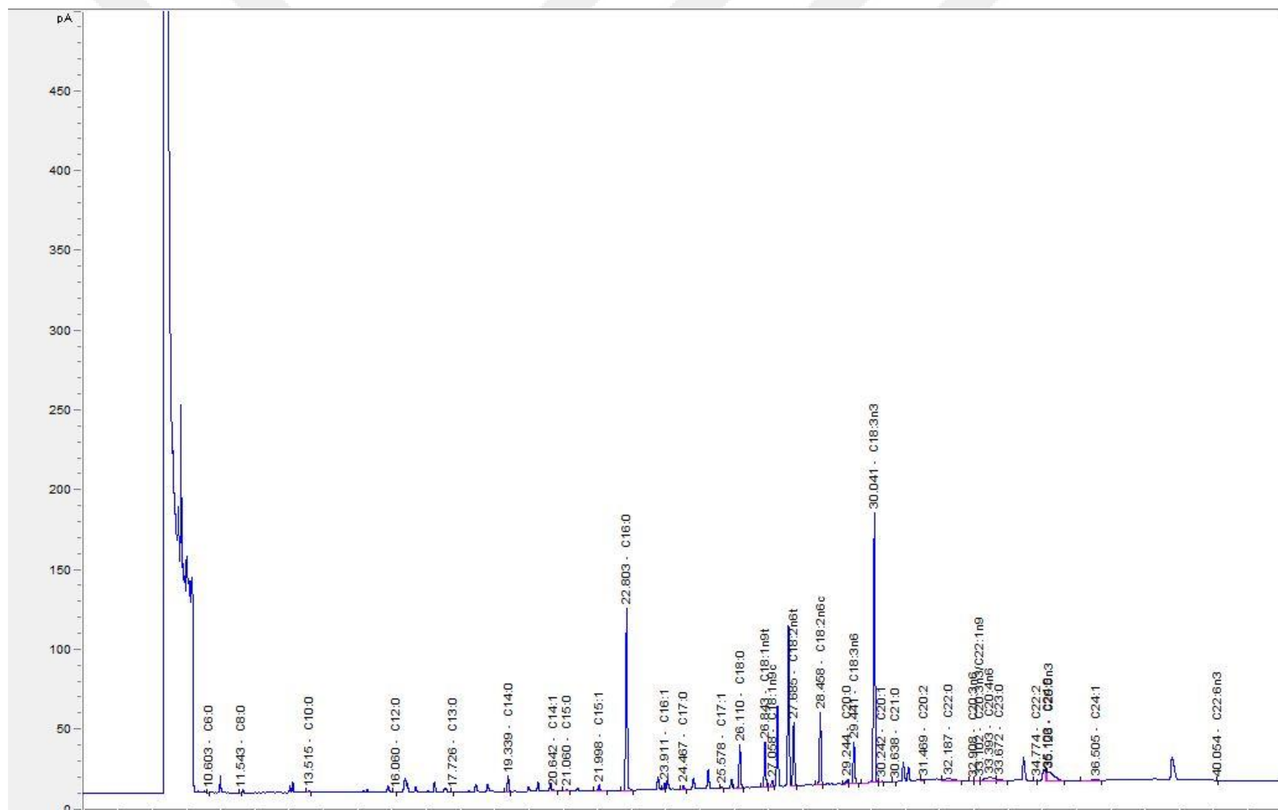

Figure B13. GC chromatogram of salinity stress condition for replicate sample-3.