LIPID CLASS SPECIFIC PRODUCTION OF EPA AND DHA IN PHYTOPLANKTON DINOFLAGELLATE *HETEROCAPSA TRIQUETRA*.

by

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Abstract

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are dietary polyunsaturated fatty acids (PUFA) with a range of health benefits. Phytoplankton are the primary marine producers of EPA and DHA through the process of photosynthesis. Measuring the effect of light intensity and temperature variation on the production of PUFA was the purpose of this study. *Heterocapsa triquetra*, a dinoflagellate, was the phytoplankton used in this study and its response to specific environmental conditions was compared to *Phaeocystis globosa*, a prymnesiophyte. *H. triquetra* were cultivated in two different light intensities (88 and 140 µmol photons m^{-2} s^{-1}) having the same temperature (24 °C) and another light intensity with a different temperature (210 µmol photons m^{-2} s^{-1} and 14 °C). For comparison with a different species, *P. globosa* was grown at the same condition as one of the *H. triquetra* (140 µmol photons m^{-2} $s^{-1}$and 24 °C). Spikes of 99% $NaH^{13}CO_3$ were added and after 6 h, cultures were harvested. Lipid classes were identified and quantified by high performance liquid chromatography (HPLC), FA proportions were measured with gas chromatography flame ionization detection (GCFID), and incorporation of ^{13}C in FA was measured by gas chromatography mass spectroscopy (GCMS).

Decreasing light intensity increased the production of DHA in digalactosyldiacyl glycerol (DGDG), while decreasing the production of DHA, 18:5n-3, and 18:3n-3 in triacylglycerol (TAG) in *H. triquetra*. Lowering the temperature allowed the measurement of production of DHA in DGDG even though a higher light intensity was used, thereby proving that lowering the temperature can possibly increase production rates of PUFA in DGDG. *P. globosa* grown under the same conditions as *H. triquetra* (24 ºC, and 140 µmol photons m^{-2} s^{-1}) was better labelled and had higher production of all PUFA in DGDG, and lower production of DHA, 18:4n-3 and 18:3n-3 in TAG, thus showing that different phytoplankton species respond to the same environmental conditions differently. This study conveys a feasible method to measure the rate of production of EPA and DHA in phytoplankton.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ANOVA	Analysis of variance
Acetyl-CoA	Acetyl coenzyme A
ACP	Acyl carrier protein
ALA	α-linolenic
CDS	CDP-diacylglycerol synthase
CI	Chemical Ionization
CDP-choline	Cytidine-diphosphocholine
CDP-ethanolamine	Cytidine-diphosphoethanolamine
CTP	Cytidine triphosphate
DAG	Diacylglycerol
DHA	Docosahexaenoic acid
DGDG	Digalactosyldiacylglycerols
DGD-synthase	Digalactosyldiacylglycerol synthase
DGAT	Acyl–CoA: DAG acyltransferase
ECT	CTP-phosphoethanolamine cytidylyltransferase
ELSD	Evaporative light scattering detector
EPA	Eicosapentaenoic acid
EPT	CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase
ER	Endoplasmic reticulum
ELOVAL	Elongase of very long-chain FA
EK	Ethanolamine kinase
FA	Fatty acid

FAE	FA elongases
FAME	Fatty acid methyl ester
FAT	Fatty acyl-ACP thioesterase
FFA	Free Fatty acid
FID	Flame ionization detector
GC	Gas chromatography
GC/MS	Gas chromatography mass spectroscopy
G3P	Glycerol-3-phosphate
GPAT	Glycerol-3-phosphate-acyltransferase
HPLC	High performance liquid chromatography
KCS	β-ketoacyl-CoA synthase
LACS	Long chain acyl-CoA synthetase
LPA	Lysophasphatidic acid
MGDG	Monogalactosyldiacylglycerols
MGD-synthase	Monogalactosyldiacylglycerol synthase
P	Production
PA	Phosphotidic acid
PC	Phosphotidylcholine
PE	Phosphotidylethanolamine
PG	Phosphotidylglycerol
PGP	Phosphatidyl glycerophosphate
PI	Phosphotidylnositol
PL	Phospholipid

SQDG	Sulfoquinovosyl diacylglycerols
TAG	Triacylglycerol
UDP-Gal	Uridine diphospho-galactose

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1. Introduction

1.1 Importance of EPA and DHA

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are dietary essential fatty acids (FA) with a range of health benefits. They are found in many parts of the body including cell membranes (Lazzarin et al., 2009), where they play a critical role in the viscosity of the membrane (Smith et al., 2011). The importance of EPA and DHA also arises in fetal development and healthy aging (Dunstan, 2007). DHA is found in abundance in the brain and retina, and is thought to be essential for the development of cognitive responses (Geppert, 2007).

It is difficult to receive sufficient amounts of EPA and DHA through our diets. Humans can convert α-linolenic (ALA), a prominent component of our diet found in many land plants, to EPA and DHA through the process of elongation and desaturation; however, this process is very inefficient, with as little as 2-10% of ALA converted to EPA and DHA (Chiu et al., 2008). Fish and fish oil supplements are the primary sources of EPA and DHA for humans. The low intake of EPA and DHA is thought to be the main reason for the increase in the inflammatory processes of the body, as well as poor fetal development, decreased cardiovascular health, and the risk of Alzheimer's disease (Grodstein, 2007).

Small fish obtain their omega-3 FAs from their diet, mainly from phytoplankton that are the primary producers of EPA and DHA. Thus, as the major source of EPA and DHA in the marine environment, it is important to understand the rates at which phytoplankton can produce EPA and DHA. Phytoplankton inhabit the upper irradiated layer of almost all oceans and bodies of fresh water on earth. Under favourable environmental conditions, phytoplankton synthesize lipids through photosynthesis, producing mainly polar lipids, such as glycolipids and phospholipids. However, under unfavorable conditions, many change their lipid biosynthetic

pathways to produce neutral lipids, mostly in the form of triacylglycerols (TAG) (Hu et al., 2008).

2. Literature Review

2.1 Long chain Fatty Acid Synthesis in Phytoplankton

Mühlroth et al. (2013) summarized FA synthesis in phytoplankton, which is initiated in two ways: 1) Photosynthesizing cells convert CO_2 to glycerol-3-phosphate (G3P) in the chloroplast. G3P is then converted to pyruvate, which is later made into acetyl coenzyme A (Acetyl-CoA) (Berg et al, 2002). This reaction is catalyzed via the pyruvate dehydrogenase complex. 2) Environmental stress, such as nutrient limitation, may disturb the citric acid cycle in the mitochondria, leading to citrate accumulation (Mühlroth, 2013). Citrate will then be released to the cytosol and converted to both oxaloacetate and acetyl-CoA, by using the enzyme ATP-dependent citrate lyase. Regardless of source, acetyl-CoA is then carboxylated to malonyl-CoA in the plastid. The formation of malonyl–CoA is catalyzed using acetyl-CoA carboxylase (a biotin-containing enzyme) (Figure 2.1).

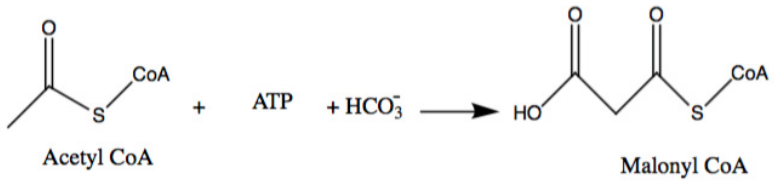

Figure 2.1 Carboxylation of acetyl CoA.

Next, malonyl–CoA and acetyl–CoA react with acyl carrier protein (ACP) to form acetyl–ACP and malonyl–ACP. Those reactions are catalyzed by the enzymes acetyl transacylase and malonyl transacylase. While malonyl transacylase is a specific enzyme, acetyl transacylase is not, and can transfer acyl groups from other carbon units. Hence, to form an odd numbered FA such as propionyl–ACP which can be synthesized from a propionyl–CoA, acetyl transacylase can be used. Acetyl–ACP and malonyl-ACP react to form acetoacetyl-ACP through

condensation (Figure 2.2).

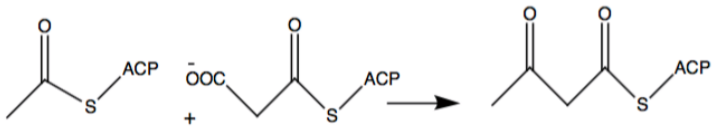

Figure 2.2 Formation of acetoacyl-ACP.

The next three steps in FA synthesis aim to reduce the C-3 carbon into a methylene group. Acetoacyl-ACP is reduced to hydroxybutyryl-ACP, then dehydrated, and finally oxidized using NADPH. This results is the formation of butyryl-ACP (Figure 2.3).

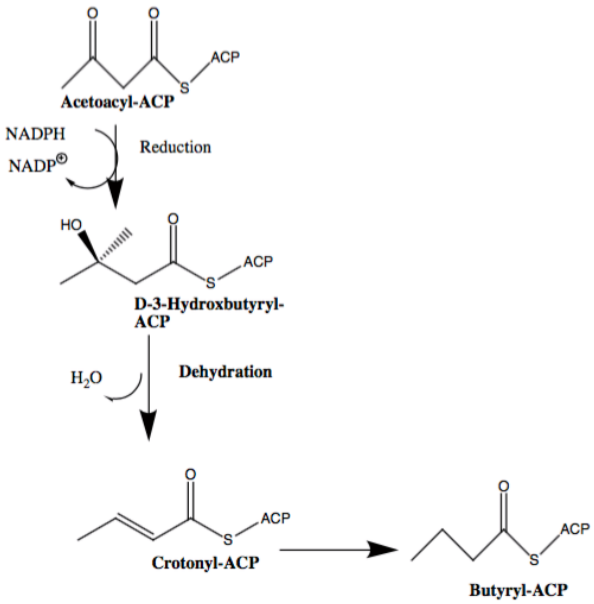

Figure 2.3 Formation of butyryl-ACP from acetoacetyl-ACP.

To form long chain FAs, malonyl–ACP will condense with butyrl –ACP to produce C-6 FA. This cycle continues until C-16 is produced (Berg et al., 2002). ACP can then be removed from the FAs using a fatty acyl-ACP thioesterase (FAT). In the chloroplast envelope, the FAs are then activated to become acyl-CoA by long chain acyl-CoA synthetase (LACS). Some FAs are then transferred to the cytosol, eventually reaching the endoplasmic reticulum (ER). In the ER, the FAs go through further elongation and desaturation in preparation for lipid synthesis. The

elongases of FAs are encoded by β-ketoacyl-CoA synthase (KCS). KCS is divided into "elongase of very long-chain FA" (ELOVL), which contributes to sphingolipids and phospholipids, and "FA elongases" (FAE), which contributes to triacylglycerols (TAG) or wax esters (Khozin-Goldberg and Cohen, 2010). FA in the ER (such as EPA and DHA) are incorporated into phospholipids and TAG via the Kennedy pathway. Desaturase enzymes may also act on the FAs, by removing two hydrogen atoms, forming a double bond at a specific location.

2.2 Lipids in Phytoplankton.

The biosynthesis of lipids is divided between two organelles: the chloroplast and the ER (Wang and Benning, 2012). In phytoplankton, the major lipids are galactolipids, specifically, monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), and sulfoquinovosyl diacylglycerol (SQDG). Phosphatidylglycerol (PG), and TAG are other major lipids found in phytoplankton. Phytoplankton may also contain phosphatidylcholine (PC) and phosphatidylinositol (PI) (Henderson et al., 1991). In the chloroplast, FAs are used to produce lysophasphatidic acid (LPA), which later is converted into phosphatidic acid (PA) via plastidial acyltransferases. PA along with diacylglycrol (DAG) are precursors for the membrane lipids synthesis such as DGDG, MGDG, and PG (Ohlrogge and Browse, 1995).

Figure 2.4. Structures of some of the lipid classes found in phytoplankton. A: DGDG, B: TAG, C: PC.

2.3 TAG Production Kennedy Pathway

TAG is synthesized in the endoplasmic reticulum (ER) through the Kennedy pathway, beginning when an acyl chain is esterified to G3P. Then, the glycerol-3-phosphate-acyltransferase (GPAT) enzyme catalyzes the formation of lysophosphatidic acid (LPA) from the G3P and a molecule of acetyl-CoA. Next, LPA and aceytl-CoA combine using the enzyme lysophosphatidic acid acyltransferase (LPAT) to form PA. PA is then dephosphorylated to form DAG, using an enzyme called phosphatase. Finally, DAG is converted to TAG using acyl–CoA: DAG acyltransferase (DGAT) (Figure 2.5) (Zienkiewicz et al., 2016).

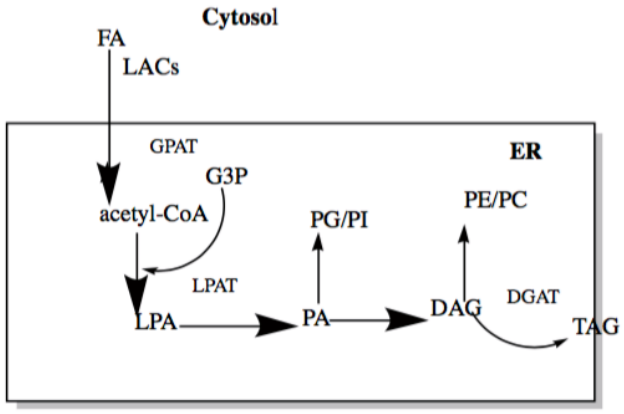

Figure 2.5 Kennedy pathway for the formation of TAG. The FA are transferred through the cytosol from the plastid. This figure also shows the precursors for PG, PI, PE, and PC. LACS is long chain acyl-CoA synthase.

2.4 PE and PC Production Kennedy Pathway

Kennedy and Weiss (1956) have proposed a pathway for the production of two phospholipids: 1) Phosphatidylethanolamine (PE) and 2) PC. The synthesis of PC and PE are divided into two branches that ultimately aim to form high energy intermediates: cytidine-diphosphocholine (CDP-choline) to synthesize PC, and cytidine-diphosphoethanolamine (CDP-

ethanolamine) for the biosynthesis of PE. As a result, those two branches are referred to as the CDP-choline and CDP-ethanolamine pathways.

In the CDP-ethanolamine pathway, the enzyme ethanolamine kinase (EK) is used to initially catalyze the phosphorylation of ethanolamine with the help of ATP. This reaction then leads to the formation of ADP and phosphoethanolamine. CTP-phosphoethanolamine cytidylyltransferase (ECT) then uses a molecule of cytidine triphosphate (CTP) and phosphoethanolamine to form a high energy intermediate called CDP-ethanolamine. This reaction also releases pyrophosphate as a by-product. Finally, CDP-ethanolamine and DAG react using the enzyme CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (EPT) to form PE, while CMP is released as a by-product (Figure 2.6). A similar pathway is followed for the synthesis of PC, with choline replacing ethanolamine in the scheme. PC can also be synthesized in both plants and algae using PE as a precursor. This pathway requires the methylation of PE using the enzyme PE methyltransferase (PEMT) (Sato et al., 2016).

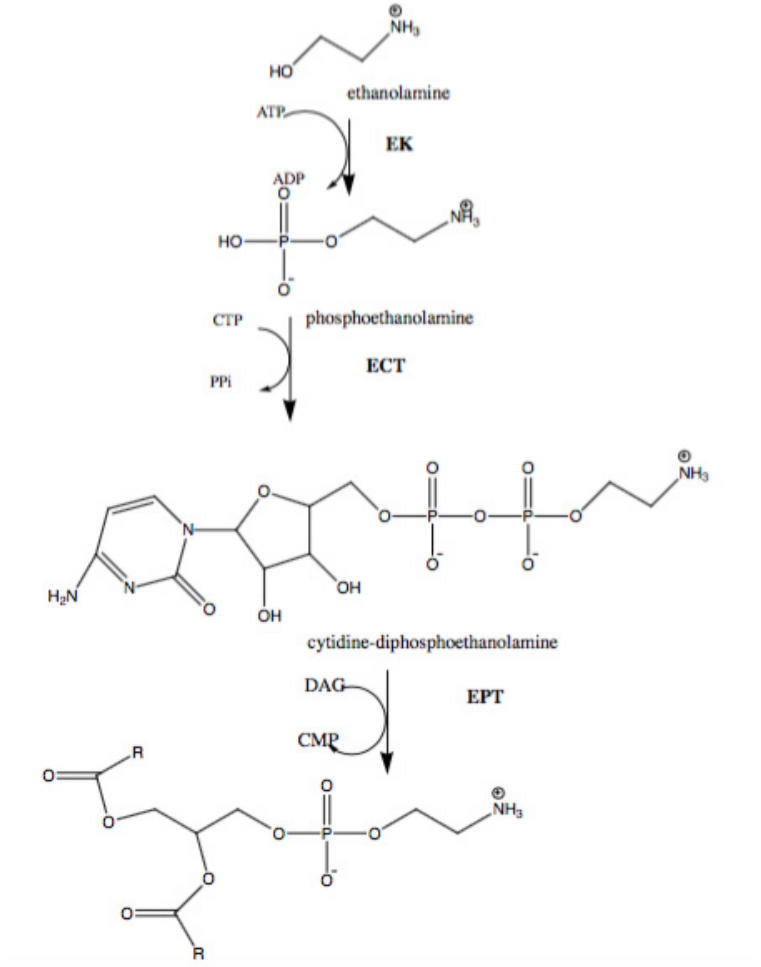

Figure 2.6 Formation of PE using the CDP-ethanolamine pathway.

2.5 Formation of the Chloroplast Lipids (DGDG, MGDG, SQDG and PG)

FAs that are synthesized in the stroma can either be transported to the ER, or stay in the chloroplast for the production of plastidal lipids. Boudiére et al. (2014) have summarized the plastidal lipid synthesis in eukaryotic cells. Plastidal lipid synthesis is initiated by the formation of G3P. PA and DAG must be available for the biosynthesis of lipids; they can either be imported from the ER, or can be newly synthesized in the chloroplast inner envelope membrane (IEM). Once PA is made it can either be used to synthesize galactolipids or PG. To synthesize PG, first PA and CTP condense, releasing pyrophosphate as a by-product. This condensation

reaction is catalyzed by the enzyme CDP-diacylglycerol synthase (CDS), which leads to the formation of CDP-diacylglycerol (CDP-DAG). Next, CDP-DAG and G3P react to form phosphatidyl glycerophosphate (PGP) using the enzyme glycerol-3-phosphate: CDP-diacylglycerol phosphatidyltransferase. Finally, phosphatidylglycerol phosphate phosphatase dephosphorylates PGP to form PG (Figure 2.7).

MGDG is synthesized using the enzyme monogalactosyldiacylglycerol synthase (MGD synthase) (Figure 2.7). MGD synthase is divided into three families (MGD1, MGD2, and MGD3) and is used to catalyze the transfer of the galactosyl residue from uridine diphospho-galactose (UDP-Gal) to DAG forming MGDG. MGDG can either be saved or used as a precursor for the production of DGDG through the addition of another molecule of UDP-Gal, using the enzyme digalactosyldiacylglycerol synthase (DGD synthase). SQDG lipids were discovered in 1959 by (Benson et al., 1959). SQDG synthesis is catalyzed by two enzymes: sulfoquinovosyldiacylglycerol synthase 1 and 2 (SQD1 and SQD2). SQD1 catalyzes the synthesis of the headgroup donor UDP-sulfoquinovose, which is formed from UDP-glucose and sulfite. SQD2 catalyzes the transfer of sulfoquinovosyl headgroup from UDP-sulfoquinovose onto a DAG to make SQDG.

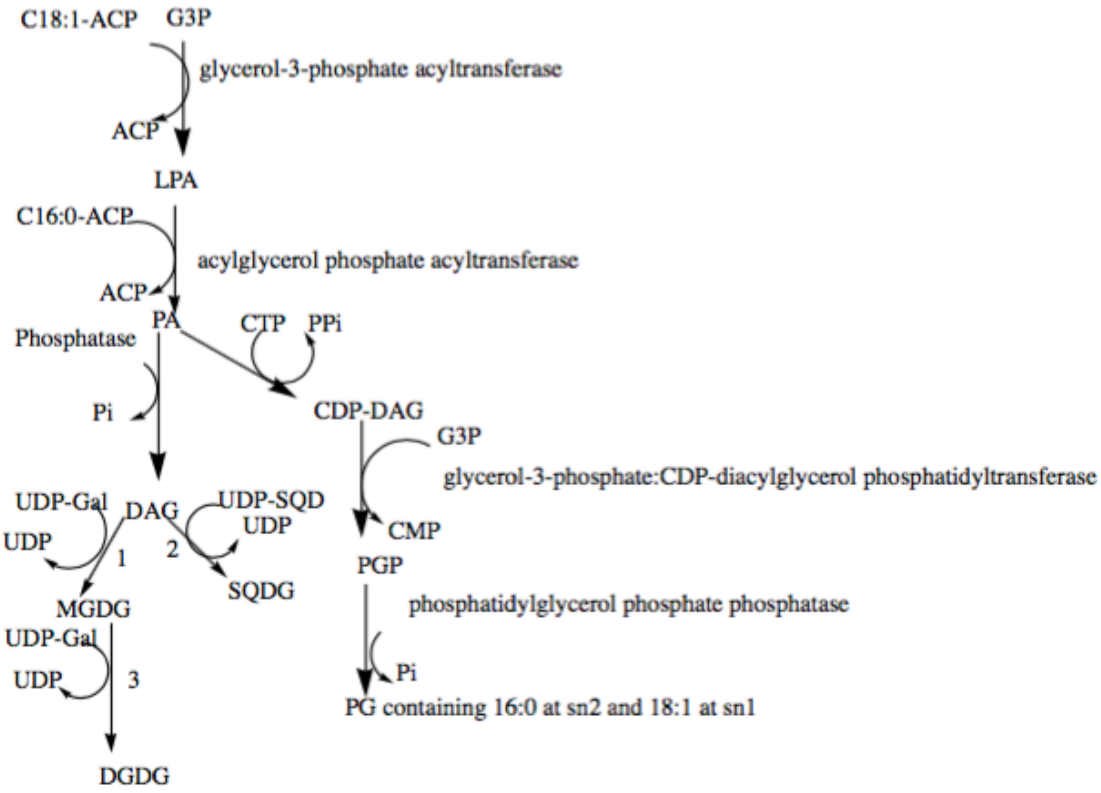

Figure 2.7 Chloroplast lipid pathway. The numbers represent the enzymes 1) MGDG synthase, 2) SQDG synthase, and 3) DGDG synthase. The right branch demonstrates the synthesis of PG. The left branch depicts the synthesis of galactolipids and sulfolipids using precursors. It has been redrawn from Boudiere et al. (2014).

2.6 Representative Phytoplankton

Two classes of eukaryotic phytoplankton were chosen in this experiment: the dinoflagellate *Heterocapsa triquetra* and the prymnesiophyte *Phaeocystis globosa*. Dinoflagellates are single-celled algae and may have two flagella, allowing the cells to be mobile. Cells are covered by a sheath that can be smooth or ornamented, and they can be autotrophs or heterotrophs (Wehr et al., 2015). Prymnesiophytes are small, and bi-flagellated. The nucleus is positioned centrally posterior to the end of the cell. They are covered with scales and may have spines or elaborated rims (Archibald, 2012).

The FA composition of phytoplankton show a number of trends. Dinoflagellates produce very little EPA and up to 43% DHA of the total FA. Many dinoflagellates also contain the unusual FA 18:5n-3 with a relative abundance of 29% (Leblond, 2000). Prymnesiophytes are rich in both DHA and EPA. Previous studies stated that prymnesiophytes were rich sources of EPA (7-34% of total FA) (Volkman et al., 1997).

2.7 Phytoplankton growth stages

Phytoplankton cell growth can be divided into four different phases: lag phase, log phase, stationary phase, and decay phase (Figure 2.8; Botana, 2014). In the lag phase, phytoplankton are introduced into a new environment and they begin to adapt. In the log phase, the cells are adapted to the new environment and are dividing and increasing in number. At this point, sufficient nutrients, light, and temperature allow the production of polyunsaturated fatty acids (PUFA), and chloroplast lipids. When nutrients are fully consumed, the stationary phase is reached. In the stationary phase, growth rates slow and the population is stabilized, with cells increasing in size but not in number. TAG is synthesized as a storage product, and waste products begin to build up. Finally, at the decay phase, the number of living cells decreases due to the buildup of waste and toxic products.

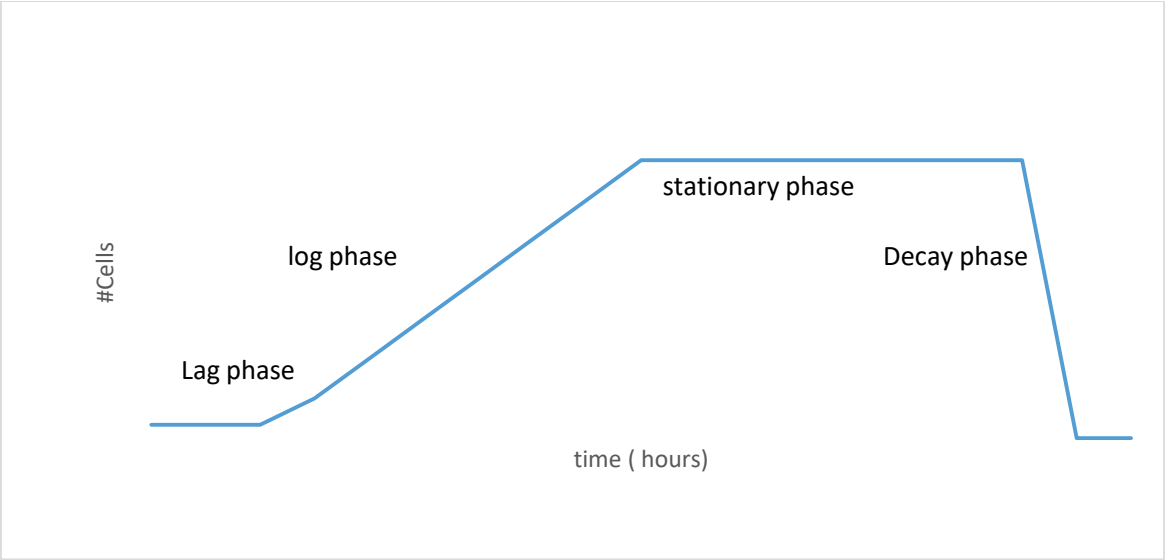

Figure 2.8 Sample plot of phytoplankton growth phases.

2.8 Variation in lipid biosynthesis with changing light intensity and temperature

Sufficient light leads to the overproduction of lipids in microalgae (Hallenbeck et al., 2015). A reason for this is given by Solovchenko et al. (2008), where it was stated that sufficient light intensity benefits the storing of photoassimilates, which is later converted into chemical energy. However, extremely low or extremely high light intensity will not favor microalgae total lipid growth (Zhu et al., 2016). For example, if the light intensity is below the compensation point i.e., the amount of light where the rate of photosynthesis equals that of cellular respiration, the biomass concentration will begin to diminish. This leads to poor growth, and thereby negatively impacting lipid accumulation. Similarly, extremely high light intensity will cause photoinhibition, damaging the photosystem, thus reducing lipid accumulation (Zhu et al., 2016). However, Wacker et al. (2016) recently evaluated the changes in FA profile in several phytoplankton species under 300 to 800 µmol photons m^{-2} s^{-1} and 20°C. They concluded that no generalization can be made for the effect of light on FA profile across different species of phytoplankton.

Temperature is thought to affect microalgae and lipid production in similar manners as light intensity. In general, increases in temperature to an optimal level will cause an increase to the total lipid content (Zhu et al., 2016). However, not all lipids experience the same increase in concentration; for example, *Tetraselmis subcordiformis* and *Nannochloropsis oculata* were found to have lower concentrations of PUFA and neutral lipids as temperature was increased (Wei et al., 2015). James et al. (2013) suggested that for the microalgae *Chlamydomonas reinhardtii,* decreasing the temperature below 25°C increased the amount of PUFA. It is important to note that the response due to change in temperature will be species-specific since optimal temperature growth varies within species. Previous studies on the effect of temperature on PUFA in plankton seston and zooplankton (Gladyshev et al., 2010) concluded that at low temperature, PUFA including EPA tend to increase.

3.0 Objectives

This study aims to evaluate the rate of production of omega-3 FA by the dinoflagellate *H. triquetra* under different light intensities and temperatures in semi-continuous cultures. It also compares the rate of production of FA in *P. globosa* with *H. triquetra* under the same growth conditions. Here, two hypotheses are tested: 1) that high light intensity causes the cell to synthesize less PUFA overall, including EPA and DHA; and 2) that low temperature causes the cell to synthesize higher levels of PUFA. At highest light intensity, it is expected that more TAG and less PUFA will be observed. Cells will increase the production of TAG in order to store the excess accessible energy. In contrast, under low light, the cell will be competing to photosynthesize. At this point, chloroplast lipid production and FA synthesis become more crucial; therefore, higher contents of chloroplast lipids, including MGDG, DGDG and PG, with higher amounts PUFA should be observed. Similar to the light effects, phytoplankton are expected to produce higher amounts of PUFA at low temperatures, possibly to maintain the fluidity of the cell (Section 2.7). This will also result in more chloroplast lipid synthesis to also maintain the cell membrane. In contrast, higher temperatures will cause the cell to produce a higher TAG content and less EPA, DHA, and chloroplast lipids. It is also expected to that the response of the two phytoplankton species will be different under the same growth conditions.

4.0 Methodology

4.1 Culture growth

All phytoplankton chosen in this study were grown by Steve Duerksen in a 12/12 light:dark L1-Si. Cultues (100 ml) were grown semi-continuously in 250 ml flasks. They were maintained in exponential growth by daily dilution using aseptic techniques until a constant growth rate and therefore, balanced growth, was achieved. *H. triquetra* (CCMP 449) was grown at a two different light levels (140 μmol photons m^{-2} s^{-1}, and 88 μmol photons m^{-2} s^{-1}) with the same temperature (24°C). A third *H. triquetra* culture was grown at a lower temperature (14°C) with the light intensity at 210 μmol photons m^{-2} s^{-1}. *P. globosa* (CCMP 628) were grown at 24°C and 140 μmol photons m^{-2} s^{-1} to analyze the rate of production within different phytoplankton species having the same growth conditions. *H. triquetra* cultures were grown in triplicate, and *P. globosa* cultures were grown in duplicate. When balanced growth was achieved, a 125 ml volume of culture was spiked with 1 ml of 100 mg L^{-1} 99% $NaH^{13}CO_3$ and then incubated for 6 hours at the irradiance in which they were grown. The entire sample was filtered on GFF filter paper and immersed in 2 ml $CHCl_3$. Unspiked samples (T0 samples) having the same growth conditions as the *H. triquetra* and *P. globosa* were also grown in triplicate and duplicate, and were used to correct the incorporation of ^{13}C into FA measured by gas chromatography mass spectroscopy (GCMS) (section 4.6).

4.2 Extraction of lipids

The lipids were extracted following the Folch et al. (1957) method using 2:1 $CHCl_3$: MeOH with 1 ml MeOH added to the 2 ml $CHCl_3$ present in the test tube. The samples were then ground with a glass rod, washed with 1 ml of 2:1 (v/v) chloroform: methanol and 0.5 ml of chloroform-extracted water, and sonicated in an ice bath for 4 minutes. They were then centrifuged for 2-3

min at > 1,000 rpm to partition the mixture into two layers, with the lower phase containing 86:14:1 (v/v) $CHCl_3$: methanol: water and all of the lipids, and the upper phase containing 3:48:47 (v/v) by $CHCl_3$: methanol: water and all the non-lipid portion of the cell. A double pipetting technique was used to recover the lower phase. The pipettes were washed with 3 x 1 ml of ice-cold chloroform, and then sonicated and centrifuged for 3 minutes. The double pipetting, washing the pipettes, sonicating and centrifuging process were done 3 times on each sample. Solvents were then evaporated to dryness using a N_2 evaporator. Samples were dissolved in 4 ml IPA: hexane (1:1) and divided into half for both high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) analysis (see below).

4.3 HPLC analysis

HPLC was used to quantify the lipids in the extract. The phytoplankton samples were concentrated to 150 µL and the HPLC was set to inject 80 µL of samples on to the column. The HPLC column was packed with YMC-Pack PVA-SIL-NP, and had the dimensions of 150 mm x 4.6 mm ID with 5 µm particle size. The detector was an evaporative light scattering detector (ELSD) with the conditions of 42 ^{o}C, 3.5 bar, and a gain 6. The syringe has a maximum injection volume of 100 µL.

Four different solvents were used in order to separate each lipid class for a total run time of 76 min. Solvent A contained hexane: ethyl acetate (98.8:1.2) (v/v), solvent B ethyl acetate with 0.1% acetic acid, and solvent C isopropanol: methanol: water (3:3:1) (v/v) with 0.1 % acetic acid and 0.05% triethylamine. The solvent gradient followed table 4.1.

Table 4.1. HPLC method for full lipid profile separation.

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)	Flow rate (mL min^{-1})
6.0	100	0.0	0.0	1.5
18	95	5.0	0.0	1.5
24	0.0	100	0.0	1.5
34	0.0	50	50	1.3
40	0.0	15	85	1.0
45	0.0	0.0	100	1.0
54	0.0	100	0.0	1.0
56	0.0	100	0.0	1.0
62	90	10	0.0	1.5
66	100	0.0	0.0	1.5
76	100	0.0	0.0	1.5

TAG, PG, PC, PE, FFA, DGDG, MGDG, DAG and MAG standards were made by dissolving each standard in 1:1 IPA: hexane. Each sample was made up to 0.5 mg ml^{-1}. TAG had a low solubility in IPA: hexane and hence was placed in a sonicating bath for 15 min. The calibration curves were made by injecting different volumes of 0.5 mg ml^{-1} standards in triplicate.

4.4 TLC separation

Thin layer chromatography (TLC) was used to separate the lipids and isolate individual lipid classes. The TLC plate was placed in a 100 °C oven for 30 minutes, and then cooled in a desiccator. Two TLC chambers were used, with chamber 1 containing $CHCl_3$: methanol: acetic acid: water (50:30:8:3) (v/v) to separate polar lipids, and chamber 2 containing hexane: diethyl ether: acetic acid (70:30:2) (v/v) to separate non-polar lipids. The samples were first evaporated to dryness to remove the IPA: hexane and then dissolved in 20 μL of methylene chloride to be spotted on the TLC plate; for comparison of retention factors, standards were made up to 30 mg ml^{-1}. Each of the standards and samples were spotted onto the TLC plate using 25 μL micropipettes.

The TLC plate was placed in chamber 1, until the solvent reached halfway up the plate. The plate was then allowed to dry for approximately 10 min, and placed in chamber 2 until the solvent reached the top of the plate. The plate then was allowed to dry again and sprayed with 4% dichlorofluorescein in ethanol to visualize under UV light. To isolate each lipid, bands were scraped off the plate and placed into a test tube. Lipids were solubilized using chloroform for non-polar lipids, and methanol: chloroform 2:1 for polar lipids.

4.5 Transesterification of lipids

FA in each band recovered from the TLC plate were converted to fatty acid methyl esters (FAME) for gas chromatography (GC) analysis. The Hilditch method (1935) was used to make FAME from FA. Hilditch reagent was made by mixing 100 ml of methanol (which was passed through a filter paper filled with anhydrous sodium sulphate) with 1.5 ml of 98% sulfuric acid. First the solvents that were used to recover each fraction from the TLC plate were evaporated. Then 1.5 ml of methylene chloride was added to each fraction along with 3 ml of Hilditch

reagent. Each fraction was then flushed with nitrogen and heated for 1 hr at 100 ^{o}C. Fractions were then allowed to cool down and 3 ml of hexane and 1 ml of distilled water was added to each fraction and vortexed. The top phase was then removed to a second tube and 1 ml of hexane was added to the first tube. Once again the top phase was removed and added to the same second tube. To the second tube, 2 ml of distilled water was added, and vortexed. The top phase from the second tube was added to a third tube which contained few scoops of sodium sulphate. The solvent of the third tube was removed and added to a final fourth tube. Samples were then concentrated down to 100 µL and placed in a GC vial.

4.6 GC and GCMS analysis

GC-FID (flame ionization detector) of FAME was used to determine the FA proportions. FAME was analyzed on a 30 m fused silica column coated with 50%-cyanopropylmethylpolysiloxane (0.25 µm film thickness), using helium (flow rate 1 ml min^{-1}) as carrier gas with detection by flame ionization. A splitless method was used to introduce 1 µL of sample to an injector maintained at 250^{o}C. The helium flow was at a rate of 1 ml min^{-1} generating a mixture of analyte and carrier gas in the column. The flow rate of air and hydrogen to the detector was 450 ml min^{-1} and 45 ml min^{-1}. The detector was kept at 270^{o}C, while the oven temperature varied, initially at 150^{o}C for 2 min, and then ramped at a rate of 5^{o}C min^{-1} until it reached 220 ^{o}C.

GCMS with the same GC conditions and operating in chemical ionization (CI) mode with ammonia as reagent gas (flow rate at 1 ml min^{-1}), transfer line temperature of 180 ^{o}C, fore pressure at 69 mTorr, and damping gas flow rate at 2.3 ml min^{-1}. GCMS was used to determine the incorporation of ^{13}C from bicarbonate ions into PUFA as atom % following Dunstan (1988). This in turn was used to calculate a rate of FA production in (ng of FA) (µg of lipid) $^{-1}$ h^{-1}:

FA production in a lipid class was calculated following (equation 1).

$$FA\ Production\ =\ \frac{\left(\frac{\left(Mass\ of\ FA\ (\mu g)\times\frac{atom\%\ FA}{atom\%\ media}\right)}{6}\right)}{total\ lipids\ (from\ the\ HPLC)(\mu g)} \qquad \text{(Equation 1)}$$

Total lipids represented the sum of acyl lipids determined by HPLC in µg per culture. Atom % ^{13}C FA was determined by GCMS (Appendix B). The atom % ^{13}C of the media was calculated based on dilution of the 1 ml spike of $NaH^{13}CO_3$ and the natural abundance of ^{13}C in seawater. The mass of FA in a given lipid class was calculated based on its mass proportion of total FA and the known mass of the lipid class it is found in. Moreover, the following steps were taken to determine the mass of all FA within a lipid class:

The molecular mass of all the FA recovered that are ≤14:0 was calculated (for example DHA's molecular mass is 328.50 g mol^{-1}, and 16:0's molecular mass is 256.43 g mol^{-1}). Next, the total mass of FA in a lipid class was assumed to be 100 g so that the number of moles of a given FA could be determined by dividing the mass % obtained from the GC by the molecular mass of the same FA.

For example, molecular mass of DHA is 328.50 g mol^{-1}, and mass % = 3.0.

$$Molar\ mass\ of\ the\ same\ FA = \frac{3}{328.50} = 0.0091$$

Then the moles of a particular lipid class that the FA was isolated from were calculated by dividing the moles of FA by the number of moles of FA within the lipid class. For example, if DHA calculated above was recovered from a TAG band in TLC, one would divide the moles of DHA calculated by 3 (TAG has three acyl groups). If it was recovered from DGDG, one would divide by 2.

For example,

$$\textit{Mol of TAG equivalent to one containing 3 DHA FA} = \frac{0.0091}{3} = 0.0030$$

The molecular mass of the lipid class if it contained only one FA structure was then calculated:

$$Molecular\ mass\ of\ TAG\ if\ containing\ only\ DHA(mass\ of\ the\ acyl\ backbone) + 3 \times\ molecular\ mass\ of DHA$$

$$= (3 \times 12.011 + 2 \times 1.008) + 3 \times 328.50.$$

$$= 985.50\ g\ mol^{-1}$$

Next, the portion due to FA was calculated by multiplying the number of moles of FA produced from 1 mole of lipid class (for TAG, it is 3), then dividing this number by the molecular mass of the lipid class. The mass of the lipid class in grams was then calculated by multiplying the number of moles by the molecular mass of the lipid class. After repeating this for all FA that are ≥14:0, the mass of lipid class in grams was added to give a total lipid mass.

The total was used to find the mass % of lipid class produced from each FA using equation (2):

$$mass\ \%\ of\ lipid\ class\ produced\ from\ each\ FA = \frac{mass\ of\ lipid\ class\ in\ (g)\ calculated\ from\ one\ FA}{Total\ mass\ of\ lipid\ class\ in\ (g)} \times 100 \qquad \text{(Equation 2)}$$

Finally, equation 3 was then followed to determine the mass due to FA from each lipid class.

$$Mass\ due\ to\ FFA\ from\ each\ lipid\ class = \left(mass\ of\ lipid\ class\ obtained\ from\ the\ HPLC\ in\ (\mu g)\right) \times \frac{mass\ \%\ of\ lipid\ class}{100} \times (portion\ due\ to\ FA) \qquad \text{(Equation 3)}$$

The portion due to FA was simply the molar mass of FA within a given lipid class relative to the molar mass of the lipid class if its acyl structure was that same FA.

4.7 Statistics

All statistical tests were carried out with SSPS software. A one-way analysis of variance (ANOVA) with post hoc tests using Tukey's comparison between subjects was used to compare the effect of temperature and light in *H. triquetra* on lipid concentration, FA proportions, and rate of production of FA.

5.0 Results

5.1 Calibration curves

Using HPLC equipped with ELSD, standard curves were created based on the major lipid classes found in dinoflagellates (Řezanka et al., 2017) and prymnesiophytes (Henderson et al., 1991). Standard curves for all the lipid classes generally had an R^2= 0.989. None of the curves had a y-intercept = 0. Appendix A shows the calibration curve for each lipid class.

5.2 Lipid classes in *H. triquetra* and *P. globosa*

With HPLC-ELSD, the lipids DGDG, TAG, FFA, and PC were consistently detected. MGDG could only be detected for the *H. triquetra* grown at 14ºC, totaling 2 µg ml^{-1}. PE and PG were detected as well; however, they were under the limit of quantification of the ELSD.

Table 5.1 Lipid class concentration expressed as a mass % of total lipids identified in *H. triquetra* and *P. globosa.*

Species	Temperature (ºC)	Light intensity (µmol photons m^{-2} s^{-1})	TAG	FFA	DGDG	PC
H. triquetra	14	210	44.95[a]	4.99[a]	44.37[a]	5.69[a]
H. triquetra	24	88	29.17[b]	22.40[b]	39.67[a]	8.75[b]
H. triquetra	24	140	50.64[a]	26.90[b]	18.36[b]	4.09[a]
P. globosa	24	140	11.17[c]	35.62[c]	27.40[c]	25.82[c]

H. triquetra: (n=3)
P. globose: (n=2)
Values with different superscripts are significantly different for ($p<0.05$)

The proportion of TAG in *H. triquetra* varied with growth conditions ($F(2,6)= 16.811$, $p= 0.003$). When temperature was held constant at 24 ºC for *H. triquetra,* post-hoc tests indicated that there was significantly more TAG at the higher light intensity (Tukey's test, $P<0.05$). However, at a lower temperature of 14ºC and much higher light intensity of 210 µmol photons m^{-2} s^{-1}, the same high proportion of TAG was found.

In contrast, growth conditions for FFA had a completely different effect ($F(2,6)= 24.29$, $p= 0.001$), with conditions of lowest growth temperature and highest light producing the smallest proportion of FFA. DGDG showed a third pattern with the most extreme conditions (low temperature/high light and high temperature/low light) generating significantly more DGDG than the more moderate condition of high temperature/medium light ($F(2,6)= 46.6$, $p= 0.001$). Last, for PC, *H. triquetra* grown at the high temperature/low light had significantly higher proportions of PC ($F(2,6)= 6.726$ $p= 0.029$). However, conditions having high light intensities (140 µmol photons m^{-2} $s^{-1}$and 210 µmol photons m^{-2} s^{-1}) had the same proportions of PC.

P. globosa grown in the same condition as *H. triquetra* (24 ºC and 140 µmol photons m^{-2} s^{-1}) had significantly different proportions of lipid classes. *H. triquetra* had significantly higher proportions of TAG ($F(1,4)= 28.926$, $p=0.006$). In contrast, *P. globosa* had significantly higher proportions of FFA ($F(1,4)= 9.346$, $p=0.038$), DGDG ($F(1,4)= 33.675$, $p=0.004$) and PC ($F(1,4)= 870.891$, $p=0.00$).

5 **3 FA proportional data within lipid classes**

The most abundant FA was 16:0 which was ~ 30% to 47% of the total FA in both *H. triquetra* and *P. globosa* under all conditions and all lipid classes. The second most abundant FA was 18:0 followed by DHA. Proportions of the other FA fluctuated depending on the conditions the phytoplankton species was grown in. FFA were consistently low in PUFA and results are not shown.

Table 5.2 Major FA (mass % total FA identified; mean ± sd) in *H. triquetra* and *P. globosa*.

Specie	Temperature (°C)	Light intensity (µmol photons m^{-2} s^{-1})	Lipid class	14:0	16:0	18:0	DHA	EPA	18:5n-3	18:4n-3	18:3n-3
H. triquetra	14	210	TAG	2.81±0.97	29.6±3.50	14.12±2.08	2.46±0.66^{a}	0.46±0.18^{a}	3.10±0.01^{a}	4.59±0.56^{a}	2.10±0.60^{a}
			DGDG	1.28±0.04	36.09±4.56	20.04±3.82	0.86±0.21^{a}	2.99±0.25^{a}	4.81±0.65^{a}	0.26±0.14^{a}	0.29±0.08^{a}
			PC	1.57±0.43	35.66±5.37	18.05±4.24	5.74±1.35^{a}	0.53±0.11^{a}	1.27±0.21^{a}	2.04±0.35^{a}	0.29±0.05^{a}
H. triquetra	24	88	TAG	2.49±0.48	36.80±4.24	15.53±2.25	4.35±0.29^{b}	0.64±0.21^{a}	1.49±0.35^{b}	0.77±0.07^{b}	2.66±0.53^{a}
			DGDG	1.30±0.44	42.83±2.20	16.99±0.94	1.85±0.32^{a}	0.29±0.067^{b}	4.55±0.50^{a}	0.80±0.05^{b}	0.93±0.17^{b}
			PC	1.25±0.21	45.90±0.64	19.62±2.66	4.31±0.69^{a}	0.26±0.081^{b}	0.27±0.024^{b}	0.74±0.01^{b}	0.34±0.05^{a}
H. triquetra	24	140	TAG	3.82±0.19	36.69±4.24	13.76±0.72	4.88±1.59^{b}	4.14±1.02^{b}	0.09±0.03^{c}	1.63±0.06^{c}	2.06±0.12^{a}
			DGDG	0.90±0.10	37.70±6.05	18.74±3.16	0.14±0.03^{b}	1.90±0.45^{c}	0.31±0.02^{b}	1.07±0.17^{c}	0.21±0.07^{a}
			PC	1.12±0.09	41.36±3.07	21.20±2.83	0.22±0.06^{b}	2.14±-0.39^{c}	0.22 ±0.09^{b}	0.86±0.03^{c}	0.24±0.09^{a}
P. globosa	24	140	TAG	2.01±0.35	46.54±2.28	22.18±2.61	2.66±0.05^{a}	0.64±0.21^{a}	0.67±0.30^{d}	1.94±0.42^{c}	1.12±0.02^{b}
			DGDG	1.37±0.47	43.35±2.44	21.82±1.28	0.38±0.04^{c}	0.81±0.01^{d}	3.63±1.38^{a}	2.38±1.52^{d}	0.86±0.11^{b}
			PC	1.12±0.01	43.03±10.18	20.99±6.44	1.80±0.06^{c}	0.66±0.24^{a}	0.63±0.04^{c}	0.66±0.01^{d}	0.55±0.03^{b}

H. triquetra: (n=3)
P. globose: (n=2)
Within a lipid class, values with different superscripts are significantly different at $p<0.05$.

In *H. triquetra*, varying the growth conditions significantly affected the FA mass% of DHA in TAG $p<0.05$ ($F(2,6)= 20.678$, $p=0.043$). Lowering the temperature caused the *H. triquetra* cells to have significantly less DHA in TAG (Tukey's test, $p <0.05$). Furthermore, no significant difference in FA proportions of DHA in TAG was found for growth conditions where the only varying factor was the light intensity. Light intensity and temperature had a different significant effect on DHA in DGDG ($F(2,6)= 10.961$, $p= 0.01$). Post hoc test shows that at the high temperature/low light intensity there was significantly more DHA in DGDG, and at high temperature/medium light intensity there was significantly less DHA in DGDG (Tukey's test, $p<0.05$). Similar to the impact of DHA on DGDG in *H. triquetra*, DHA in PC was also affected by both light intensity and temperature ($F(2,6)=21.896$, $p=0.002$). No difference in FA proportions of DHA in PC was found at the low temperature/high light intensity and high temperature/low light intensity (Tukey's test, $p<0.05$). However, there was significantly less DHA in PC as the light intensity was increased keeping the same temperature.

Other PUFA proportions have also been affected by varying light intensity and temperature. For example, the higher light intensity had a substantial effect on the EPA levels in *H. triquetra*, as elevating the light intensity while keeping the same temperature has caused EPA levels to raise by 83-85% in all TAG and DGDG. In all conditions in *H. triquetra,* 18:5n-3 levels in TAG were significantly different as shown by one-way ANOVA ($F(2,6)= 144.896$, $p= 0.000$). Raising the temperature and light intensity caused the levels of 18:5n-3 to be lower in TAG. In *H. triquetra*, the effect of light and temperature on levels of 18:5n-3 in DGDG was similar to that effect on DHA in PC ($F(2,6)= 93.611$, $p=0.00$); post hoc tests show no difference in the FA proportions of 18:5n-3 at the low temperature/high light intensity and high temperature/low light intensity (Tukey's test, $p<0.05$). However, there was significantly less 18:5n-3 in DGDG at the

high temperature/medium light intensity (Tukey's test, $p<0.05$). In TAG, 18:4n-3 was significantly impacted by light intensity and temperature ($F(2,6)=122.687$, $p=0.000$), as raising the light intensity caused 18:4n-3 to rise significantly. Finally, no significant difference was seen for the levels of 18:3n-3 in TAG and PC across all conditions as shown by one-way ANOVA ($F(2,6)=0.994$, $p=0.424$). However, lowering the light intensity caused the levels of 18:3n-3 in DGDG to rise.

P. globosa grown in the same conditions as *H. triquetra* had significantly different PUFA levels in all lipid classes (Table 5.2). For instance, DHA and EPA in TAG was significantly higher in *H. triquetra* than *P. globosa* grown in the same conditions as noted by one-way ANOVA ($F(1,4)=10.361$, $p=0.032$) and ($F(1,4)= 48.01$, $p= 0.002$). In contrast, DHA and EPA in DGDG was significantly higher for *P. globosa* compared to *H. triquetra* grown in similar conditions ($F(1,4)=90.096$, $p= 0.001$) and ($F(1,4)= 18.192$, $p= 0.013$). Finally, DHA and EPA in PC demonstrated a different trend as DHA in PC was significantly higher for *P. globosa* ($F(1,4)= 18.309$, $p= 0.13$), while EPA was significantly lower for *P. globosa* in PC ($F(1,4)= 84.962$, $p= 0.$

5.4 Incorporation of ^{13}C into FA

Table 5.3 Incorporation of ^{13}C in FA (atom% ^{13}C; mean ± sd) in *H. triquetra* and *P. globosa*.

Species	Temperature (°C)	Light intensity (µmol photons m^{-2} s^{-1})	Lipid class	DHA	EPA	18:5n-3	18:4n-3	18:3n-3
H. triquetra	14	210	TAG	1.4±0.1[a]	N/A	N/A	0.8±0.01	2.5±0.4[a]
			DGDG	3.5±0.3[a]	N/A	N/A	N/A	N/A
			PC	2.7±0.5[a]	N/A	N/A	N/A	N/A
H. triquetra	24	88	TAG	7.1±0.7[b]	4.6±0.2	12.6±0.2	4.7±0.2	10.5±0.4[b]
			DGDG	9.1±0.3[b]	5.7±0.9	1.1±0.6	1.6±0.6	10.9±0.9
			PC	7.2±0.1[b]	N/A	N/A	N/A	N/A
H. triquetra	24	140	TAG	4.2±0.4[c]	N/A	5.1±1.0	6.2±0.8	11.3±1.3[b]
			DGDG	N/A	N/A	N/A	N/A	N/A
			PC	10.9±0.3[c]	N/A	N/A	N/A	N/A
P. globosa	24	140	TAG	25.0±3.9[d]	17.6±0.1	21.3±1.3	26.3±0.6	21.4±1.3[d]
			DGDG	10.8±0.3[c]	14.3±0.5	24.7±3.3	29.3±3.3	21.6±3.3
			PC	22.8±0.4[d]	14.3±0.4	N/A	N/A	N/A

H. triquetra: (n=3)
P. globose: (n=2)
Values with different superscripts are significantly different for ($p<0.05$)
"N/A" is not available, as the peak size was insufficient to allow measurement of atom% ^{13}C

Since atom% ^{13}C was corrected for natural abundance of ^{13}C, any FA with a value >0 was synthesized during the incubation period and represented new production of FA (Table 5.3). There are many gaps in this data because for a number of PUFA, the incorporation of ^{13}C was below the limit of detection. Despite those gaps, a number of patterns can still be observed. For instance, atom% ^{13}C for DHA in all lipid classes is much lower for high light intensity and low temperature, and the highest labelling in *H. triquetra* was found at high temperature/low light intensity condition. The only newly incorporated EPA in *H. triquetra* was found in TAG and DGDG grown at low light intensity and high temperature. No newly incorporated PUFA was detected in DGDG for *H. triquetra* grown in medium light intensity and high temperature. Finally, overall, the incorporation of ^{13}C in all FA was much higher in *P. globosa* than *H. triquetra*.

DHA was detected for most lipid classes grown in different conditions and can be used for comparison. In *H. triquetra*, there was a significant effect on the labelling of DHA in TAG $F(2,6)=110.161$, $p=0.00$). Post hoc test revealed that there was significantly higher labelling of atom% ^{13}C in conditions of high temperature/low light intensity, and significantly lower labelling of DHA in TAG in conditions of low temperature/high light intensity (Tukey's test, $p<0.05$). Atom % ^{13}C of DHA in PC varied significantly in *H. triquetra* as well ($F(2,6)=913.904$, $p=0.00$). Increasing the light intensity while keeping a constant temperature caused an increase in labelling of DHA in PC. Decreasing the temperature and further increasing light intensity also caused a decrease in atom % ^{13}C in DHA in PC for the *H. triquetra*.

5.5 Rate of production

Following the equations in section 4.6, Table 5.4 has been created to show the rate of PUFA production.

Table 5.4 Production (P; mean ± sd, in (ng) (µg lipid)$^{-1}$ (h)$^{-1}$) of PUFA in *H. triquetra* and *P. globosa*.

Species	Temperature (°C)	Light intensity (µmol photons m^{-2} s^{-1})	Lipid class	DHA	EPA	18:5n-3	18:4n-3	18:3n-3
H. triquetra	14	210	TAG	0.38±0.16^{a}	N/A	N/A	0.17±0.02^{a}	0.28±0.06^{a}
			DGDG	9.9±2^{a}	N/A	N/A	N/A	N/A
			PC	4.5±1.2^{a}	N/A	N/A	N/A	N/A
H. triquetra	24	88	TAG	2.3±0.01^{b}	0.24±0.01	0.26±0.03^{a}	8.1±0.95^{b}	9.1±1.1^{b}
			DGDG	18.1±1.0^{b}	1.6±0.9	4.2±2.1	17.5±0.1	18.7±0.1
			PC	5.2±0.2^{a}	N/A	N/A	N/A	N/A
H. triquetra	24	140	TAG	3.2±0.2^{c}	N/A	0.53±0.16^{b}	0.98±0.01^{b}	2.7±1.0^{c}
			DGDG	N/A	N/A	N/A	N/A	N/A
			PC	0.39±0.04^{b}	N/A	N/A	N/A	N/A
P. globosa	24	140	TAG	0.20±0.02^{a}	12.9±11.2	0.71±0.30^{b}	0.14±0.06^{d}	7.2±0.1^{b}
			DGDG	2.8±0.2^{c}	7.9±0.6	5.3±0.2	1±0.03	7.6±0.6
			PC	38±4^{c}	9.6±0.6	N/A	N/A	N/A

H. triquetra (n=3)
P. globose: (n=2)
Values with different superscripts are significantly different for ($p<0.05$)
"N/A" is not available, as the peak size was insufficient to allow measurement of atom% ^{13}C

DHA is the only FA that had detectable production levels in all conditions and different patterns were observed for different lipid classes. In *H. triquetra*, the effect of light and temperature on DHA production in TAG was significant at $p < 0.05$ ($F(2,6)= 5.033$, $p=0.021$). Post hoc tests show that the highest production of DHA in TAG was at high temperature/medium light intensity, and significantly lowest production of DHA in TAG was observed at low temperature/high light intensity. Furthermore, production of DHA in DGDG could not be measured for high temperature and medium light intensity conditions. However, the effect of light and temperature was also significant ($F(1,4)= 5.539$, $p=0.016$), as the highest production of DHA in DGDG was found at high temperature/low light intensity (Tukey's test, $p<0.05$).

Finally, the effect of light intensity and temperature was also significant for DHA in PC in *H. triquetra* ($F(2,6)= 5.093$, $p= 0.031$). Post hoc test show the lowest production of DHA in PC was at high temperature/medium light intensity, and there was no significant difference in *H. triquetra* grown at high temperature/low light intensity and low temperature/high light intensity for DHA in PC.

Unlike DHA, EPA production was only measurable in *H. triquetra* grown at low light intensity and high temperature in the lipid classes DGDG and PC. EPA rate of production was also measurable in all lipid classes in *P. globosa*. 18:5n-3 however was measurable in TAG for the *H. triquetra* samples grown at a constant temperature with different light intensity, as the light variation caused a significant effect on production rates of 18:5n-3 in TAG ($F(1,4)= 12.832$, $p=0.023$). Higher levels of 18:5n-3 was observed for conditions grown at the medium temperature and higher light intensity (Tukey's test $p<0.05$).

P. globosa produced significantly less DHA ($F(1,4)= 6.736$, $p=0.042$), 18:4n-3 ($F(1,4)= 9,121$, $p=0.039$) and 18:3n-3($F(1,4)= 39.890$, $p=0.003$) than *H. triquetra* grown in the same

condition. No significant difference was found in the production of 18:5n-3 in TAG between the two species. *P. globosa* had some FAs detectable in PC and DGDG.

6.0 Discussion

Here, for the first time the effects of light and temperature on the rate of production of PUFA have been described. The dinoflagellate *H. triquetra* was chosen to evaluate this effect because it is a DHA producer. For comparison, PUFA production was also determined in *P. globosa*, another DHA producer. To gain a better sense of the organelle where production was occurring, PUFA production within lipid classes was measured. This required the extraction and separation of lipid components of the phytoplankton species. In this study, four major lipids were detected: TAG, DGDG, FFA and PC.

Many previous studies have reported the lipid profile of dinoflagellates. This phytoplankton class tends to be abundant in TAG (Parrish et al. 1993, 1994) and a similar result was found in this work where TAG was ~30 – 50% of the total lipids. Similarly, galactosyl lipids (DGDG and MGDG) are among the most abundant lipids in nature and account for ~30 – 50% of the total lipids in dinoflagellates (Siegenthaler, 2004). In agreement with this, DGDG was present in *H. triquetra* at ~ 18 – 45% of the total lipids; however, MGDG was below the detection limit of the ELSD, except at the lower temperature. Flaim et al. (2012) offer a possible explanation for these low MGDG levels. They found that in psychrophiles (organisms that are capable of growth and reproduction in low temperatures) MGDG is more important for maintaining membrane fluidity than DGDG at low temperatures. Similarly, at higher temperatures, Leblond et al. (2010) found that in the dinoflagellate *Pyrocystis sp.* grown between 25 and 35 ^{o}C, DGDG tend to respond more to temperature changes than MGDG, which could explain the decrease in DGDG content as the temperature was raised.

The third major lipid class that was detected in the dinoflagellate was FFA. It was surprising to see such high levels of FFA at 5-35% (Table 5.1); FFA are typically only generated

when lipids have been either chemically or enzymatically hydrolyzed. Normally in the cell, the levels of FFA should be <5% (Virtue et al., 1993). Levels above 5% are generally thought to indicate degradation of samples due to mishandling (Virtue et al., 1993). In general, lipase activity seems to be stimulated by the process of algae filtration during sample collection (Berge et al., 1995; Budge et al., 1999) but this effect was thought to be limited to diatoms. The results in the current study suggest that the problem is more wide-spread. To avoid this, samples should have been treated with a small aliquot of boiling seawater just before completion of filtration; this has been found effective in deactivating lipolytic enzymes, which is the likely cause of the acyl lipid degradation (Budge et al., 1999). The presence of FFA is also problematic in determining the concentration of lipid classes, as other lipid classes concentrations determined in this study could be larger than the values reported. Also, it is hard to determine the preferential lipid class substrate for the enzymes.

Phospholipids (PE, PC and PG) were also found with PC being the major phospholipid; unlike PC, PE and PG were under the limit of quantification. This agrees with early work evaluating the lipid profiles of 10 dinoflagellates species (Anesi et al., 2016). That study found PC as the major phospholipid class, and concluded that PG and PE were not present in all dinoflagellates. Other studies also found PC to be the major phospholipids in dinoflagellates (Řezanka et al. 2017).

For *P. globosa,* Maat et al. (2013) have found similar lipid class results, with PC being the most abundant lipid. That study also found TAG at lower proportions than DGDG, with minor contributions from PE and PG. However, MGDG was found to be the second most abundant lipid in *P. globosa.* Maat et al. (2013) grew *P. globosa* at 15^{o}C and 90 µmol photons m^{-}

^{2} s^{-1} and the differences in MGDG composition may have been due to a similar effect as found in the *H. triquetra* (Flaim et al., 2012).

The second part of the work was to evaluate the FA composition of each lipid class. Major FAs in dinoflagellates reported in the literature (Harvey et al., 1988; Reitan et al., 1994) are 16:0, 18:4n-3, EPA, and DHA. In this study, high levels of 18:0 was observed and the PUFA were relatively low (~ 9%). Although findings from Harvey et al. (1988) and Reitan et al. (1994) contradicts this study by observing high levels of PUFA and low 18:0, other studies on *H. triquetra* grown in similar conditions (100 µmol photons m^{-2} s^{-1} and 22^{o}C) have reported 18:0 to be as high as 29.6% and PUFA to be as low as 12.8% of the total FA (Matsuyama and Suzuki, 1998). The difference from Harvey et al. (1988) and this study may be due to the growth conditions, where the samples in the literature were grown at 15 ^{o}C and 160 µmol photons $m^{-2}s^{-1}$.

Prymnesiophytes *Isochrysis galbana* and *Pavlova lutheri* were found to be dominated by 14:0, 16:0, 18:1 and DHA (Hamm et al. 2003). Here, all lipid classes in *P. globosa* were found to be dominated by 16:0, and 18:0. Normally, Prymnesiophytes and more specifically *P. globosa* tend to be high in 18:1n-9 (>20 %) but in the current work, 18:1n-9 only represented 5% of the total FA composition in DGDG. Nonetheless, the higher presence of 18:5n-3 and DHA corresponds with the literature. Previous studies by Tang et al. (2001) have also concluded that *P. globosa* tend to be low in PUFA.

Thus, overall, the general lipid class data (TAG, DGDG, and PC) for both *H. triquetra* and *P. globosa* are quite similar to those reported in the literature. The same is true for FA profiles, with the data here meeting expectations for compositional analyses. This similarity to literature provides confidence that the results relating to PUFA production are also generally applicable to similar phytoplankton species.

When production is estimated relative to lipid, total lipid in a plankton cell will of course affect the calculated rate of production (Equation 1). The total lipid is influenced by the concentration of each lipid class in the cell. As DGDG is synthesized in the chloroplast where photosynthesis takes place, more DGDG at a low light intensity was expected (due to the cell competition for photosynthesis), while in high light, the cell would store excess energy in the form of TAG; thus, more TAG under higher light intensity was anticipated. Although surprisingly high levels of FFA were present, the results do agree with that expected, as *H. triquetra* grown at 88 μmol photons m^{-2} s^{-1} and 24 °C had lower TAG and higher DGDG levels than the ones grown at 140 μmol photons m^{-2} s^{-1} and 24 °C. Similarly, one would expect to see higher DGDG levels at lower temperatures to maintain the fluidity of the cell membrane (Harwood and Jones, 1989). Indeed, *H. triquetra* cells did have more DGDG at lower temperature (14 °C) even though the light intensity was raised. The high levels of TAG observed at the lower temperature might be due to the cell's tendency to store the excess energy from the high light intensity used in the lower temperature. This suggests that light intensity is a major factor influencing TAG levels in the cell. PC levels were also affected by the change in light intensity with PC concentrations highest at the lowest light intensity. This shows that at low light, the cell favors the conversion of PC, likely from DAG. PC is a major part of the eukaryotic cell membrane, so the higher PC concentrations at low light is likely to aid in maintaining the fluidity of the cell membrane.

H. triquetra grown at the same conditions as *P. globosa* produced more TAG and less DGDG, suggesting that *P. globosa* are better acclimated to higher light intensity. This also indicated that *P. globosa* cells were reacting similarly as *H. triquetra* grown at the lower light intensity, showing that effects of environmental conditions are species- specific.

Another factor influencing the productivity of PUFA is the mass of FA which is calculated from the proportional FA data determined by GCFID (Equation 1). Higher levels of PUFA for conditions in low light and low temperature was expected; however, this did not hold for all PUFA. In this study, when temperature was held constant, FA in DGDG responded to the increase in light levels by having less DHA, 18:5n-3 and 18:3n-3. At the same time, EPA and 18:4n-3 increased in proportions as the light intensity increased. Wacker et al. (2016) have examined the effect of light intensity in four different taxonomic classes of phytoplankton species: a bacillariophyte (*Asterionella Formosa*), a chrysophyte (*Chromulina* sp.), a cryptophyte (*Cryptomonas ovata*) and a zygnematophyte (*Cosmarium botrytis*). They observed an increase in 18:4n-3 and EPA in DGDG as the light intensity was increased in *Chromulina* sp and *Cryptomonas ovata*. This indicates that in different light environments, not all PUFA are important in maintaining membrane functions. Thus, the GC results suggest that in a controlled setting, manipulating the light levels for the purpose of increasing the production of both EPA and DHA might not work.

DHA, 18:5n-3, and 18:3n-3 are important FA that are typically associated with DGDG and MGDG in the thylakoid membrane (Hardwood and Jones, 1989). Previous studies have shown that those n-3 FA play key roles in increasing membrane fluidity which in turn increases the electron flow in photosynthesis (Horváth et al., 1987; Mock and Kroon, 2002). For this study's results, at low temperature and high light levels, more DHA in DGDG was observed than at high temperature and medium light levels, indicating that lowering the temperature does have an effect on increasing the DHA levels in the cell. A better designed study where the light level is kept constant and with varying temperature would give a definitive answer. *P. globosa* had different proportions of FA compared to *H. triquetra* grown in similar conditions and produced

different proportions of PUFA in lipid classes. For example: higher DHA mass proportions in TAG were found in *H. triquetra*, while DHA was higher in both DGDG and PC in *P. globosa*. The different proportions of PUFA in different lipid classes reinforces the species- specific nature of FA profiles.

Incorporation of ^{13}C determined by GCMS is another factor influencing the FA productivity. Knowing FA proportions alone does not explain the process within the cell, i.e. which FA is being produced under certain environmental condition. The CI GCMS used in this experiment was much less sensitive than the GC-FID and as a result FA with low concentrations did not produce a peak of sufficient size to measure atom % ^{13}C. This led to many gaps in the data and explains the many N/A in tables 5.3 and 5.4. However, a general trend can be noted for *H. triquetra*; the lower light intensity had the highest labeling of ^{13}C in all FA present in DGDG, meaning newly synthesized ^{13}C was created. The highest labeling of ^{13}C in TAG was observed under higher temperature/ medium light levels. The atom% data in *H. triquetra* show high labeling of 18:3n-3 which does not correspond to the FA proportional data (Table 5.2); this is explained by the synthesis of longer chain FA, by elongation and desaturation, as 18:3n-3 is being used as a substrate for production of longer chain PUFA. *P. globosa* in (Table 5.3) was much better labeled than *H. triquetra*; this could be due to the fact that the conditions in which *P. globosa* was grown favored higher growth rates, meaning that more lipid with labelled ^{13}C was being produced in the given time than *H. triquetra.*

Finally taking all the above data along with equation 1, production can be calculated. It would have been more valuable to have determined production relative to total carbon or dry weight rather than lipid content. Sample preparation loss was not taken into account when initially it was thought that using HPLC to measure total lipid would be valid. However, total

carbon or dry weight of sample should be noted for future work. Nonetheless, the results in table 5.4 show that more PUFA in DGDG are produced under low light. This is an expected result, as the *H. triquetra* cells have adapted to the low light levels by putting more resources into photosynthesis, and creating more FA (since FA production starts in the chloroplast).

Taking into account PUFA associated with a specific lipid class, DHA and 18:5n-3 production in TAG was higher for the 140 μmol photons m^{-2} s^{-1} vs 88 μmol photons m^{-2} s^{-1}. Lowering the temperature was expected to have a higher production of PUFA to maintain membrane fluidity (Al-Hansan et al., 1991**).** Although the PUFA production was lower for the colder temperature, the cell was still able to produce sufficient DHA in DGDG to be detected by GCMS; thus, it is possible that lowering the temperature have caused the cell to increase the rate of photosynthesis. *P .globosa* had high production rates in DGDG, especially in 18:5n-3 and 18:3n-3. This might be due to the fact that *P. globosa* blooms in tropical oceans where temperature is high and adequate sunlight is available; thus, they may have been grown in optimal conditions in this study (Baumann et al., 1994; Medlin et al., 1994). High concentrations of 18:5n-3 and 18:3n-3 are also known to be a characteristic of *P. globosa*, and could account for the higher production rates in those two FA (Table 5.4) (Hamm et al., 2003).

Production of 18:4n-3 in *H. triquetra* in DGDG was highest under low light. This high production value may be related to the use of 18:4n-3 as a precursor in the formation of other PUFA; one round of elongation and desaturation applied to 18:4n-3 will yield EPA with further steps yielding DHA. High production of 18:4n-3 may be necessary to ensure sufficient substrate for synthesis of EPA and DHA. However, EPA generally had the lowest production, suggesting that much of the synthesized 18:4n-3 is serving as a precursor for DHA. Interestingly, EPA would normally be considered the precursor for 18:5n-3 by chain-shortening (Joseph 1975;

Volkman et al. 1981); however, its low production suggests that 18:5n-3 is likely produced by a different pathway. Phytoplankton are known to possess enzymes capable of far more desaturase activity than animals and terrestrial plants (Jónasdóttir. 2019), so the cells in *H. triquetra* maybe synthesizing 18:5n-3 directly from 18:4n-3 through the use of a Δ4 desaturases, an enzyme not present in animals or terrestrial plants.

Overall, this study shows that it will be difficult to predict the FA production in a sample of phytoplankton in a specific oceanic region, as the light intensity and optimal temperature growth may differ from one species to another. This study could aid in creating a model for the production of PUFA in the dinoflagellate *H. triquetra* living in areas of different light intensity and temperatures. Such models are important because the growth of phytoplankton will influence the aquatic food web, as they are an important primary sources of EPA and DHA, which play a crucial role in the growth of zooplankton. Zooplankton in turn are consumed by small fish which eventually find their way to humans and terrestrial animals (Müller-Navarra et al., 2000). Fish serve as primary source of EPA and DHA for humans, and the production of EPA and DHA in phytoplankton will affect the efficiency of the consumption of EPA and DHA in humans; thus, it is important to understand the environmental effect on the production of PUFA.

7.0 Future Work

This study could be improved in a number of ways. The greatest short-coming was in the lack of complete atom% ^{13}C data. This was a direct result of the poor sensitivity of the CI-GCMS techniques. Had this problem been known, it could have been prevented simply by growing larger volumes of cultures with the same cell density. This would have in turn enabled that calculation of the full production data.

A second obvious problem with this work was the confounding of data. To effectively compare temperature and light effects, one of those variables must be held constant while the other is changed. That was accomplished for the combination of 24 ^{o}C, 88 and 140 μmol photons m^{-2} s^{-1} light with *H. triquetra*. However, when temperature was modified to 14 ^{o}C, light was also changed; this prevents any firm conclusions about temperature from being made. Future work should focus on sound experimental design, avoiding this type of error.

A third important area of improvement would be to treat samples with boiling water near the end of filtration. This would have prevented lipolysis and resulted in FFA values near zero. FFA are present as a result of lipolysis; thus, the estimated lipid class content could be larger than the reported value. It is also impossible to know which lipid class was preferentially broken down by enzymatic activity, and definitive comparison of results are difficult.

Last, the production data would have benefitted from initial measurements of either culture dry mass or carbon content, so that production could be expressed relative to those measures. Because culture volume was limited, the entire sample was used for lipid analyses, with none available for those measurements. While no estimates of FA production exist on any basis, with those data, comparison could have at least been made to carbon production under similar growth conditions.

8.0 Conclusion

The results of this study generally confirmed the initial hypotheses. Overall, as the light level increased, higher production of PUFA was found in TAG in *H. triquetra*. With lower light intensity, PUFA production was higher in DGDG and PC in *H. triquetra*. Temperature had an effect on PUFA by increasing the levels of DHA in DGDG in *H. triquetra*. EPA production in *H. triquetra* was only measurable at low light intensity and had the smallest production value. *P. globosa* grown in similar conditions as *H. triquetra* responded differently, as it was able to more efficiently photosynthesize, creating more PUFA in both DGDG and PC. Finally, the light intensity could also alter which FA are produced in the cell, as the chloroplast FA composition changes with different light intensity and temperature. Demonstration of this technique to estimate FA production within lipid classes paves the way for more rigorous studies that will evaluate the effects of growth conditions on PUFA production in phytoplankton.

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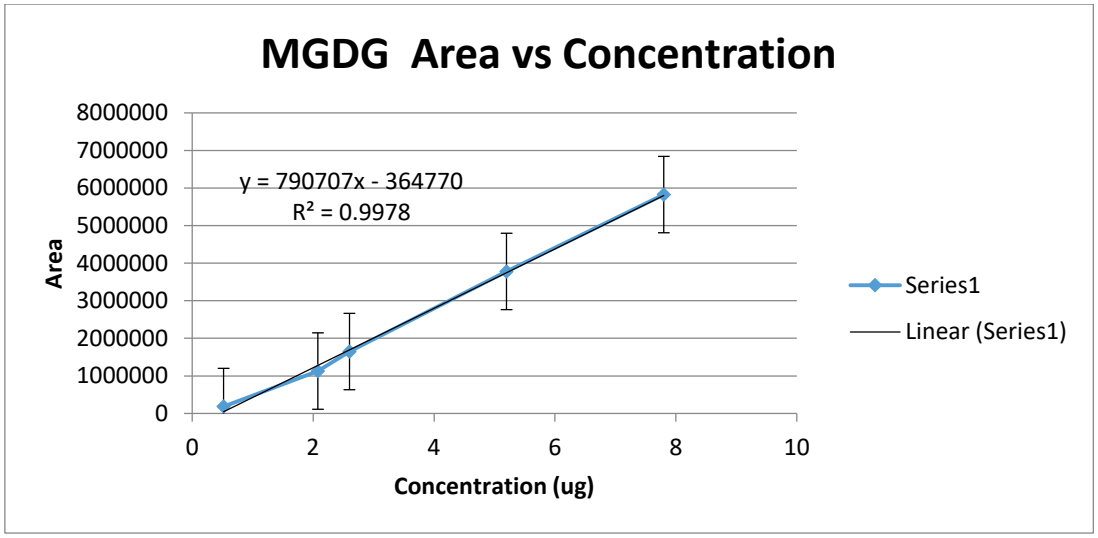
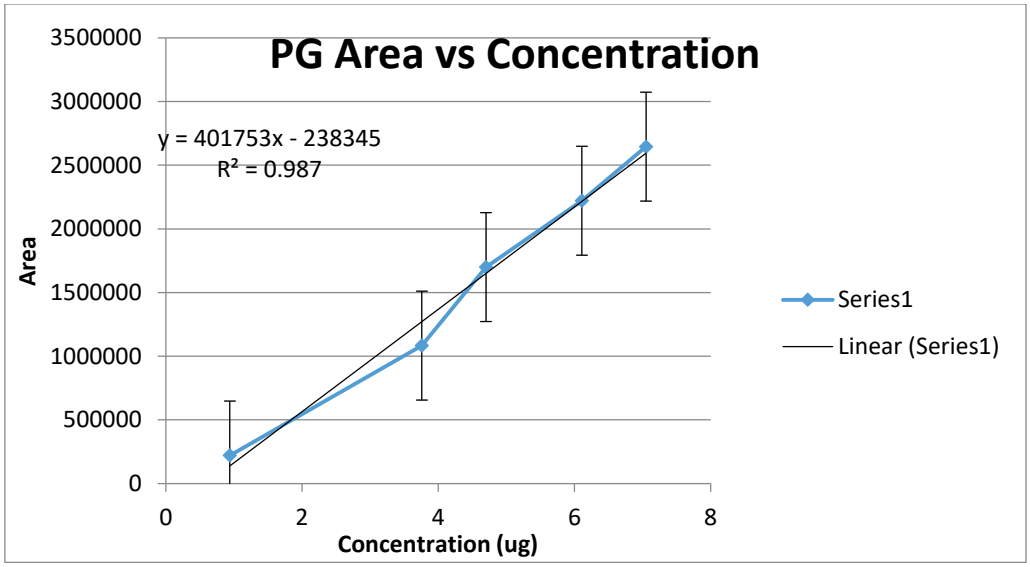
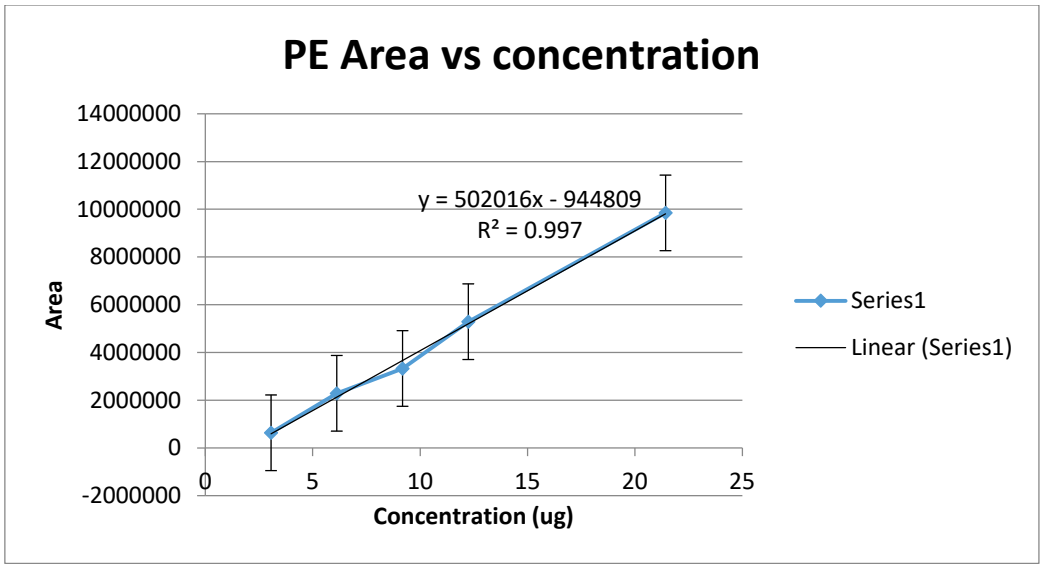
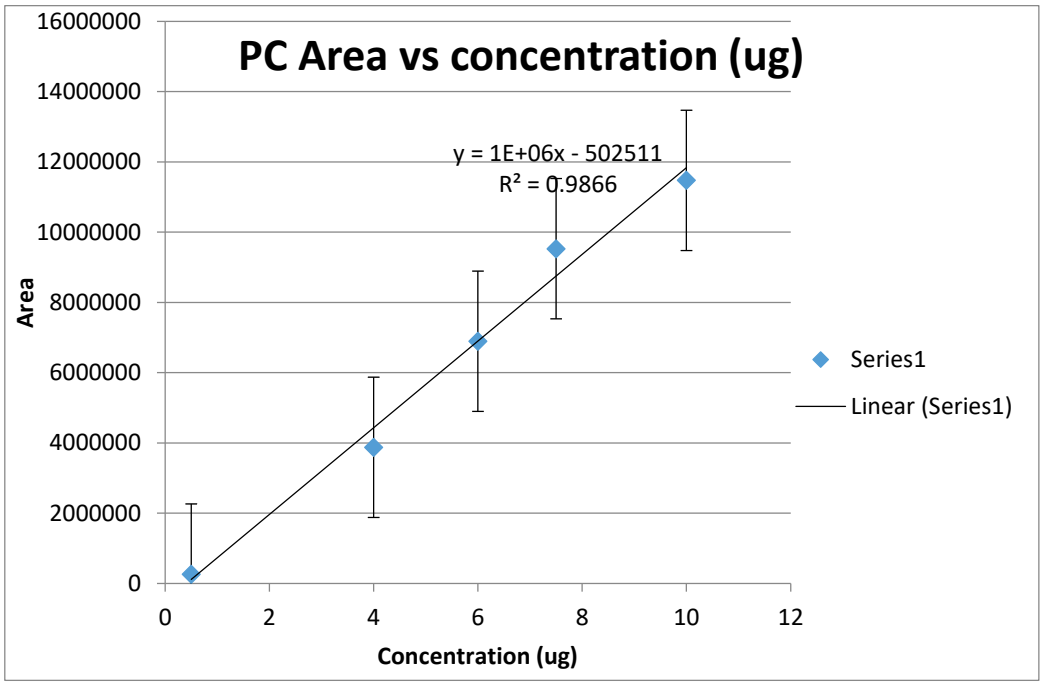
Appendix A

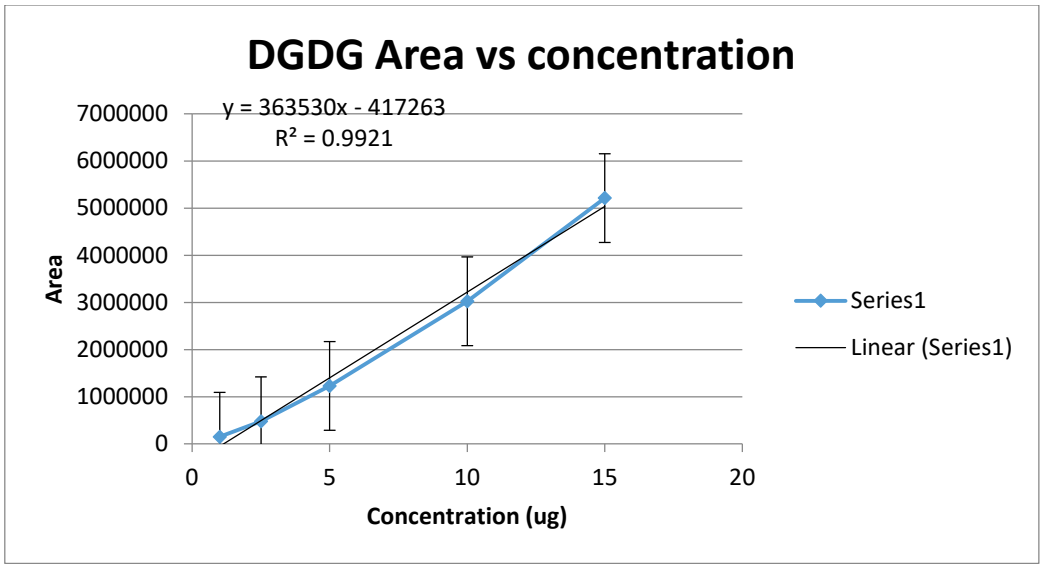
Standard curves for determination of lipid class concentration by HPLC.

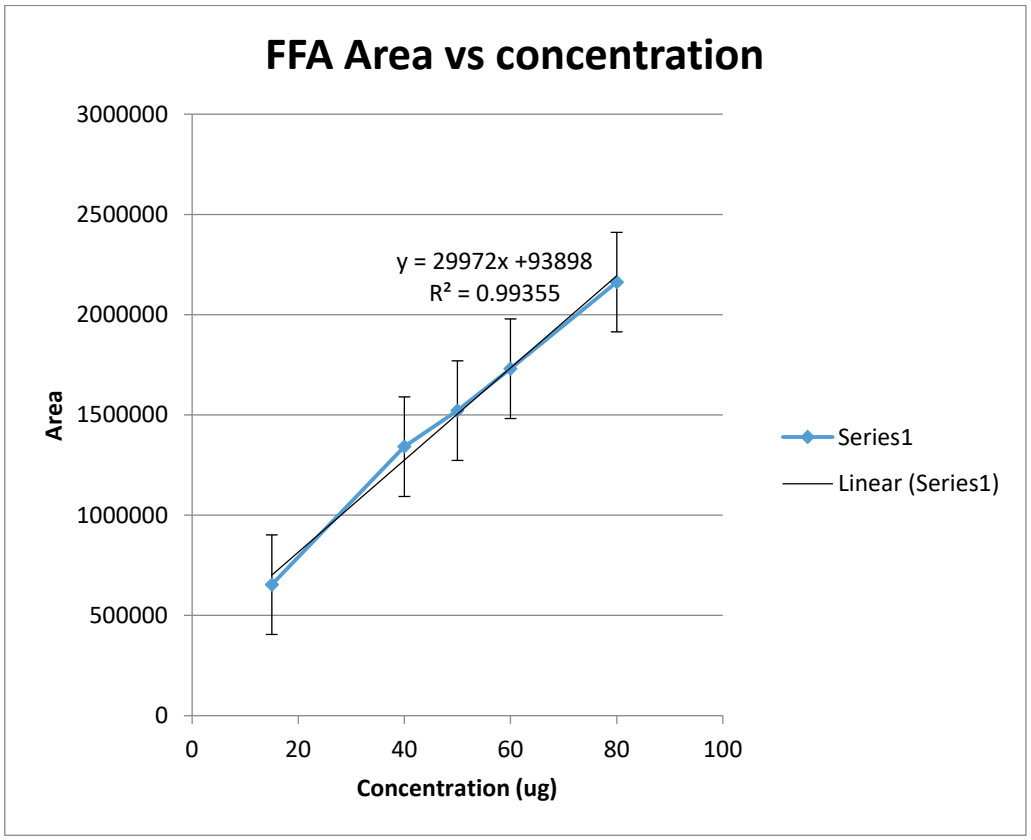


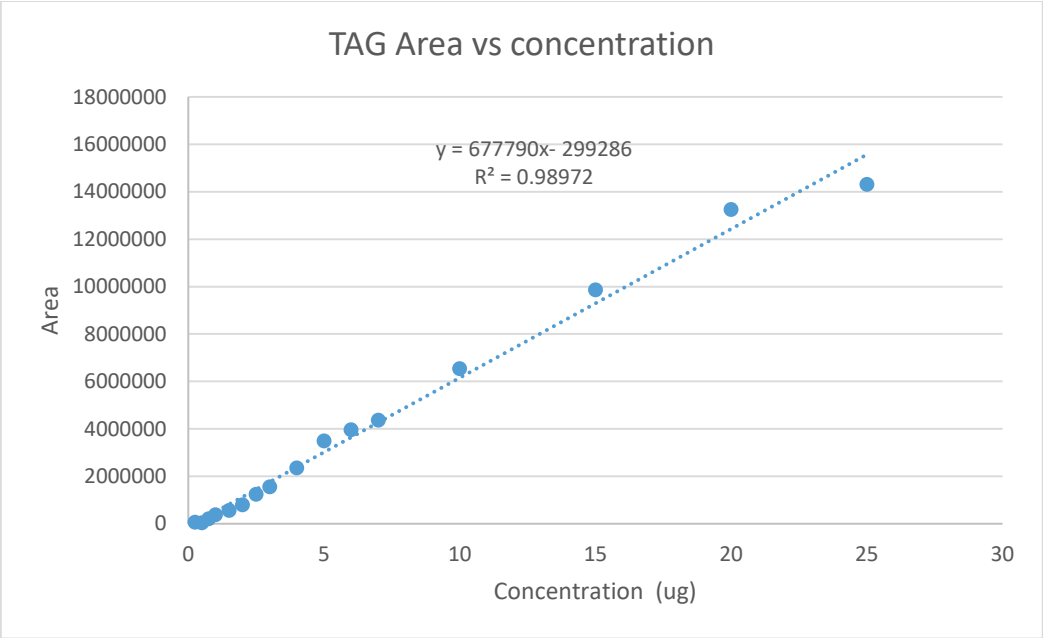



PE Area vs concentration
14000000
12000000
10000000
8000000
6000000
4000000
2000000
0
-2000000
Area
0
5
10
15
20
25
Concentration (ug)
y = 502016x - 944809
R² = 0.997
Series1
Linear (Series1)


PC Area vs concentration (ug)
16000000
14000000
12000000
10000000
8000000
6000000
4000000
2000000
0
Area
0
2
4
6
8
10
12
Concentration (ug)
y = 1E+06x - 502511
R² = 0.9866
Series1
Linear (Series1)


DGDG Area vs concentration
y = 363530x - 417263
R² = 0.9921
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
Area
0
5
10
15
20
Concentration (ug)
Series1
Linear (Series1)


FFA Area vs concentration
y = 29972x +93898
R² = 0.99355
3000000
2500000
2000000
1500000
1000000
500000
0
Area
0
20
40
60
80
100
Concentration (ug)
Series1
Linear (Series1)


TAG Area vs concentration
y = 677790x- 299286
R² = 0.98972
Area
18000000
16000000
14000000
12000000
10000000
8000000
6000000
4000000
2000000
0
0
5
10
15
20
25
30
Concentration (ug)

Appendix B

To calculate the atom%, T0 samples were run first on the GCMS in order to account of the natural abundance of ^{13}C in each labelled samples. First, the ratio

of $\frac{I_{m+n}}{I_0}$ is taken

where I_0= the intensity of the molecular ion consisting only of ^{12}C and natural abundance of ^{13}C (i.e., it is the molecular ion typically associated with an unlabelled FA);

m= the mass of the molecular ion being tested;

n= the increase in mass of the molecular ion due to incorporation of ^{13}C from $NaH^{13}CO3$; and

I_{m+n}= the intensity of the molecular ion where the molecular mass equals m+n.

Next, equation 4 is used in order to correct for the relative abundance of ^{13}C (RA) between the labelled and unlabelled samples for all possible number of carbons in a FA:

$$RA = (I_{m+n}) - \frac{I_{m+n}}{I_0} \times (I01)$$

(Equation 4)

I_{01}= the intensity of the molecular ion consisting only of ^{12}C in the labelled samples.

Equations 5 and 6 are used to calculate the relative compositions of ^{12}C and ^{13}C.

^{12}C: $RA \times (\#C\ in\ FA - n)$ $(Equation\ 5)$

^{13}C: $RA \times (n)$ $(Equation\ 6)$

Finally, the above steps are repeated for all possible molecular mass of the FA. For example, DHA methyl ester with ammonia as a reagent gas (GCMS reagent gas) has a molecular mass of 360 and has 22 carbons in the FA, and the above steps will be repeated for I_{360+1}, I_{360+2}, I_{360+3},….. I_{360+22}

Then the sum of all values of ^{12}C (equation 5) and ^{13}C (equation 6) along with equation 7 will yield the ^{13}C atom%.

$$^{13}\text{C atom\%} = \frac{(Sum\ of\ equation\ 6)}{(Sum\ of\ equation\ 5)+(Sum\ of\ equation\ 6)} \times 100 \quad \text{(Equation 7)}$$

Appendix C

Table C1 Lipid class concentrations in *H. triquetra* and *P. globosa* as an average. The lipid extracted were reduced in volume to 150 µL; the volume injected onto the HPLC column was 80 µL. indicated values are a representative of the lipids in the algae.

Specie	Temperature (°C)	Light intensity (µmol photons m^{-2} s^{-1})	TAG (µg)	FFA (µg)	DGDG (µg)	PC (µg)	Total acyl lipid recovered (µg)/ 150 µL
H. triquetra	14	210	10.8±1.7	1.2 ± 0.1	10.6±0.2	1.4±0.2	27.8±4.4
H. triquetra	24	88	6.5± 0.6	4.9±0.6	8.7±1.1	1.9±0.1	25.4±0.9
H. triquetra	24	140	20.6± 3.9	11.0 ± 2.2	7.5±0.6	1.7±0.1	47.6±11.4
P. globosa	24	140	8.5±1.0	27.2±2.2	21.4±0.2	19.7±1.5	80.3±3.0

No measurements of C or biomass for each culture was taken, thus it is impractical to use (as a means of comparison within growth conditions). To account for this, (Table 5.1) was constructed by taken the ratio of each lipid class to the total acyl lipids.