The Response of Fatty Acids and Pigments to Variations in Temperature and Irradiance in the Marine Diatom *Thalassiosira pseudonana*

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Biology by Frank F. Shang

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2011
The thesis of Frank F. Shang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2011
DEDICATION

To the ones I love.
EPIGRAPH

Winning is about heart
not just legs.
It’s got to be in the right place
—Lance Armstrong
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VITA

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<td>2009</td>
<td>B. S. in Molecular Biology</td>
<td>University of California, San Diego</td>
</tr>
<tr>
<td>2010</td>
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<td>University of California, San Diego</td>
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The Response of Fatty Acids and Pigments to Variations in Temperature and Irradiance in the Marine Diatom *Thalassiosira pseudonana*

by

Frank F. Shang

Master of Science in Biology

University of California San Diego, 2011

B. Greg Mitchell, Chair

Eric Allen, Co-Chair

In this study, we set out to understand environmental effects on basic cell physiology as well as on the relationship between fatty acid and pigment constituents. Closed culturing environments were applied to grow *Thalassiosira pseudonana* under a temperature and irradiance matrix. We detected an inverse relationship between the expression of fatty acids and pigments from exponential to stationary phase growth; pigments were expressed at the highest concentrations during exponential growth and with the onset of stationary phase growth, there was a significant decrease in pigment concentrations that were mainly attributed to a sharp decline in chlorophyll-a. During stationary phase, changes in irradiance had the most dramatic effect at 18.0°C with 55% increase in lipid concentration from low to high irradiance. This increase in lipids was also reflected in a 20% increase in the average cell sizes from 4.8µm to 5.4µm and a 50% decrease in chlorophyll-a concentrations. Temperature and irradiance also had a significant effect on both fatty acid and pigment moieties. A synergistic relationship was observed for temperature and saturated fatty acids and the inverse was detected for polyunsaturated fats. The ratio of photoprotective pigments to photosynthetic pigments (PP/PS) increased as a function of both temperature and irradiance during
stationary phase, increasing most dramatically at higher temperatures and irradiances. These findings were able to give some insight in cellular lipid biochemistry in response to fluctuations in temperature and irradiance.
Chapter 1

Introduction
1.1 The Potential of Algae for Bioenergy Production

Alternative fuels are becoming increasingly relevant due to the finite supply of petroleum, the environmental consequences of exhaust gases from petroleum-fueled engines and issues of national security related to imported fuel supplies. Terrestrial plants such as corn, sugar cane and soy have proved impractical due to the competition with food crops, the slow generation times and minimal net energy yields [25]. The increased interest in the development of sustainable, carbon-neutral, non-food based, bioenergy feedstock has opened many new avenues of research. Many directions have been explored from which bioenergy from microalgal feedstock has emerged as a field with unique promise. Microalgae are efficient photoautotrophic organisms capable of growing rapidly, producing higher energy yields per area than terrestrial plants and providing important auxiliary benefits of wastewater treatment and carbon dioxide uptake to combat global warming.

Microalgae embody an exceptionally diverse yet highly specialized group of photoautotrophic microorganisms. These unicellular microorganisms have developed physiological adaptations to thrive under a wide range of conditions including various freshwater, marine and hyper-saline habitats, as well as a range of temperatures, salinities, pH and nutrient availabilities [20]. Many microalgae have the ability to produce substantial amounts (e.g. 20-50% dry cell weight) of triacylglycerols (TAG) as a storage lipid under photo-oxidative stress or other adverse environmental conditions [21]. Fatty acids of various carbon chain lengths are incorporated into TAGs and accumulate as cytosolic lipid bodies, a survival mechanism by which algal cells store energy to cope stress conditions [13]. The ability of algae to survive or proliferate over a wide range of environmental conditions is largely reflected in the adaptiveness of their cellular biochemistry, including the ability to modify lipid metabolism efficiently in response to changes in environmental conditions [9].

Microalgae represent a plausible and promising model meeting the bioenergy feedstock criteria with many applications towards energy production. Marine
microalgae generate lipids, alcohols, hydrogen, and polysaccharides that can all be transformed into fuel [12]; the byproducts and waste biomass of which, can then be further extricated for energy via digestion to yield biomethane gas [8]. Novel research is being conducted to more efficiently use the solar energy harvested by photosynthetic organisms, applying nanoprobes to extract photosynthetic electrons from *Chlamydomonas reinhardtii*. This bypasses the inefficiencies of cellular metabolism all together and collects the energy directly from the source of photosynthesis [27]. There is incredible energy potential in algae, however, to fully capitalize on this potential, a solid foundation based upon the physiology and biochemistry of the microalgal cell must first be established.

1.2 The Physiological Impact of Variations in Temperature and Irradiance on the Diatom *Thalassiosira Pseudonana*

As the focus of this study, the marine diatom *Thalassiosira pseudonana* is a valid prospect because of both its bioenergy potential and the large body of literature to guide interpretation. *T. pseudonana* is a widely studied diatom species that has been demonstrated to synthesize and accumulate lipids in relatively large quantities [21]. The complete sequencing of the *T. pseudonana* genome [4] and the ever developing improvements on systems for genetic manipulation will allow a more systematic approach to understanding and enhancing both the efficiency and yields for lipid production. However, in order to best utilize these genomes and genetic tools, we must first construct a solid foundation based on the understanding of both physiology and ecology. In order to carry out lipid anabolism and bioenergy production at the efficiencies necessary for industrial scale, a thorough understanding of the environmental effects on cell physiology and biochemistry must first be established. An understanding of how the cell adapts to environmental changes is critical in gaining the ability to exercise the control needed for further genetic studies and eventual industrial scale production.
Both temperature and irradiance (as photosynthetically available radiation; PAR) are important factors influencing the growth and physical state of diatoms [10]. Temperature and irradiance flux has a direct effect on the balance between energy production and expenditure and modifies the biochemical functions of diatoms. The study of variations in temperature and irradiance and their effect on diatom physiology will improve our ability to pair ecological niches to species with the best chances for thriving. It is understood that the production and accumulation of lipids occurs in diatoms under conditions of nutrient stress, such as nitrate and silicate deficiency [3] but the effect of growth environment on stressed induced lipid accumulation is less understood. Although a variety of environmental stresses induce the accumulation of lipids, there have not been systematic comparisons of the yields and biochemical makeup of the cell with respect to modifications in temperature and irradiance.

Of particular interest to this study is the relationship between fatty acids and pigments. It is understood that in *T. pseudonana* fatty acid and pigment biosynthesis are co-localized in the chloroplast [16]. A majority of the enzymes involved in plastid biochemistry are expressed from nuclear DNA and then transported to the chloroplast. However, fatty acids and pigments share similar lipid structures, related functionality and are synthesized in close proximity suggesting that there may be connections in both expression and regulation. To test the hypothesized relationship between fatty acids and pigments, we took measurements of the fatty acid profile, concurrently with the pigment profile under various temperature and irradiance parameters during both exponential and stationary phases of the logistic growth curve. In addition to testing fatty acids and pigments expression, we hoped to gain a better overall understanding of the biochemistry of diatoms from which a framework of genetic studies can be built. This understanding can further be applied towards increasing the effectiveness of algae for industrial scale, bioenergy applications.

In this study, the diatom *Thalassiosira pseudonana* was grown in the laboratory with orthogonal combinations of irradiance and temperature, employing irradiance levels 83±11 µmol photon·m$^{-2}$·s$^{-1}$ (low PAR) and 237±11 µmol
photon·m$^{-2}$·s$^{-1}$ (high PAR) in combination with the temperatures 14.0 ± 1°C, 18.0 ± 1°C and 22.0 ± 1°C (14, 18 and 22 respectively). For *T. pseudonana*, the limit of growth rate as a function of irradiance is about 150 µmol photon·m$^{-2}$·s$^{-1}$ [22]. Low PAR and high PAR treatments were selected to compare growth and cellular composition above and below saturated PAR levels. Cells were taken at the point of highest specific growth rate (maximum metabolic activity) referred to as mid-log or exponential phase, and again when cell growth was limited by the depletion of nutrients (minimum metabolic activity), referred to as stationary phase. The suite of cellular analyses incorporated measurements of particulate organic carbon, growth rates, cell sizes, pigment and fatty acid content. The influence of temperature, irradiance and growth rate on lipid and pigment constituents was examined to gain insight as to how the cell is able to buffer environmental fluctuations to maintain steady-state cellular functions. The influence of temperature and irradiance on algal physiology and biochemistry, as determined by the parameters stated, is discussed with particular emphasis on the correlation between fatty acids, pigments and environmental parameters.
Chapter 2

Material and Methods
2.1 Cell Culturing and Growth Tracking

*Thalassiosira Pseudonana* was obtained from the Provasoli-Guillard National Center for Culturing of Marine Phytoplankton (CCMP), Bigelow Laboratory for Ocean Sciences (West Boothbay Harbor, ME, USA). Stocks were maintained at both 18°C and 22°C under a 12:12 light, dark cycle at an intensity of 20-30 µmol photons · m⁻²·s⁻¹ in artificial seawater (ASW). Light was provided with General Electric fluorescent tubes and attenuated by distance and neutral-density screens.

To make ASW, distilled water was autoclaved and then used as the solvent according to the recipe of the North East Pacific Culture Collection (NEPC). The nutrient enriched water was autoclave sterilized a second time. Then to the nutrient enriched water, heat sensitive supplements (glycerophosphate, bicarbonate, trace metals and vitamins) were added after filter sterilization (0.22 µm Millipore). Initial nutrient concentrations are listed in Table 2.1.

A small aliquot of ASW stock culture was used to inoculate batch cultures in 18 1-liter tubes to achieve an initial density of 1 × 10⁴ cells · mL⁻¹ and grown to 5 × 10⁶ cells · mL⁻¹, after which cell division was arrested by nutrient limitation. Triplicate cultures were grown in six combinations of three temperatures (14.0 ± 1°C, 18.0 ± 1°C and 22.0 ± 1°C) and two irradiances (83 ± 11 µmol photon·m⁻²·s⁻¹ and 237 ± 11 µmol photon·m⁻²·s⁻¹) over a period of 12 to 16 days to achieve the complete growth phase profile. A second independent trial was conducted 2 months following the same experimental protocol, but with an additional irradiance treatment of 420 ± 11 µmol photon·m⁻²·s⁻¹.

Cell density was monitored daily by reading optical density at 750nm using Varian Cary 1E Spectrophotometer (Varian Inc., Santa Clara, CA, USA) and additional monitoring was done with daily haemocytometer cell counts. Daily pH monitoring was performed using Thermo Orion Star LogR Meter (Thermo Fisher Scientific Inc., Beverly, MA, USA). Samples were taken at mid-log phase and stationary phase of the growth curve for analytical analysis of total fatty acids, total pigments and particulate organic carbon and nitrogen (POC and PON). At the time of experimental sampling, cell size was determined using a Coulter particle counter (Beckman Coulter Inc., Fullerton, CA) equipped with a 30 µm aperture.
Table 2.1: A table showing the concentrations of nutrients in artificial seawater.

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock Solution (g•L⁻¹)</th>
<th>Quantity Used</th>
<th>Concentration in Medium (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anhydrous Salts</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>-</td>
<td>20.8 g</td>
<td>3.55 x 10⁻¹</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>-</td>
<td>3.5 g</td>
<td>2.46 x 10⁻²</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>110.9</td>
<td>9.0 ml</td>
<td>9.00 x 10⁻³</td>
</tr>
<tr>
<td>KCl</td>
<td>223.6</td>
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<tr>
<td>NaHCO₃</td>
<td>78.0</td>
<td>1.0 ml</td>
<td>9.28 x 10⁻⁴</td>
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<tr>
<td>KBr</td>
<td>85.0</td>
<td>1.0 ml</td>
<td>7.14 x 10⁻⁴</td>
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<tr>
<td>H₃BO₃</td>
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<td>1.0 ml</td>
<td>4.04 x 10⁻⁴</td>
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<tr>
<td>NaF</td>
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<td><strong>Hydrous Salts</strong></td>
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<td>MgCl₂•6H₂O</td>
<td>-</td>
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<td>SrCl₂•6H₂O</td>
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<td>Na₂SeO₃•5H₂O</td>
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<td><strong>Macronutrients</strong></td>
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<td>NaNO₃</td>
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<td>Na₂SiO₃•9H₂O</td>
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<td>Na₂β-glycerophosphate•H₂O</td>
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<td>FeCl₂•6H₂O</td>
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<td>Na₂MoO₄•2H₂O</td>
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<td>ZnCl₂</td>
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<tr>
<td>CoCl₂•6H₂O</td>
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<td><strong>Vitamins</strong></td>
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<td>Thiamine</td>
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<td>Vitamin B₁₂</td>
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<td>Na₂EDTA•2H₂O</td>
<td>5.58</td>
<td>1.0 ml</td>
<td>1.59 x 10⁻⁵</td>
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calibrated against spherical particles 10 µm in diameter.

Differences in growth rates, cell concentrations, cell densities, fatty acid concentrations and pigment concentrations were examined using separate 2-way Model I analysis of variance (ANOVA), followed by Tukey’s HSD post hoc comparisons (THSD).

2.2 Total Fatty Acid Analysis

Assays for fatty acid composition and quantification in biological materials are commonly carried out by gas chromatography (GC). However, it is difficult to perform in situ analysis directly via GC due to the high polarity, low volatility and high tendency to form hydrogen bonds of typical lipids [2]. Therefore, the conversion of lipid material into corresponding methyl esters (FAME) via suitable derivatization reactions is required before GC analysis. This process increases the volatility of lipid components, thus providing better separation and resolution of lipid compounds.

2.2.1 Gas Chromatography Standardization

Gas chromatography (GC) was performed using a HP 5890 Series II (Hewlett-Packard Company, Wilmington, DE, USA) with flame ionization (FID) and mass selective (MSD) split detection. The GC-MS/FID was calibrated at five concentration points using mixed fatty acid methyl ester standards GLC 68A and GLC 68D (Nu Chek Prep, Elysian, MN, USA) incorporating 14 FAMES including all FAME signatures of *T. Pseudonana*. The concentration calibration was optimized for the concentration range of the sample extracts. For maximum precision, specific volumes were estimated by mass on a microbalance. The GLC 68A and GLC 68D standards were mixed with a pentadecanoic fatty acid methyl ester standard (C15 FAME) at a ratio of 20:1 by volume. The mixed calibration standard (CAL-STD) was diluted by weight in hexane; the concentrations were calculated to be 0.2958 mg·mL⁻¹, 1.5826 mg·mL⁻¹, 4.8977 mg·mL⁻¹, 14.2504 mg·mL⁻¹, and 19.5841 mg·mL⁻¹. To prepare the GC-MS/FID standards (GC-STD), 1 mL of each
CAL-STD was pipetted into a glass autosampler vial and approximately 45 µL of a tridecanoic/nonadecanoic fatty acid methyl ester standard (C13/C19 FAME). 1 µL of each GC-STD was injected at concentrations of 0.7064 mg·mL⁻¹, 1.8759 mg·mL⁻¹, 4.8902 mg·mL⁻¹, 13.5870 mg·mL⁻¹, 18.3245 mg·mL⁻¹ with a 50:1 split. The C13 FAME, C15 FAME and C19 FAME were all obtained from Nu Chek Prep (Elysian, MN, USA).

A slope was obtained from the peak area of each fatty acid from which a R² value and response factor were calculated. The response factor was used to correlate the peak areas of each sample to the C13/C19 FAME concentration standards.

### 2.2.2 Lipid Extraction

Samples for total fatty acids were collected twice during the growth curve, during mid-exponential phase and again at no more than 36 hours into stationary phase. 100ml of cells were pelleted and the supernatant decanted. The pellets were then frozen and stored at −80°C for at least 24 hours. The frozen pellets were then prepared for anhydrous solvent extraction via lyophilization using a freeze dryer. As an efficiency standard, 200 µl of 1.098mg/ml pentadecanoic acid (C15 FA) in methanol stock was added (by mass) to the dried pellet. The C15 FA was tracked through the extraction process and the percentage of C15 fatty acid methyl ester (C15 FAME) recovered was used to calculate the efficiency of the extraction and normalize total fatty acid concentrations. The pellet was then resuspended in 2 ml of 2:1 chloroform:methanol (v/v) containing 0.01% butylated hydroxytoluene (BHT) (w/v). After vigorous homogenization in a vortex for 15 seconds, and sonication for 15 minutes, 1 ml of water was added, which lead to phase separation; the chloroform/lipid layer (bottom layer) was recovered, and the chloroform was evaporated under a steady stream of nitrogen. The residual lipid film was then derivatized in accordance with the American Oil Chemists’ Society (AOCs) method Ce 1b-89 [6] as follows: a preliminary alkaline hydrolysis of fats with 0.5 M sodium hydroxide, at 70°C for 15 minutes, followed by acidic transmethylation with 12% (v/v) boron trifluoride (BF₃) in methanol, at 70°C
for 30 minutes. Phase separation was achieved with adding 1 ml of hexane; the supernatant (organic phase) was recovered. 450\(\mu l\) of the organic phase was added to a GC autosampler vial along with 50\(\mu l\) of C13/C19 FAME standard.

2.2.3 GC-MS/FID Analysis

The assay of FAME was carried out with a HP 5890 gas chromatograph system from Hewlett-Packard (Wilmington, DE, USA), equipped with a fixed outlet capillary column splitter to utilize both mass selective, and flame ionization split detection on one column. Separation was achieved in a DB-Wax column (30m, 0.25mm, and 0.25\(\mu m\)) from Agilent Technologies, Inc. (Santa Clara, CA, USA) with a helium mobile phase at a flow of 1.4 ml.min\(^{-1}\). The GC oven temperature was programmed to hold at 140\(^\circ C\) for 1 minute, increase from 140 to 160\(^\circ C\) at a rate of 10\(^\circ C\) min\(^{-1}\), hold at 160\(^\circ C\) for 2 minutes, increase from 160 to 210\(^\circ C\) at a rate of 15\(^\circ C\) min\(^{-1}\), hold at 210\(^\circ C\) for 3 minutes, and increase from 210 to 260\(^\circ C\) at a rate of 20\(^\circ C\) min\(^{-1}\) with a final hold at 260\(^\circ C\) for 5 minutes. The injector, FID and MS source temperatures were set to 220\(^\circ C\), 300\(^\circ C\), and 230\(^\circ C\) respectively. 1 \(\mu l\) per sample was injected and injections were performed under a 50:1 split.

Hexane blanks were ran at the beginning and end of each algae sample sequence. Identification of fatty acids in \textit{T. Pseudonana} samples was performed by comparison of GC/FID retention times with retention times obtained from known FAME standards (i.e., GLC 68A and 68D) as well as comparison of the mass spectrometry profile of each peak to those in the National Institute of Standards and Technology (NIST) library. Quantification of C14 to C18 and C20 to C24 fatty acids was performed relative to known concentrations and measured peak areas of C13 and C19 FAME internal standards, respectively. The response factor for each specific compound (in GLC 68A and 68D) of interest was applied to the peak area to calculate the final concentration of each peak from the standard compounds. The fatty acid methylation reaction (i.e., transesterification) efficiency was calculated and performed with the C15 FA internal standard added to each lyophilized algal pellet. Effects of temperature and irradiance on specific fatty acid concentrations were reported when specific patterns were observed.
2.3 Pigment Analysis

The analysis of photosynthetic pigments is commonly carried out by high-performance liquid chromatography (HPLC) as it allows the separation and quantification of taxon-specific chlorophylls and carotenoids [24]. The HPLC method is based on a reversed-phase monomeric octylsilica (OS, C\textsubscript{8}) column and pyridine-containing mobile phases [15]. With adequate gradient profiles, the monomeric C\textsubscript{8} column and optimized mobile phase is capable of separating most polar and non-polar chlorophylls and most taxon-specific carotenoids found in marine phytoplankton. This method was able to obtain good resolution in terms of both pigment composition and quantification for \textit{T. Pseudonana}.

2.3.1 HPLC Standardization

Pure calibration standards were obtained from DHI Laboratories (Hørsholm, Denmark) for the 23 major algal pigments. The analytical accuracy of the HPLC was first determined by comparing a 5 point HPLC calibration with concentrations of the pure pigments. The HPLC method accurately determined pigment concentrations of the standards for all 23 pigments ($r^2 > 0.9997$). The calibration was optimized for the concentration range of the sample extracts.

2.3.2 Pigment Extraction

Biomass for pigment extractions were harvested during mid-log phase and stationary phase by filtering under reduced vacuum onto 25 mm diameter GF/F filters (Whatman Ltd, USA). The filters were placed in cryogenic vials and frozen in liquid nitrogen until extraction. Frozen culture samples were extracted in Teflon-lined screw-capped tubes with 5 ml 95% acetone. The tube was then homogenized with a vortex for 30 seconds and placed in an ice bath for 15 minutes of sonication. Extracts were then filtered through 2 mm diameter polypropylene syringe filters (0.2 µm pore size) to remove cell and filter debris. A 1 ml aliquot of acetone extract was mixed with 0.6 ml water as an ion-pairing reagent to avoid shape distortion of earlier eluting peaks [14]. Each sample was injected no later than 2
hours after water addition, as a sharp decrease in non-polar pigment concentrations was observed when diluted extracts were held for longer than this. The injection volume was 200 µl. All samples were prepared under subdued light to minimize chlorophyll degradation.

### 2.3.3 HPLC Analysis

The assay of pigments was performed using Waters Alliance HPLC (Waters Corp, Milford, MA, USA), including a Waters 996 diode-array detector (1.2 nm optical resolution) interfaced with a Waters 474 scanning fluorescence detector and Waters variable wavelength detector. The analytical separations were performed using a Waters Symmetry C$_8$ column (150 · 4.6 mm, 3.5 µm particle size, 100 Å pore size). The mobile phase was a combination of 3 solvents; solvent A, B and C. Solvent A was a mixture of methanol:acetonitrile:aqueous pyridine solution (0.25 M pyridine) at a ratio of 50:25:25 respectively (v:v:v) while solvent B was a 20:60:20 ratio of methanol:acetonitrile:acetone respectively (v:v:v) and solvent C was a 80:20 mixture of acetonitrile:acetone respectively (v:v). All solvents employed to prepare mobile phases were HPLC-grade. To prepare the 0.25 M aqueous pyridine solution, 10 ml of glacial acetic acid and 20 ml of pyridine were added to 900 ml of HPLC grade water in a 1 volumetric liter flask and were mixed using a magnetic stirrer. The solution was then titrated with acetic acid until the pH was 5.0. The mixture was then brought to 1000 ml with HPLC grade water and filtered (0.22 µm Millipore). The flow rate was fixed at 1 ml · min$^{-1}$.

Chlorophylls and carotenoids were detected by diode-array spectroscopy (350-750 nm). Chlorophylls were also detected by fluorescence (excitation: 440 nm, emission: 650 nm). Pigments were identified based on comparison of each absorbance spectrum with a spectral library previously created as well as co-chromatography with pure standards and by diode-array spectroscopy (wavelength range: 350 to 750 nm, 1.2 nm spectral resolution). Absorbance chromatograms were extracted at wavelengths of maximum absorbance (430, 440 and 450 nm).
Chapter 3

Results
3.1 Tracking of Cell Growth and Size in the Light and Temperature Culturing Matrix

Cells were grown in a growth environment matrix of low and high irradiances at temperatures 14°C (hereafter, 14 Low and 14 High respectively), 18°C (hereafter 18 Low and 18 High respectively) and 22°C (hereafter, 22 Low and 22 High respectively). Cell growth was quantified with hemacytometer cell counts which were tallied in conjunction with optical density measurements at 750nm (Fig. 3.1; OD750); cell counts (Fig. 3.2) and OD750 were highly correlated (Fig. 3.3 A; \( r = 0.87 \)). The specific growth rate (\( \mu \)) for each experimental parameter was calculated from three time points during exponential growth. Under these growth conditions, *T. pseudonana* achieved \( \mu \) ranging from 0.043 hour\(^{-1} \) to 0.051 hour\(^{-1} \) (18 Low and 18 High respectively) with an average \( \mu = 0.0479 \pm 0.0066 \) hour\(^{-1} \) (mean ± 1SD). Maximum cell densities were quantified 10 days after inoculation (213 hours) when all treatments were verified to be in stationary phase. The maximum cell density of \( 4.4 \cdot 10^6 \) cells \( \cdot \) mL\(^{-1} \) (0.448 absorbance) was achieved at 22 High (Fig. 3.2). After the 10th day, significant aggregation of cells was observed microscopically, accounting for the high degree of standard deviation in hemacytometer cell counts during late stationary phase; cell clumping is characteristic of diatom nutrient depletion [19]. Final optical densities increased significantly with irradiance (\( F_{1,12} = 4.84, P = 0.048 \)) and temperature (\( F_{2,12} = 14.91, P < 0.001 \)), however, changes in temperature and irradiance had no significant influence on specific growth rates (\( F > 0.2, P > 0.3 \) for all main effects).
Figure 3.1: Optical density at 750 nm as a function of light and temperature. L and H refer to low and high PAR respectively. The error bars represent standard deviation between triplicate conditions. Absorbance is presented on a log scale.
Figure 3.2: Cell counts of the 6 treatments represented as cells $\cdot$ ml$^{-1}$. Hema-
cytometer cell counts were used to track cell growth. L and H refer to low and
high PAR respectively. Error bars represent the standard deviation between the
triplicate conditions.
The pH of all treatments was tracked daily but was not controlled. As expected, pH increased with log-phase growth, presumably with the uptake of inorganic carbon from the medium [1]. Cultures were inoculated at pH $7.7 \pm 0.2$ and sparged with 0.2 $\mu$m filtered atmospheric air. The stock *T. pseudonana* cells were maintained under atmospheric CO$_2$ concentrations so they were low CO$_2$-adapted. The pH steadily increased from pH 7.7 at inoculation and the highest pH (pH 9.2) was measured at hour 165 in the 22 High condition (Fig. 3.4). The cells were bubbled with air however there was still a steady increase in pH during exponential growth signifying that atmospheric CO$_2$ levels were not enough to offset the pH shift. 14 Low and High, and 18 Low and High cells reached maximum pH values of 8.2 and 8.6 respectively at hour 189 and showed little variation between low and high irradiance treatments. After hour 189, there was a steady decrease in pH in 22 Low and High treatments, dropping to pH 7.9 by hour 357. Temperature treatments, 14 and 18 showed only a slight decrease in pH with the onset of stationary phase which was independent of irradiance. The decreases in pH after hour 190 correlated with the arrest of cell division and the transition into stationary phase growth. The pH in this experiment was an uncontrolled variable.

Figure 3.3: Specific growth rates of cells in growth environment matrix are represented.
and it is very likely that the change in pH had some effect on cell growth and biochemistry. In future experiments, we expect to control pH using regulated CO$_2$ input.

Figure 3.4: The daily pH measurements of each experimental parameter. The averages of each triplicated treatment is represented along with the standard deviations. pH varied between 7.50 and 9.25.
The sizes of cells at 18 Low and 18 High were analyzed during both mid-log and stationary phase with respect to irradiance. At mid-log phase when the cells are most metabolically active, there was little variation between cell sizes under low light and high irradiances averaging 4.7 µm and 4.5 µm respectively. During stationary phase, cells exposed to irradiance levels below-saturation (low PAR) showed little change in size, increasing from 4.7 µm to 4.9 µm on average (Fig. 3.5). The slight increase in cell size can partially be explained by the higher numbers of cells detected microscopically in the range of 8 to 10 µm. The detection of cells at sizes about 8 µm can attributed to an arrest in cell-separation at the M phase of the cell cycle [17]. Cell cycle arrest in M phase is characteristic of nutrient limitation and the onset of early stationary phase in T. pseudonana [18]. However at irradiance levels above-saturation (high PAR), cells showed a 20% increase in cell size from 4.5 to 5.4 µm. The increase in cell size is presumably from the increased accumulation of lipids at higher irradiances during stationary phase (Fig. 3.6).

An addition irradiance level of 420 µmol photon·m$^{-2}$·s$^{-1}$ was added to see if any further increases in cell size could be observed at supersaturated irradiances. When the sizes of cells in stationary phase at 18°C were analyzed at the 3 irradiance levels, changes in cell size were only observed as a function of light saturation (180 µmol photon·m$^{-2}$·s$^{-1}$). There was a 20% increase in cell size from 80 to 240 µmol photon·m$^{-2}$·s$^{-1}$ (low PAR to high PAR) from 4.8 to 5.4 µm average. However the sizes of cells during stationary phase at 240 and 420 µmol photon·m$^{-2}$·s$^{-1}$ showed very little variation with average cell sizes of 5.4 µm and 5.5 µm respectively (Fig. 3.7).
Figure 3.5: Cell Sizes of the 18 Low cells during mid-log and stationary phase. Mid-log and stationary samples were diluted 1:20 and 1:29 respectively. The bell curve represents the number of cells counted as a function of size.

Figure 3.6: Cell Sizes of the 18 High cells during mid-log and stationary phase. The bell curve represents the number of cells counted as a function of size.
Figure 3.7: Cell Sizes of the 18°C, at 3 irradiance levels 80, 240 and 420 µmol photon·m⁻²·s⁻¹ during stationary phase determined with a Coulter counter. Samples from irradiance levels 80, 240 and 420 µmol photon·m⁻²·s⁻¹ were diluted 1:33, 1:29 and 1:29 respectively.
3.2 Fatty Acid Profiling as a Function of Growth Phase and Culturing Environment

3.2.1 Gas Chromatography Standardization

To establish a quantitative gas-chromatography method, the flame ionization detector first had to be standardized with fatty acids of known concentrations. Quantitative gas-chromatography analysis was first calibrated with a 5 point concentration standard with fatty acid methyl ester (FAME) components encompassing signatures of those expected in algal samples. Hexane was used as the solvent for dilution of all standards to concentrations shown in table 3.1. The concentration range of 0.71 to 18.32 mg FAME·ml⁻¹ hexane defined the range of expected FAME concentrations in hexane extracts. Tridecanoic acid methyl ester (C13 FAME) and nonadecanoic acid methyl ester (C19 FAME) were concentration standards and were added to the prepared standards before injection at a ratio of 1:10. Pentadecanoic acid methyl ester (C15) was a proxy representing the pentadecanoic acid that is initially added to each sample to track the recovery and efficiency of the derivatization reaction. The standard curves obtained for each FAME showed good a linear correlation of peak area to concentration ($r^2 > 0.971$). C15 was selected as an internal standard specifically because it is not naturally produced by *Thalassiosira pseudonana* making it a good tracer molecule. The standardization data was also used to calculate a response factor that correlates C13/C19 FAME standard peak areas to peak areas of the sample. The GC-FID chromatograms of each of the 5 concentrations were used to quantify peak areas and calculate FAME concentrations. Fig. 3.8 is a sample chromatogram displaying the retention times of each standard.
Table 3.1: A table showing the 5 concentration standards that were used to calibrate GC-MS/FID. GC-STD1-5 represent the 5 standard concentrations used for the calibration between 0.71mg/ml to 18.32mg/ml. All components of the standard was present in equal concentrations except for the C16 spike, which concentration was double the concentration of the other FAMEs. C13 and C19 are concentration standards and C15 is a recovery standard.

<table>
<thead>
<tr>
<th>Component</th>
<th>GC-STD1</th>
<th>GC-STD2</th>
<th>GC-STD3</th>
<th>GC-STD4</th>
<th>GC-STD5</th>
<th>GC-STD6</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16 STD</td>
<td>1.071G</td>
<td>0.0185G</td>
<td>3.611G</td>
<td>0.0014G</td>
<td>4.391G</td>
<td>0.0250G</td>
</tr>
<tr>
<td>C14-1</td>
<td>0.0145</td>
<td>0.0145</td>
<td>0.0145</td>
<td>0.0145</td>
<td>0.0145</td>
<td>0.0145</td>
</tr>
<tr>
<td>C14-2</td>
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<td>0.0145</td>
<td>0.0145</td>
<td>0.0145</td>
<td>0.0145</td>
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</tr>
<tr>
<td>C16-1</td>
<td>0.0145</td>
<td>0.0145</td>
<td>0.0145</td>
<td>0.0145</td>
<td>0.0145</td>
<td>0.0145</td>
</tr>
<tr>
<td>C16-2</td>
<td>0.0145</td>
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<td>0.0145</td>
<td>0.0145</td>
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<td>0.0145</td>
</tr>
<tr>
<td>C16-3</td>
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<td>0.0145</td>
<td>0.0145</td>
<td>0.0145</td>
<td>0.0145</td>
<td>0.0145</td>
</tr>
<tr>
<td>C16-4</td>
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<td>0.0145</td>
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<td>0.0145</td>
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<tr>
<td>C16-5</td>
<td>0.0145</td>
<td>0.0145</td>
<td>0.0145</td>
<td>0.0145</td>
<td>0.0145</td>
<td>0.0145</td>
</tr>
<tr>
<td>Total %</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
Figure 3.8: A sample chromatogram from flame ionization detector analysis of the FAME standards used for GC calibration. This chromatogram represents a standard concentration of 13.58mg/ml (GCSTD4) injected at a 50:1 split. FAMEs were eluted in 7 to 19 minutes of a 20 minute temperature program.

### 3.2.2 Fatty Acid Concentration During Stationary Phase as a Function of Growth Environment

Hexane extracted samples were analyzed for total fatty acid content during exponential and stationary phase using GC-MS/FID as a means of identification and quantification. Significant levels of fatty acids were accumulated during stationary phase for all temperature and irradiance parameters. With the accumulation of lipids during stationary phase, trends in the fatty acid composition became evident. Figure 3.9 A, B and C show FID chromatograms for hexane extracts from stationary phase at 14 High, 18 High and 22 High respectively. Peak areas from FID chromatograms were first normalized to the C15 ISTD to calculate total fatty acid concentrations within the hexane extracts. The hexane extracts were then normalized to particulate organic carbon (POC) to calculate the proportion of fatty acids to the total organic carbon of the cells (Fig. 3.10). The major saturated fatty acid (SFA) was C16:0 and there were also significant levels of C14:0 detected. The dominant monounsaturated fatty acid (MUFA) was C16:1 with trace levels of C18:1 detected. The polyunsaturated fatty acids (PUFA), stearidonic acid (SDA; C18:4) and eicosapentaenoic acid (EPA; C20:5) were detected at significant levels
with trace levels of docosahexaenoic acid (DHA; C22:6). Regardless of temperature and irradiance, the major fatty acids remained C14, C16, C16:1 and the LC-PUFAs SDA and EPA.
Figure 3.9: Sample chromatograms from flame ionization detector analysis of the extracts from 14, 18 and 22°C are represented by figures A, B and C respectively. The displayed chromatograms are visualized from extracts taken from stationary phase cultures at high PAR. C13 STD and C19 STD were standards added to samples prior to injection at a ratio of 1:10. C15 ISTD was the internal standard used to track derivatization efficiency.
Figure 3.10: Fatty acid profile and concentrations at stationary phase expressed in ug/mg POC. The temperatures 14, 18 and 22°C are represented by figures A, B and C respectively. Error bars represent the standard deviation of triplicate cultures at each treatment.
There were a number of fatty acid moieties common to cells regardless of changes in the experimental protocol. For instance, the predominant fatty acids were C16:0 and C16:1. Levels of C16:0 and C16:1 hold significance because previous studies have demonstrated that C16:0 and C16:1 along with C18:4 and C20:5 are the major fatty acid constituents of storage triacylglycerols [5]. The neutraceutical and pharmaceutically relevant ω-3 long chain poly unsaturated fatty acids (LC-PUFA), stearidonic acid (SDA, C18:4), eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) were also accumulated in high percentages during stationary phase (Fig. 3.11 B). In addition to the detection of high levels of SDA, EPA and DHA, the fatty acid profile analysis also showed the presence of other PUFAs, including C16:2 and C16:3 but at levels less than 5% (Fig. 3.10). The ω-3 fatty acids, SDA and EPA were the main PUFAs detected at all culturing conditions.

Fatty acid patterns dependent on temperature and irradiance were also observed during stationary phase growth. Most generally, levels of saturated fatty acids changed conversely with unsaturated fatty acids with respect to temperature and irradiance parameters. The ratio of saturated fatty acids (C14, C16 and C18) to total fatty acids significantly increased with temperature (two way ANOVA, $F_{2,12} = 33.9, P < 0.001$) and irradiance ($F_{1,12} = 12.35, P = 0.004$). The ratio of ω-3's to total fatty acids held a significant inverse relationship to temperature (Fig. 3.11; two way ANOVA, $F_{2,12} = 22.38, P < 0.001$). This pattern was primarily due to the effects of temperature variation on the concentration of SDA; there was no significant effect of irradiance ($F_{1,12} = 1.35, P = 0.26$). As well, the ratio of C16:0 to C16:1 increased significantly in a stepwise fashion as a result of increasing temperature and irradiance (Fig. 3.12 A; two-way ANOVA: temperature, $F_{2,11} = 122.66, P < 0.001$; irradiance, $F_{1,11} = 37.29, P < 0.001$). All temperature dependent trends were consistent with the maintenance of lipid fluidity; as temperature increases, to maintain a consistent fluidity in lipids, the proportion of saturated fatty acids must increase relative to unsaturated fatty acids.
Figure 3.11: Light and temperature dependent relationships in saturated and unsaturated fatty acids during stationary growth. Figure A represents the synergistic relationship of saturated fatty acid (saturated FA) accumulation and temperature/irradiance as a fraction of the total fatty acids (TFA). Error bars represent the standard deviation between triplicate cell cultures.
Figure 3.12: Environment dependent relationships of C16 to C16:1. Error bars represent the standard deviation between triplicate cell cultures.
Fatty acid concentrations from the hexane extract samples were normalized to total organic carbon (POC) for the purpose of establishing trends in carbon partitioning with respect to temperature and irradiance (Fig. 3.13). There were no significant trends in fatty acid profiles during exponential phase growth. However during stationary phase, there was an interactive effect of temperature and irradiance on the concentration of total fatty acids as a percent of POC (temperature \( \times \) irradiance: \( F_{2,11} = 5.28, P = 0.025 \)). Total fatty acid concentration was highest at 18 High accounting for 33.4% of POC. 18 High, 22 Low and 22 High all showed similar levels of lipid accumulation during stationary phase. Irradiance had little effect on the accumulation of lipids except at 18.0°C. Interestingly, fatty acid concentrations increased 55% from low to high irradiance at 18.0°C (Fig. 3.13). This significant increase in lipids was consistent with our Coulter counter data (Fig. 3.7) which showed a 20% increase in average diameter from 18 Low to 18 High during stationary phase (Fig. 3.7). 14 Low, 14 High and 18 Low all showed similar levels of lipid accumulation.
Figure 3.13: Total fatty acid (FA) concentrations at stationary phase for all treatments expressed in terms of percentage of total organic carbon (POC). Low and High PAR represent the respective irradiance levels. Error bars represent the standard deviation between triplicate cell cultures.
3.2.3 Comparison of Fatty Acid Profiles with Respect to Growth Phase

Changes in overall fatty acid chain length and degree of unsaturation were revealed by a comparison of the ratio of unsaturated fatty acids/saturated fatty acids and the ratio of (ω-3 fatty acids)/(C16:0 + C16:1) during the exponential and stationary phases. Table 3.2 shows the fatty acid composition during the exponential and stationary phases of growth for *T. pseudonana* in each of the six growth conditions. During exponential growth, there was no significant effect of experimental treatment on the composition of fatty acids (two-way ANOVA for ω-3 to total fatty acids, $F_{11} < 4, P > 0.10$; saturated fatty acid to total fatty acid, $F_{11} < 3, P > 0.1$). During stationary phase, the higher temperature resulted in an increase in the ratio of saturated over unsaturated fatty acids (Table 3.2). However, ratio analyses such as these can be dominated by changes in the most abundant fatty acids. As a result, changes in lower abundance fatty acids can be masked; during stationary phase EPA concentration increased rather than decreased at higher temperature and irradiance (Fig. 3.10) but this increase was masked by the greater accumulation of C16. Interestingly DHA was only accumulated during stationary phase of growth and the C18 family of fatty acids were rarely above the detectable limits during exponential phase growth.
Table 3.2: Fatty acid composition for exponential and stationary phase growth for the experimental parameters. Each value represents the mean ± SD of three replicates. The notation L and H represent low PAR and high PAR respectively. EXP and STA represent exponential phase and stationary phase growth respectively. 14, 18 and 22 are the experimental temperatures (in °Celsius). ND denotes that the fatty acid was not detected. The column 'unsat./sat.' represents the ratio between unsaturated fatty acids and saturated fatty acids. \((\text{omega-3})/(\text{C16:0 + C16:1})\) is the ratio between the LC-PUFAs (SDA, EPA, DHA) and C16 fatty acids (C16:0, C16:1).

<table>
<thead>
<tr>
<th></th>
<th>C14</th>
<th>C16</th>
<th>C16:1</th>
<th>C16:2</th>
<th>C16:3</th>
<th>C18</th>
<th>C18:1</th>
<th>C18:4</th>
<th>C20:5</th>
<th>C22:6</th>
<th>unsat./sat.</th>
<th>((\text{Omega3})/(\text{C16 +C16:1}))</th>
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<tbody>
<tr>
<td>14 Low EXP</td>
<td>13.82%</td>
<td>14.47%</td>
<td>31.50%</td>
<td>5.30%</td>
<td>9.09%</td>
<td>ND</td>
<td>ND</td>
<td>9.78%</td>
<td>16.03%</td>
<td>ND</td>
<td>2.53%</td>
<td>0.56%</td>
</tr>
<tr>
<td>14 High EXP</td>
<td>14.36%</td>
<td>17.81%</td>
<td>29.58%</td>
<td>3.80%</td>
<td>6.54%</td>
<td>4.57%</td>
<td>ND</td>
<td>8.33%</td>
<td>13.74%</td>
<td>ND</td>
<td>1.69%</td>
<td>0.47%</td>
</tr>
<tr>
<td>18 Low EXP</td>
<td>12.78%</td>
<td>14.03%</td>
<td>29.89%</td>
<td>4.95%</td>
<td>10.20%</td>
<td>ND</td>
<td>2.47%</td>
<td>9.67%</td>
<td>16.01%</td>
<td>ND</td>
<td>2.73%</td>
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<td>18 High EXP</td>
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<td>17.50%</td>
<td>31.18%</td>
<td>3.74%</td>
<td>7.35%</td>
<td>2.11%</td>
<td>ND</td>
<td>7.55%</td>
<td>14.70%</td>
<td>ND</td>
<td>1.93%</td>
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<tr>
<td>22 Low EXP</td>
<td>17.20%</td>
<td>15.38%</td>
<td>26.11%</td>
<td>7.00%</td>
<td>12.27%</td>
<td>ND</td>
<td>ND</td>
<td>6.03%</td>
<td>16.01%</td>
<td>ND</td>
<td>2.07%</td>
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<tr>
<td>22 High EXP</td>
<td>17.20%</td>
<td>15.38%</td>
<td>26.11%</td>
<td>7.00%</td>
<td>12.27%</td>
<td>ND</td>
<td>ND</td>
<td>6.03%</td>
<td>16.01%</td>
<td>ND</td>
<td>2.07%</td>
<td>0.53%</td>
</tr>
</tbody>
</table>

The table shows the fatty acid composition for exponential and stationary phase growth for the experimental parameters. Each value represents the mean ± SD of three replicates. The notation L and H represent low PAR and high PAR respectively. EXP and STA represent exponential phase and stationary phase growth respectively. 14, 18 and 22 are the experimental temperatures (in °Celsius). ND denotes that the fatty acid was not detected. The column 'unsat./sat.' represents the ratio between unsaturated fatty acids and saturated fatty acids. \((\text{omega-3})/(\text{C16:0 + C16:1})\) is the ratio between the LC-PUFAs (SDA, EPA, DHA) and C16 fatty acids (C16:0, C16:1).
3.3 Pigment Analysis as a Function of Growth Phase and Experimental Condition

The major photosynthetic pigment was chlorophyll a; chlorophyll C1 and chlorophyll C2 were detected in much lower concentrations (Fig. 3.14). Chlorophyll a accounted for up to 66% of total pigments (dependent on condition) and comprised up to 6.53% and 1.53% of total organic carbon during exponential and stationary growth, respectively; concentrations of chlorophyll a, as a percentage of total organic carbon, was much higher during exponential growth than stationary growth (Fig. 3.15) with all conditions except 14 Low. The major carotenoids detected were fucoxantin and diadinoxantin with fucoxantin concentrations always higher than diadinoxantin. Diatoxantin and β-carotene were detected at much lower concentrations. These detected pigments are characteristic of the diatom pigment profile[26].

The total pigment concentration decreased significantly from exponential to stationary growth in all tested conditions except 14 Low (Fig. 3.16; 3-way ANOVA, \( P < 0.005 \)). During exponential phase total pigments increased inversely with irradiance (Fig. 3.16; 3-way ANOVA, \( P < 0.001 \)). However, with the onset of stationary phase, there was no significant effect of irradiance but total pigment content was much higher at 14°C than at 18 and 22°C. With the onset of stationary phase, cellular metabolic activity decreases relative to exponential phase growth and cell division. With the decreased energy demands, the maintenance of photosynthetic pigments are no longer necessary and cellular carbon is partitioned away from pigment biosynthesis.
Figure 3.14: Pigment profiles and concentrations at stationary phase expressed in µg/mg POC under 6 growth conditions. The temperatures 14, 18 and 22°C are represented by figures A, B and C respectively. Error bars represent the standard deviation of triplicate cultures at each treatment.
Figure 3.15: Chlorophyll a concentrations during exponential growth (A) and stationary growth (B) expressed in ug/mg POC under 6 growth conditions. Error bars represent the standard deviation of triplicate cultures at each treatment.
Figure 3.16: Total pigment concentrations are displayed for exponential phase growth (A) and stationary phase (B) in all growth environments expressed as a percentage of POC. Error bars represent the standard deviation between triplicate cell cultures.
The total cellular pigments were divided, based upon organic structure, into carotenoids and chlorophylls to better understand the regulation of pigment biosynthesis with respect to changes in irradiance [28]. The chlorophylls included chlorophylls \(a\), C1 and C2. Carotenoids included fucoxantin, diadinoxatin, diatoxanthin and \(\beta\)-carotene. During stationary phase, the increase of both temperature and irradiance had a synergistic effect on the relative of amount of carotenoids to chlorophyll pigments; the ratio of carotenoids to chlorophylls significantly increased during stationary phase at 18 High and 22 High. This ratio was also higher at 22 Low than at lower temperatures (Fig. 3.17; 3-way ANOVA, \(P < 0.001\); \(P = 0.02\)). Chlorophyll concentrations decreased significantly during stationary phase at 18 High and 22 Low (\(P < 0.02\)) and decreased inversely with light at higher temperatures (\(P < 0.001\)) showing the most significant reduction at 22°C (Fig. 3.17; \(P < 0.008\)).
Figure 3.17: Pigment composition for 2 stages of growth expressed in terms of carotenoids and chlorophylls. Figure A represents cells in exponential phase and figure B represents cells in stationary phase. 14 18 22 corresponds to culturing temperatures. Low PAR and High PAR represent low and high levels of irradiance. Error bars represent triplicate conditions.
Chapter 4

Discussions
The availability of a complete genome sequence and its ability to accumulate lipids in large intracellular oil globules has made *T. pseudonana* a model candidate for diatom research as well as a valid prospect for biodiesel feedstock. However, in order to maximize the potential of this and other species for bioenergy production, it will be important to understand not only the production of specific fatty acids under different growth environments but also the related changes in cell biochemistry. The overall goal of this research was to utilize a controlled culturing environments to investigate the changes in algal cell physiology and biochemistry in orthogonal combinations of temperature and irradiance. The data that is presented in this study suggests that both the growth phase and experimental parameters have a significant effect on the physiology of the *T. pseudonana* cell and its potential efficiency in bioenergy production. The environmental conditions for cell growth are fundamental in determining the characteristics of any organism. For the unicellular diatom *T. pseudonana* the relationship between the major environmental factors, temperature and irradiance, together with cell biochemistry still requires considerable research but this study provides relevant data on the fatty acids and pigments of the cell as a function of both growth phase and major environmental factors.

During exponential growth, *Thalassiosira pseudonana* showed similar specific growth rates for all experimental conditions. However, final cell densities were synergistically influenced by both temperature and irradiance. With increased cell densities, we also observed similar trends in pH; pH increased linearly throughout exponential growth with all environmental parameters but peak pH values corresponded to final cell densities. Large pH increases is associated with higher levels of photosynthesis and energy production. The observed pH increase is consistent with the uptake of dissolved inorganic carbon from the media which is associated with cell growth and proliferation [1]. There is a high degree of correlation between peak pH values and peak biomass yields. However the trend was not as evident when comparing peak pH values to fatty acid concentrations suggesting that energy expenditure was not only focused towards lipid accumulation during stationary phase.
It is well established that microalgae accumulate lipids during stationary phase in response to nutrient stress [11]. We did not detect any significant trends in fatty acid profiles during exponential phase growth so our analysis of the lipids portion of this study focused on the trends of fatty acid accumulation during stationary phase. The lack of common moieties during exponential growth can presumably be attributed to the nonessential nature of lipid accumulation when the cells are most metabolically active. We demonstrated that under conditions favorable for lipid accumulation, the fatty acid profile of *T. pseudonana* was significantly affected by both temperature and irradiance. With GC-MS/FID we detected C14, C16:0 and C16:1 as the predominant fatty acids regardless of growth phase. This data supports previous studies which have found that diatom lipid globules are predominantly composed of triacylglycerols (TAGs) having C14, C16 and C16:1 fatty acid substituents [5]. Our study also supports the finding that PUFAs C16:3, C18:4, C20:5 and C22:6 are incorporated into TAGs for storage during stationary phase [5].

With the higher temperatures, we detected higher fatty acid concentrations containing a lower concentration of PUFAs and a higher concentration of saturated FAs (fig. 3.11). The concentration of ω – 3 fatty acids were inversely related to temperature whereas the concentration of saturated fatty acids (C14, C16, and C18) showed strict correlation to temperature. A second temperature dependent trend detected was the ratio of C16 to C16:1(fig. 3.12). This ratio appeared to be under strict regulation of both temperature and irradiance; with the increase of both temperature and irradiance, we detected a significant stepwise increase of C16 with respect to C16:1. The temperature dependent ratio of saturated to unsaturated fatty acids is most likely attributed to the biochemical adaptiveness of the cell. One idea is, as the temperature increases, the lipid viscosities must change to maintain homeostasis in the cell as a buffer for cell metabolism.

The main photosynthetic and carotenoid pigments were chlorophyll-a and fucoxantin respectively. The pigment signature detected was characteristic of diatoms, consisting of the photosynthetic pigments chlorophyll *a*, chlorophyll C1 and chlorophyll C2, and the carotenoids fucoxantin, diadinoxantin, diatoxantin, and β-
carotene [26]. Along with the function of auxiliary pigments in pigment-protein complexes, the carotenoids also have an important photoprotective function in the cell by sequestering the photo-induced triplet-state chlorophylls and the oxygen radicals derived from them [20]. The total pigment concentration decreased significantly from exponential to stationary phase in all tested conditions except 14 Low. Chlorophyll $a$ constituted up to 6.53% of the total organic carbon during exponential phase and only 2% during stationary phase. There was a significant increase in the relative concentration of carotenoid pigments during stationary phase at the higher irradiance. This finding is consistent with the understanding that cells maximize their light harvesting potential during exponential phase for functions necessary to cell division [7]. By the end of exponential phase, the capacity of the cell for photosynthesis has declined considerably and no longer focused on multiplication but rather becoming increasingly diverted towards energy storage.

We observed an inverse relationship between pigment and fatty acid concentrations during stationary phase. From 14 to $22^\circ C$ there is a general trend of increasing fatty acid concentrations and decreasing pigment concentrations with specific attention to the photosynthetic chlorophyll pigments. A similar trend is also observed from low to high irradiance. Although the total pigment concentration decreases during stationary phase, there was an increase in the relative proportion of carotenoids. The shift in the pigment profile from a photosynthetic to photoprotective focus as cells enter stationary phase supports the understanding that the cell is regulating the partition of carbon between the two biochemical pathways to maximize carbon storage as lipids; the cell reduces its metabolic activity during stationary phase warranting less need to harvest light [7]. But with the maintenance of the carotenoids, it appears that the need for continuous protection from photo-oxidation is recognized.

Observations of cell sizes at the $18^\circ C$ temperature for the the irradiance levels of 80, 240 and 500 $\mu$mol photon$\cdot$m$^{-2}$ revealed that cell size at the lowest irradiance did not change between exponential and stationary phase. At the higher irradiance levels stationary phase cells were approximately 20% larger than at low irradiance and during exponential phase. Very interestingly, a similar shift in lipid
accumulation is observed between low and high irradiance levels at 18°C with a 55% increase in the accumulated lipids (fig. 3.13). Comparable concentrations of lipids were detected at conditions 18 Low, 14 Low and 14 High as well as for conditions, 18 High, 22 Low and 22 High, only at 50% higher concentrations. When comparing cells under low and high irradiance at 18°C with respect to pigment composition, there was a significant shift in the ratio of carotenoids to chlorophyll. The ratio of carotenoids underwent a shift from 32% to 50% of total pigments at 18°C where cells in treatments above and below this threshold behaved similarly to each other. The shifts in lipid accumulation, cell size and pigment composition at 18°C between low and high irradiance suggests that this temperature is a limit, having significant influences on cell sizes and the biochemical regulation of both fatty acid accumulation and pigment composition.

Profiling the basal fatty acid and pigment moieties under a matrix of environmental parameters for *T. pseudonana* provides a study of cell biochemistry that is required for maximizing the effectiveness of diatoms as a bioenergy feedstock. Generally the ideal biodiesel composition contains a high proportion of medium chain, monounsaturated fatty acids (C16:1, C18:1), and the reduced presence of PUFAs for high energy yield, and superior oxidative stability [23]. However the ω – 3 PUFAs hold great pharmaceutical value, and can be used to supplement the economics of production by yielding a higher value oil. With this study, we showed that depending on the environmental parameters, the composition of fatty acids and the distribution of carbon to fatty acid and pigment biosynthesis varies substantially. Still unexplored is the effect of increased carbon dioxide levels on the parameters analyzed; an additional 2% CO₂ input has been shown to increase cell densities and increase lipid yields in green algae [1], however there was no analysis of fatty acid and pigment compositions. As well, cellular carbon is distributed between lipids, proteins and carbohydrates; a future study of diatom biochemistry incorporating all three components would give a more complete picture of lipid production and more importantly, a better understanding of diatom environmental adaptiveness.
Bibliography


