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Nerissa L. Fisher for the degree of Master of Science in Microbiology presented on June 10, 2015.

Title: Photosynthetic Energy Allocation Strategies of *Thalassiosira pseudonana* During Light Limited Growth

Abstract approved:

________________________________________________________________________

Kimberly H. Halsey

Photosynthetic energy allocation strategies were investigated in the marine diatom, *Thalassiosira pseudonana*, grown under a wide range of light limitation. Steady-state, continuous cultures were established at three light-limited growth rates. Simultaneous measurements of photosynthetic activity were made that targeted different points in photosynthetic energy flow from gross to net photosynthesis. Cells maintained high photosynthetic efficiencies across all growth rates by increasing chlorophyll content and PSII reaction centers as light became increasingly limited. Across all growth rates 65-80% of gross photosynthesis (*GPP*$_{O2}$), was maintained as net primary production (*NPP*$_c$). A constant 5% of *GPP*$_{O2}$ was allocated for the direct reduction of nitrate and sulfate across all light limited growth rates. At low light limited growth rates, a higher fraction of energy was allocated to light dependent respiration and mitochondrial respiration, reflecting greater requirements for maintenance energy in the form of ATP. In contrast, fast growing light limited cells allocated a greater fraction of *GPP*$_{O2}$ to reductant (NADPH) needed for biosynthesis of biochemically reduced macromolecules. Lipids were a small fraction across all light limited growth rates for short-term
biofractionation measurements but a higher fraction for only high and medium light limited growth rates in biomass biofractionation measurements. However, these behaviors were not reflected in the biochemical reduction state of biomass. The nearly four-fold greater C\textsubscript{r}/C\textsubscript{o} ratio in cells acclimated to the lowest light level was driven by a predominance of protein relative to carbohydrates. Significant differences in rates of mitochondrial respiration (MR) were observed in the light and dark. Lower rates of MR in the light may reflect a form of pathway gating that preserves carbon storage compounds in the light by switching to non-carbon pathways for ATP generation. This strategy may be linked to their ecological success particularly following prolonged periods of darkness following deep mixing events. Behaviors in photosynthesis-irradiance (PE) relationships that are characteristic of photoacclimation [namely, constant light limited slopes (\(\alpha^*\)) and variable maximal rates of photosynthesis (\(P_{\text{max}}\))] were observed at the level of PSII but not when PE curves were generated from short-term \(^{14}\text{C}\)-uptake rates. These unexpected growth rate-dependent shifts in \(\alpha^*\) add complexity to interpretations of PE curves that are commonly used for describing phytoplankton responses to environmental variables. Our results also demonstrate that photosynthetic metabolism is markedly different depending on the limiting resource (e.g., light vs. nutrients). These data are discussed in the context of the adaptive growth strategies thus far understood for diatom species.
Photosynthetic Energy Allocation Strategies of *Thalassiosira pseudonana* During Light Limited Growth

by

Nerissa L. Fisher

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I understand that my thesis will become part of the permanent collection of the Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Nerissa L. Fisher, Author
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CONTRIBUTION OF AUTHORS

All experiments were performed in the laboratory of Dr. Kimberly Halsey, who served as major professor. Dr. Kimberly Halsey was involved in the editing of this manuscript and instrumental in the experimental design of this thesis research. Dr. Bethan Jones assisted with protocol design for some experiments in addition to offering advice along with Drs. Michael Behrenfeld, Jason Graff, and Allen Milligan.
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Chapter 1 – Introduction

Photosynthesis is undeniably one of the most important processes on Earth as it produces the oxygen for aerobic life to exist as we know it today. Photosynthesis originated billions of years ago when the oceans covered the entire Earth’s surface. It is often assumed that the majority of primary production is accomplished by terrestrial plants, but it is the single celled marine prokaryotic and eukaryotic microalgae, called phytoplankton, that inhabit these vast bodies of water and contribute over half the global net primary productivity (Field et al., 1998) and produce oxygen at rates that equal, or even exceed, those of terrestrial plants (Behrenfeld et al., 2001). It is also remarkable how fundamental the process of photosynthesis is, and yet there is much we still do not understand about the underlying mechanisms that control photosynthetic metabolism. Since photosynthesis is, by definition, a light-dependent process, one challenge for current researchers are the widely varied measurements and associated units used to report photosynthetic activities. One simple example is the unit conversions needed to describe light intensity, which can be reported in units of footcandles (typical for literature in the 1940s) that measures brightness, to the current standard units of µmol photons m\(^{-2}\) s\(^{-1}\) that is a measure of light energy and intensity. A more complicated example that challenges researchers in the field of photosynthesis research is that there are multiple methods to measure photosynthesis (e.g., the rate of photosynthesis can be determined as the amount of oxygen produced or the amount of carbon dioxide (CO\(_2\)) fixed into organic matter), and the measurements cannot be easily compared with any certainty.
Particularly when comparing studies on phytoplankton photosynthesis, the culture conditions must be carefully documented and checked by researchers, as the units of measurements are often different. Furthermore, microalgae are extremely responsive to resource limitation, and species differences are commonly noted. For example, one diatom species may have adaptive strategies for photosynthetic activity under very low light, while another diatom species under the same experimental conditions can give completely different physiological responses. Such is the “paradox of the plankton.” The concept of the paradox explains how so many species are able to coexist and thrive in the ocean, essentially occupying the same niche space. As a marine microbiologist, I am interested in understanding how these microbes are able to survive on variations in resources needed for growth. Do phytoplankton species have different photosynthetic metabolisms to allow their growth and persistence?

These microscopic photosynthesizers have withstood billions of years of evolution, but many of their fundamental responses to resources required for growth, namely light and nutrients, are still largely unknown. It is fascinating that an organism so small and so vital can still baffle the scientific community on even the simplest level (i.e., how are diatoms able to be one of the most successful groups of phytoplankton, especially in coastal systems?) The intricacies of photosynthetic metabolism, adds impetus to the importance of their study, especially during a time of climate change. Phytoplankton are the base of the aquatic food web and their responses to global climate change could have worldwide effects. In an era of global climate change, the study of phytoplankton ecophysiology is more important than ever. Global climate change is having a significant influence on eutrophication and sedimentation in coastal waters,
which affects both of the environmental factors that limit phytoplankton growth: light and nutrients. Several studies highlight the effects of nutrient limitation on phytoplankton primary production but fewer have investigated, in detail, how light limitation alters the efficiency at which light energy is converted to biomass.

This research investigates the relationships between photosynthesis and light limitation using a coastal diatom species, *Thalassiosira pseudonana*. The first objective of this work is to measure how this organism uses the energy harvested through photosynthesis to fuel the cellular processes required for growth. The second objective is to determine how this energy use changes depending on the degree of light limitation. Knowledge about photosynthetic energy use in response to changing light availability will be useful in efforts to model current and future primary production.
Chapter 2 – Literature Review

Isotopic fractionation of the oldest carbon in rocks on Earth suggests that life evolved about 3.8 billion years ago (Mojzsis et al., 1996). However, there is no direct evidence that photosynthesizers supported this life, but there is still debate that the earliest life forms were autotrophic nonphotosynthetic (Wachterhauser, 1990) or photosynthetic cells (Granick, 1965; Hartman, 1975, 1998; Mauzerall, 1992). There is direct evidence of life on Earth from microfossils in 3.5 billion-year-old stromatolites that suggest biological activity from fossilized casts of bacteria was photosynthetic as “they are morphologically remarkably similar to certain groups of modern cyanobacteria, raising the possibility that these organisms were capable of oxygen evolution” (Schopf, 1992, 1993). The evolution of photosynthesis is important because it allowed for the oxygenation of Earth, which changed the course of evolution on this planet. Bacteria were the first photosynthesizers to emerge using water and light as energy sources to reduce carbon dioxide and making oxygen as a by-product, but it was the eukaryotic algae that became dominant and are considered responsible for changing Earth’s atmosphere. According to geological evidence, by about 2.2 billion years ago significant quantities of oxygen had accumulated in the atmosphere (Rye and Holland, 1998; Canfield et al., 2000). Prior to this time, the oceans were also saturated with Fe$^{2+}$, which was immediately oxidized to Fe$^{3+}$ as a result of increasing oxygen concentrations from algal photosynthesis. The oxidation of Fe$^{2+}$ resulted in the banded-iron rock formations that developed between 3.5 to 1.8 billion years ago (Schopf, 1992). It is remarkable that even with these dramatic changes in Earth’s atmosphere, from a more CO$_2$-rich to a more oxygenated atmosphere, no oxygenated photosynthetic organism abandoned the primary
enzyme responsible for carbon fixation, Ribulose bisphosphate carboxylase oxidase (RUBISCO) for another, more primary carboxylation mechanism (Geider and Osborne, 1992) even though RUBISCO has a low specificity for CO$_2$. Thus, photosynthetic organisms have overcome many obstacles during their evolution. The poor substrate specificity of RUBISCO and evolution of active carbon concentration mechanisms are examples of adaptive strategies that can alter how photosynthetic energy is used under different environmental conditions.

Phytoplankton account for approximately 50% of global primary production (Field et al 1998). Phytoplankton are essential and irreplaceable sequesters of CO$_2$ from the atmosphere as well as major players in the microbial loop, biogeochemical cycling, cloud formation, petroleum reserves, and, most recently, an excellent resource for generating biofuels. Phytoplankton are found ubiquitously in aquatic environments ranging from polar waters to the tropics, lakes and marshes, and oligotrophic open ocean to nutrient enriched coastal waters. Evolutionary selection of these microorganisms has driven conservations of genes that best suit them to survive in dynamic environments. Complex mechanisms, sometimes a combination of plant and animal genes (Falkowski et al., 2004; Armbrust et al., 2004), allow phytoplankton to sense and respond to changes in abiotic factors such as temperature, nutrients, salinity, light, and pH. The main drivers of phytoplankton growth are light and nutrients, thus understanding how their photosynthetic machinery influences energy allocation to different metabolic processes leading to growth and how these processes change in response to these particular factors is paramount.
Of the broad range of phytoplankton groups, diatoms are the most recent to evolve and are responsible for approximately 40% of all phytoplankton primary production (Treguer et al., 1995) making them an extremely important group to study. This is especially important during a time of ongoing climatic change. Climate change is expected to increase eutrophication and sedimentation and thus affect nutrient and light availability, respectively. Diatom genomes have a combination of animal and plant genes, thus proving that their evolutionary history is connected to a secondary endosymbiosis event where a red algae cell was engulfed by an unknown heterotrophic cell (Falkowski et al., 2004). The diatom evolutionary history helps explain how they are able to dominate most phytoplankton communities as they are apparently well equipped to adapt to a plethora of environmental changes; however these endosymbiotic events also make deciphering and interpreting the diatom genome difficult. Aside from photosynthetic prokaryotes (e.g., Prochlorococcus and Synechococcus species), diatoms are the most successful class of eukaryotic unicellular algae in environments with replete nutrients and low temperatures (Monstant, 2007). Although diatoms have also been observed to withstand variations in temperature (Litchman, 1998; Nicklisch et al., 2008; Cabrerizo et al., 2014). For the purposes of this thesis, I will focus on the sequenced genome of the marine diatom, Thalassiosira pseudonana, with particular attention to information about what the genome has revealed about the genetic mechanisms that are thought to be responsible for allowing this species to survive under dramatically varying light regimes on timescales that range from minutes to seasons.

Sequenced Genome of Thalassiosira pseudonana:
In 2004, Armburst et al. published the sequenced genome of the marine diatom, *Thalassiosira pseudonana*, using a whole-genome shotgun approach with 14-fold sequence coverage to encompass nuclear, mitochondrial, and plastid sequences. *T. pseudonana* is a centric diatom isolated in 1958 from Moriches Bay located in Long Island, New York. In summary, the nuclear genome contained 34 million base pairs composed of 24 diploid chromosomes, the plastid genome contained 129 thousand base pairs, and the mitochondrion had a genome size of 44 thousand base pairs (Armburst et al., 2004). This particular diatom species was an appealing choice for genomic sequencing not only because of its ecological importance and relative ease of manipulation at the genetic and molecular level, but also because of its predicted small genome size observed using flow cytometry after staining the DNA with a fluorescence dye (PicoGreen or SYTOX) (Veldhuis et al., 1997; Grossman, 2005). While many novel genes corresponding to various metabolic pathways and mechanisms had already been identified, there was still much about the inner workings of this diatom that required further investigation (and still does). The evolutionary history of diatoms is unique to that of higher plants that dominate photosynthesis on land. This distinctive character and was further confirmed through genome sequencing. While no DNA/RNA sequencing was performed for this thesis research, I have collected culture samples for both nutrient limited and light limited cultures of *T. pseudonana* for later analysis of their transcriptomes with the goal of linking gene expression data to the physiological behaviors I report in Chapters 2, 3, and 4. Therefore, I look forward to staying involved in this project well past my graduation.
Endosymbiotic Events Creating the Present-Day Diatom:

Diatoms have unique genes that code for various cellular functions. The presence of these genes are explained by the history of diatom evolution. The diatom genome is unlike any other photosynthetic organism sequenced to date and even the genomes of centric versus pennate diatoms are quite distinct. The best explanation for the unusual set of genes discovered through whole genome sequencing is that an endosymbiotic event occurred where a heterotrophic host first engulfed a cyanobacteria cell giving rise to red algae and then a second endosymbiotic event occurred where another heterotrophic host engulfed a red algal cell thereby creating the diatom group (Rynearson & Palenik 2011
Fig. 1 Secondary endosymbiosis of eukaryotic phytoplankton. The inheritance of the plastid was derived from a red algal cell that was engulfed by a heterotrophic host via an endosymbiotic event. This combination of heterotrophic host and red algal plastid allowed for much of the genetic transfer that has allowed the diatom group to be so successful in highly turbulent conditions. (figure taken from Armbrust et al., 2004)
Nuclear genomes of diatoms were found to be a combination of genes from several evolutionarily distinct organisms, which strengthened the hypothesis of a secondary endosymbiotic event where the progenitor plant cell from the first endosymbiotic event created the red algae group and then at a later point in evolutionary history a red algal cell was engulfed by a second heterotrophic host. As confirmed by the sequenced genome, the red algal nucleus and mitochondrion were lost, but the presence of a four-membrane thylakoid in present day diatoms supports the engulfment of a cyanobacteria and red algae cell (Smith et al., 2012). This unique evolutionary history is thought to allow diatoms to be very successful in low light and temperature environments, facilitating their ability to outcompete other algal groups under these conditions.

*Light Attenuation in Aquatic Environments and Diatom Light Utilization:*

Green light penetrates to the greatest depths in coastal waters, which is consistent with identification of genes for synthesizing rhodopsin in green algae, but, interestingly, no such genes were identified in the diatom genome. However, cryptochrome receptors for the perception of blue light as well as a possible phytochrome photoreceptor for perceiving red/far-red light were found in the diatom genome (Montsant, 2007). The presence of these receptors is consistent with previous physiological studies that show diatoms preferentially absorb light in the blue and red/far-red wavelengths of the light spectrum (Armbrust et al., 2004; Yanovsky and Kay, 2003; Leblanc et al., 1999, Fisher et al., 1996). Diatoms likely inherited this phytochrome-encoding gene from the secondary heterotrophic host rather than the red algal endosymbiont as no such gene is found in red
algae genome sequences. This suggests that some components of diatoms more closely resemble their animal-like heterotrophic progenitor rather than the plant-like counterpart. This further indicates that the diatom plastid had to develop new mechanisms to facilitate interactions with its distantly related genetic partners. Unfortunately, this regulatory network in diatoms is not yet known (Wilhelm, 2007). This mixture of plant and animal genes from the two endosymbiotic events likely explains why diatoms are best suited in well-mixed waters, where turbulent velocity and turbidity, along with changes in light attenuation and spectral quality are major factors affecting growth (Tozzi et al., 2004).

Light attenuates rapidly in the water column (Fig. 2), so compared to terrestrial environments where light is often saturating, phytoplankton must tune their photosynthetic machinery in response to lower light environments. Photosynthesis is saturated for most diatoms at photon irradiances ranging from 200-300 µmol photons m⁻² s⁻¹ (Brand and Guillard, 1981; Richardson et al., 1983; Geider et al., 1985; Litchman, 2000) and growth rate remains constant beyond this irradiance (Litchman et al., 2003; Bittar et al., 2013), not excepting *T. pseudonana* whose growth is photoinhibited at around 250 µmol photons m⁻² s⁻¹ (Nelson et al., 1979 and Brand and Guillard, 1981).
Fig. 2 Attenuation of light in the water column comparing two typical water types at various wavelengths. As described by Austin and Petzold (1986), type I water is characterized by clear water with low phytoplankton concentration (solid line); type III water is characterized by turbid water with high phytoplankton concentration (dashed line). (figure modified from Depauw et al., 2012)
For my research study, I focused how *T. pseudonana* differentially utilizes its harvested photosynthetic energy depending on steady-state growth under light conditions that approximated the light intensity at the upper, middle, and lowest levels of the euphotic zone of the water column.

**Maintaining Optimal Position in the Euphotic Zone:**

All photosynthetic organisms require light to generate the energy required for cellular metabolism and therefore they must fulfill two requirements: [1] capturing enough photons to run photosynthetic electron transport and [2] avoiding the damaging effects photons can have. Diatoms are no exception to these fundamental photosynthetic requirements, so they possess mechanisms that allow them to maintain optimal position in the euphotic zone with respect to the balance between sufficient light and an oversupply of light. This is a difficult task for diatoms as their dense silica frustules that form protective glass walls also increases sinking rates. One compensatory strategy is that diatoms are able to increase drag by extending chitin fibers from the pores in the frustule and thus remain in the illuminated portion of the water column (Armburst et al., 2004). Genome sequencing identified enzymes for chitin biosynthesis and nearly 22 chitinases-related genes suggesting chitin fiber length may be dynamic and controlled by the diatom according to the needs of the cell at any given time (Armburst et al., 2004). Reducing chitin fiber length would decrease the thickness of the boundary layer around the cell thereby increasing nutrient accessibility to the cell surface. On the other hand the
propagation of chitin fiber length would keep the cell suspended in the euphotic layer of
the water column for longer periods.

In addition to an active regulation of cellular buoyancy in the water column, diatoms are also faced with physical properties of water including vertical mixing, horizontal movement from currents, waves, wind, eddies, etc., which further forces changes in diatom positioning in the water column. These events will often push diatoms away from zones of ideal light intensities. On shorter time scales, the time of day, weather, and cloud cover change the intensity of light cells experience. Therefore, they must adapt to changes in light rapidly to efficiently maintain photosynthetic efficiency and ultimately growth.

*Xanthophyll Cycle:*

When diatoms are exposed to different light conditions the carotenoids, pigments that play a significant role in light harvesting and photo-protection, undergo chemical changes through a process called the xanthophyll cycle. Diatoms differ from terrestrial plants in that they possess a simple xanthophyll cycle, which involves a one-step conversion of diadinoxanthin (Ddx) to diatoxanthin (Dtx) as opposed to higher plants that possess a more complex xanthophyll cycle (Goss & Jakob, 2010). It is worth noting that when diatoms are exposed to prolonged high light conditions they are able to perform the conventional xanthophyll cycle observed in terrestrial plants. Diatoxanthin is required for the non-photochemical quenching activity in diatoms. This positive correlation between diatoxanthin and non-photochemical quenching (NPQ) has been reported to occur under high light whereas no diatoxanthin could be detected under low light conditions. This
indicates that under low irradiance, the change in pH across the thylakoid membrane was not sufficient to activate the conversion of diadinoxanthin to diatoxanthin (Zhu & Green, 2010). However, just minutes after exposure to high light there was a significant amount of diatoxanthin produced. This pigment increase was inversely related to the decrease of diadinoxanthin (Zhu & Green 2010). Recent findings concerning the xanthophyll cycle were discovered by RNA extraction, reverse transcribed RNA into cDNA for qRT-PCR, and protein extraction, which were then cross-referenced with the genomic sequences and chromosome locations for T. pseudo nana to explain the gene expressions observed under different light treatments. More broadly, NPQ is a mechanism utilized by photosynthetic organisms to protect themselves from the damaging effects of high light intensity, when light energy absorption surpasses the capacity for light utilization in photosynthesis. NPQ acts to dissipate excess energy generated by excited electrons as heat through molecular vibrations (Depauw et al., 2012). Unfortunately, there are many components missing that would provide a clearer understanding of the xanthophyll cycle, such as the functional and biochemical characterization of diatom-specific variants that might be involved in the chromist-specific diadinoxanthin cycle, the mechanistic aspects of the diadinoxanthin-dependent non-photochemical quenching, and the exact binding site of diadinoxanthin (Depauw et al., 2012).

Light Harvesting Complexes:

Diatoms live in an extremely dynamic environment especially giving the fact that planktonic organisms cannot swim against currents or control their position in the water column when vertical mixing forces them to unfavorable conditions for growth.
Therefore, diatoms must possess mechanisms for such adapting their physiology to such situations to explain their dominance in well-mixed water columns. Photoreceptors found in the light harvesting complexes are able to detect changes in light through the capturing of photons to then trigger a cellular response. There has been some progression in the understanding of the molecular organization of the light-harvesting complexes found in the thylakoids called fucoxanthin-chlorophyll a/c-binding proteins (FCP) but less is known about the main photosynthetic components of photosystem (PS) I and PSII (Bailleul et al., 2010). In diatoms, three main groups of light harvesting complex (Lhc) proteins have been identified: Lhcf, Lhcr, and Lhcx (Grouneva et al., 2011). Diatoms lack the homologue PsbS (PSII S subunit protein) found in higher plants (Smith et al., 2012) thus it is predicted that Lhcx proteins may provide a similar function in diatoms. Lhcx gene expression is detectable when *T. pseudonana* was exposed to low light conditions with one Lhcx protein shown to have a direct connection with PSI. The detectable expression of genes encoding Lhcx proteins under high and low light treatments suggests a general light stress response-related function of these particular proteins extending beyond NPQ (discussed above). Lhcr proteins were detectable when *T. pseudonana* was exposed to high light treatments, which suggests this protein is involved in photoprotective mechanisms. Lhcf proteins appear to be unaffected by high light treatments but gene expression for this protein was high over longer low light exposure (Zhu and Green, 2010). This suggests that a variety of light harvesting proteins allow diatoms to adapt quickly to changes in light as certain proteins were activated under different light treatments. Subsequent work on a pennate diatom, *Phaeodactylum tricornutum*, showed similar responses in light harvesting protein expression under the different light
treatments as was observed in *T. pseudonana* (Nymark et al., 2013). Although my research study did not focus on the effects of excess light energy on photosynthetic energy allocation, it is nonetheless an important and advanced feature of diatoms.

*Photoacclimation:*

Phytoplankton photoacclimation encompasses a range of physiological mechanisms that allows cells to reduce their energetic and maintenance costs under conditions of limiting energy resource supply (Richardson et al., 1983). To clarify, photoacclimation refers to physiological processes while photoadaptation refers to evolutionary processes. This distinction is important because sometimes these terms are used synonymously in the literature (Falkowski and LaRoche, 1991). On that note, photoacclimation involves changes in the minimum quantum requirement for photosynthetic oxygen evolution (Dubinsky et al., 1986), respiration (Geider et al., 1986; Langdon, 1993), and growth rate (Laws and Bannister, 1980).

Myers introduced the concept of photoacclimation in 1946. Following that initial introduction, he demonstrated distinguishing responses such as a decrease in pigment concentration before light saturation of growth rate and the independence of the Chl a-specific light limited (initial) slope (α∗) with growth irradiance (Myers, 1970).

Technological advances in culturing methods from batch to continuous culture systems enabled features of photoacclimation to be studied (Myers and Clark, 1944) without interference from factors that varied during growth in traditional batch cultures (Beardall and Morris, 1976) as cells were maintained in balanced growth. Specifically, in a batch culture, the biomass constantly increases, thereby changing the light field throughout the
period of growth. In contrast, a continuous culture is maintained at a constant biomass, thus the light field remains constant.

There are two broad categories of physiological responses to light limitation that algae utilize, including (1) responses that improve the cells’ light energy harvesting and utilization abilities and (2) responses that reduce the energy requirements for growth (Thompson, 1991). When a photon hits PSII, there are only three pathways for the absorbed photon to take: fluorescence, thermal dissipation, or charge separation (Behrenfeld et al., 2004). Charge separation is, by definition, the total sum of energy available to the cell to use.

Photosynthesis-irradiance (PE) curves are important because they provide a relatively simple means of distinguishing between light-limited and light-saturated photosynthesis (MacIntyre et al., 2002). The variables that characterized the PE curve are the light-limited slope, \( \alpha \), the light-saturated rate, \( P_{\text{max}} \), of photosynthesis (MacIntyre et al., 2002; Behrenfeld et al., 2004), and the light-saturation parameter, \( E_k (= P_{\text{max}}/\alpha) \) (Talling, 1957; Platt et al., 1977). In oceanography, these variables will often be normalized to chlorophyll a concentration because of the specificity of this readily measured variable to the phytoplankton (MacIntyre et al., 2002) and chlorophyll a is detectable and estimated from satellite imaging (Behrenfeld et al., 2002). Chlorophyll normalization is denoted by the addition of a superscripted asterisk or b following the reported parameter. For example, chlorophyll-normalized \( \alpha \) and \( P_{\text{max}} \) become \( \alpha^* \) and \( P_{\text{max}}^* \). Behrenfeld et al. (2004) described variability in PE curves associated with variations in \( E_k \). One form of PE curves was associated with independent changes in \( \alpha^* \) and \( P_{\text{max}}^* \) that alter \( E_k \) (termed “\( E_k \)-dependent variability”) and the other having parallel
changes in $\alpha^*$ and $P_{\text{max}}^*$ that did not change $E_k$ (termed “$E_k$-independent variability”). It was well known that $E_k$-dependent variability was characteristic of photoacclimation responses; where $E_k$ varies as a result of changes in the cell content of light harvesting pigment that is regulated separately from the processes downstream of photosynthetic electron transport (Behrenfeld et al. 2004). However, it was not until the work of Halsey et al. (2010) that $E_k$-independent variability was the result of changes in growth rate dependent carbon metabolism under varying degrees of nutrient limitation.
Fig. 3 Photosynthesis-irradiance (PE) curves showing two fundamental descriptors of photosynthesis: $E_k$-dependent variation (A) and $E_k$-independent variation (B) when photosynthesis is normalized to chlorophyll (Behrenfeld et al., 2004).
For this research study, I focused on light intensity rather than quality because “photoacclimation responses to changes in light quantity play a more important role than physiological responses to variation in light quality in determining photosynthetic performance” (Falkowski and LaRoche, 1991; Morel et al., 1987). We also chose to expose our cultures to constant light rather than diel cycles because photoacclimation is not a physiological acclimation to a light-dark cycle (Post et al., 1984). Although PE curves are commonly used as the basis for models of phytoplankton productivity (Platt et al., 1977; Fasham and Platt, 1983), there have been few direct comparisons of PE curves determined by carbon assimilation and oxygen evolution (Platt et al., 1987; Kana, 1992). My research measured behaviors in both carbon and oxygen production in an effort to obtain a more complete understanding of photophysiology in *T. pseudonana*.

*Previous work on photosynthetic energy allocation in algae in response to light limitation:*

Several studies have measured changes in energy allocation in response to nutrient limitation, but the studies to date involving energy allocation strategies in response to a wide range of light intensities are sparse. The literature on physiological responses to light focuses on two extremes: fluctuating vs. constant light exposure at low and excessively high irradiances, and the molecular changes that occur upon exposure to extreme light exposure. This study hopes to fill the gap in the current knowledge by providing a cellular level view of the physiological changes that are induced under the
different light-limiting conditions that cells are likely to experience in the euphotic zone with vertical mixing.

Other studies have found net primary production remained remarkably constant once cells were fully photoacclimated over a wide range of light intensities (Falkowski et al., 1985; Laws and Bannister, 1980). These results have lead to the proposition that there is a tight coupling between growth and respiration to the photosynthetic electron transport chain such that the rate at which photons can be effectively harvested adjusts as cells photoacclimate thus optimizing growth efficiency for an algal cell (Langdon, 1987, 1988).

Over diel cycles, net photosynthesis and carbohydrate accumulation are limited to the illuminated part of the day, but protein synthesis often continues in darkness for batch cultures of *Dunaliella tertiolecta* (Cuhel et al., 1984). In the field, the Arctic species *Laminaria solidungula* accumulates carbohydrate reserves during the summer when light is more available but inorganic nutrients are low, and growth occurs at the expense of stored carbohydrates from winter when nutrient concentrations were high but light was limiting (Chapman and Lindley, 1980; Dunton, 1985).

*Growth rate response of Thalassiosira pseudonana to light:*

When designing this experiment, there were many factors of light that needed to be considered. One such factor was the influence of growing *T. pseudonana* under a light:dark cycle or continuous light. Nelson et al. (1979) found that the growth rate of *T. pseudonana*, in addition to another diatom (*P. tricornutum*), versus light intensity whether grown in fluctuating or constant light yielded the same maximum growth rates
(Fig. 4). This provided a maximum light intensity for photosynthetic saturation that could be applied to the current research, which focused on photosynthetic efficiencies at light limiting growth rates. With the conversion equation (1 ly min\(^{-1}\) = 3485 µE m\(^{-2}\) s\(^{-1}\)), photosynthetic saturation of \(T.\) \textit{pseudonana} occurs at approximately 250 µE m\(^{-2}\) s\(^{-1}\). This information helped determine the highest light limited growth intensity to grow \(T.\) \textit{pseudonana} to generate the results that answer the research questions.
Fig. 4 Growth rate ($\mu$) of *Thalassiosira pseudonana* (filled circles) and *Phaeodactylum tricornutum* (open circles) versus light intensity ($I$) over a 14:10 light:dark cycle (a) and continuous light (b) (Nelson et al., 1979).
Methods for Measuring Primary Production:

Measuring primary productivity is important for understanding phytoplankton dynamics, but it is also complicated because photosynthetic energy harvested at PSII fuels multiple cellular processes. Thus, gross primary production (GPP) measures the total energy harvested at PSII and net primary production (NPP) is the difference between GPP and energy lost to respiratory (ATP generating) processes. Further complicating these measurements are the metabolic pathways that consume oxygen, reduce inorganic compounds (e.g., nitrogen and sulfur), and those that catabolize carbon. Thus, the most informative approaches to understand primary production simultaneously address these different pathways to obtain a comprehensive view of photosynthetic metabolism.

Steemann-Nielsen introduced the $^{14}$C technique in 1952 to measure phytoplankton production. This method was hailed as an approach that overcame the sensitivity problem encountered with conventional gas exchange measurements from light-dark bottle methodologies (Riley, 1939) that are still used today. Nevertheless, there is debate over whether $^{14}$C-uptake measures gross, net, or an intermediate rate of primary production (Lloyd et al., 1977; Williams et al., 1996; Laws et al., 2000; Marra, 2009). In an extensive review of $^{14}$C uptake studies, it is clear that $^{14}$C-based estimates of GPP were considerably underestimated as they did not account for recycled respired carbon, and it is still unclear whether the $^{14}$C method accurately estimates NPP (Pei and Laws, 2013). Nonetheless, the radioactive tracer $^{14}$C has been instrumental in determining the biochemical pathways of CO$_2$ fixation (Bassham and Calvin, 1957) and generating photosynthetic irradiance curves that are fundamental in making broader-scale estimates
of aquatic primary production. Much progress has been made in developing an incubation-free method to derive NPP from phytoplankton carbon measurements (Graff et al., 2012, 2015) that can be combined with models of growth rate. Similarly, chlorophyll concentrations are more closely related to GPP than NPP, and over the past 15 years, the triple oxygen isotope method has become more advanced and has helped provide good estimates of GPP (Juranek and Quay, 2013).

Morris et al. (1974 and 1980) introduced biochemical fractionation of assimilated $^{14}$C to phytoplankton research building from methodologies developed for *Escherichia coli* (Roberts et al., 1963). These early studies set the stage for identifying the role of nutrient availability in carbon metabolism over the short-term (less than a cell cycle). In a series of studies, Halsey and colleagues identified shifts in carbon metabolism across the cell cycle to cause variability in $^{14}$C uptake rates (Halsey et al. 2010, 2011, 2013). They used $^{14}$C-pulse-chase experiments to track the incorporation of $^{14}$C into different end products. These experiments also gave information about how pools of those initial products changed over 24 h time-course experiments. These data were used to develop a mathematical model that was based on growth rate-dependent turnover of newly fixed carbon. All of those studies were done using nutrient-limitation to establish growth rates. What was unknown, was how light limited growth rates affected $^{14}$C-uptake rates. My research fills this important knowledge gap.

Radiolabeled carbon can also be used to measure carbon lost from the cell as dissolved organic carbon ($\text{DO}^{14}\text{C}$). Extracellular release of $\text{DO}^{14}\text{C}$ is a normal occurrence of algal metabolism and accounts for a varying portion of photosynthesis in many microalgae (Leboulanger et al. 1998). $\text{DO}^{14}\text{C}$ production has been observed to continue at
a constant rate during light and dark transitions (Mague et al., 1980), suggesting that this
loss of cell material is the result of passive diffusion. When performing DO\textsuperscript{14}C
experiments, it is important that the sample is not contaminated by organic \textsuperscript{14}C sources
leached from cells during filtration and cell fragments that are smaller than the size of the
filter pores (Sharp, 1977).

Another useful isotope in evaluating the roles of respiration, photorespiration, and
other non-carbon respiratory pathways (e.g., the Mehler reaction, midstream oxidases,
and the malate valve) is the oxygen isotope, \textsuperscript{18}O\textsubscript{2} (Geider and Osborne, 1992). This
isotope is useful because it allows for the direct evaluation of GPP (as gross O\textsubscript{2}
production) (Bender et al., 1987; Grande et al., 1989) and respiration in illuminated algae
(Radmer and Ollinger, 1980) where respiration can be estimated from the consumption of
\textsuperscript{18}O\textsubscript{2} while simultaneously measuring photosynthesis from the evolution of \textsuperscript{16}O\textsubscript{2} (Peltier
and Thibault, 1985).

**Major pathways used in photosynthetic metabolism:**

The total energy a photosynthetic cell has available to carry out the sum total of
metabolic processes, ultimately leading to growth, is obtained from the splitting of water
after a photon of light is absorbed and funneled to PSII; a process termed GPP. We
measure GPP as the total O\textsubscript{2} produced so this important photosynthetic rate is designated
as GPP\textsubscript{O2}. Following GPP\textsubscript{O2}, energy can be ‘lost’ through light dependent respiration
(LDR), an oxygen reducing process that produces ATP during periods of illumination.
The term ‘lost’ is not entirely accurate because while electrons are utilized during
respiratory processes, the cell obtains ATP through their transfer to O\textsubscript{2}. Moving through
the photosynthetic metabolism of a cell, energy in the form of electrons can also be used to reduce the primary substrates, nitrate and sulfate, that are needed for nucleic acid and amino acid synthesis. The remaining energy (electrons) are used for carbon fixation. The total amount of carbon fixed through the Calvin cycle is gross carbon production (GPc). Some of this carbon is respired through mitochondrial respiration (MR), thus this processes represents the loss of energy at the expense of ATP production for cellular maintenance and it occurs in the light and the dark. Net oxygen production, NP2, is the oxygen produced after losses from LDR and MR, both oxygen-reducing processes, have been accounted for. NP2 can be used to measure the amount of energy used for MR and the amount of energy used for biosynthesis of reduced forms of carbon. Energy for biosynthesis of reduced carbon forms is a carbon catabolic process. Through this carbon catabolism, electrons are released and reinvested to make proteins, lipids, and nucleic acids, and other carbon components of the cell that are more biochemically reduced than the initial product of carbon fixation. Dissolved organic carbon, DOC, is the energy the cell excretes because there was no use for that energy or it was lost accidentally. Particulate organic carbon, POC, measurements should equate NPPc which is a measure of biomass production. This is the energy that is remaining for higher trophic levels.

To my knowledge, the only studies that have conducted comprehensive analyses of photosynthetic energy allocation under light limitation have used fluctuating light conditions that compare saturating and non-saturating regimes. I discuss, in detail, how my results compare with those studies (see Discussion). Briefly, Wagner et al (2006) found higher productivity rates in diatoms grown under higher light intensities and Jakob et al. (2007) found higher protein in diatoms grown under lower light intensities. Both
studies also observed higher chlorophyll concentration with decreasing irradiance.

Neither study looked at the effect of photosynthetic energy allocation in response to a range of light intensities where photosynthetic electron transport is not inhibited (photoinhibition), which is one of the ways my research is unique.
Fig. 5 Photosynthesis energy flow schematic. A photosynthetic cell starts with all the energy it has for maintenance and growth with the splitting of water at PSII. This production of energy is called gross primary production, GPP\textsubscript{O2}. The colored terms on top of the arrow, light dependent respiration (LDR), gross carbon production (G\textsubscript{Pc}), mitochondrial respiration (MR), net oxygen production (Net \textsubscript{O2}), Biosynthesis, and POC/DOC, represent the areas where energy is lost for oxygen reduction and carbon catabolism from the total energy harvest initiated by light hitting PSII. The words underneath the arrow explain how the energy loss processes above were measured and/or how they were measured. The * signifies more reduced carbon compounds and thus a higher energy requirement to synthesize. The final product is cell growth, or biomass, termed net primary production, NPP\textsubscript{c}. The faded arrow represents the dissipation of energy that occurs from GPP\textsubscript{O2} to NPP\textsubscript{c}. 
Future Work/Implications:

Even with the technological advances in genome sequencing, there are still major gaps in current understanding of how phytoplankton are able to adjust their physiology in response to changes in light so that the cell is able to capture enough photons and remain energetically favorable in low light but also avoid photo-damage at high light. Although much has been learned since the genome of *T. pseudonana* was sequenced in 2004 there are still many fundamental questions left unanswered that may be useful for predicting how changes in light will affect the proliferation and succession of this diatom and other diatom species. After reviewing the research completed on the subject of *T. pseudonana* responses to light, the majority of publications came to the same conclusion: there are more questions than answers on this subject at the present time. The mechanisms and corresponding genes studied since the original sequencing of *T. pseudonana* have added essential details to the genes and their gene products that were already identified. For example, one of the fundamental steps in the process of photosynthesis involves concentration and delivery of CO$_2$ to the active site of the carbon-fixation enzyme RUBISCO (Armburst et al., 2004). However, this processes is still not clearly understood. The closest explanation in the literature is that *T. pseudonana* possesses multiple carbonic anhydrases, though none appear to be localized to the plastid where photosynthesis occurs, which suggests that an alternative mechanism (including C4 metabolism, Reinfelder, 2011) may deliver CO$_2$ to RUBISCO (Parker et al., 2008). A decade since the genome of *T. pseudonana* was sequenced several metabolic pathways have been discovered, but questions pertaining to mechanisms underlying diatom
viability, growth, and persistence remain. This realization further proves that because technologies for sequencing genomes are only getting faster, more accurate, and less expensive, the limiting factor lies with the manpower available to analyze the data generated, and suggests that experiments that connect diatom physiology with genomic information are a promising approach to revealing key mechanisms involved in their global success.

Light is not just compulsory as the energy source that photosynthetic organisms need to generate energy for cellular metabolism; it is also one of the most important signals used by organisms to obtain information about the surrounding environment (Li et al., 2009). While progress has been made for various mechanisms defining T. pseudonana physiology, the molecular mechanisms controlling diatom responses to light are still largely unknown (Depauw et al., 2012). While this is surprising, it further highlights the uniqueness of diatoms and the importance for understanding mechanisms that control their productivity, especially given their abundance and richness in aquatic environments across the globe. There is much more work to be done on those phytoplankton species that have been sequenced already and more effort should be put into sequencing other phytoplankton species to compare genomic information. Such comparisons will provide further insights and potentially provide predictive indicators for which species will be better adapted for the imminent changes ahead. Unveiling the mechanisms responsible for major processes, such as responses to resource limitation can be a slow process but combining detailed culture studies with next-generation sequencing approaches for different diatom species can provide a more holistic view of photosynthetic metabolism than currently exists.
Research Objectives:

This research study is unique in that it explores cell physiology over a wide range of steady-state light limited growth rates. Ensuring cells are in steady state growth is essential for unambiguous assessment of photoacclimation (Beardall and Morris, 1976). We assessed the use of total energy available to cell from the harvesting of light at PSII through the splitting of water, to the various forms of production ultimately leading to growth, measured as biomass. An important take home message is that whether diatoms are growing in low or high light limiting environments they are able to adjust their photosynthetic machinery and downstream carbon metabolism to maintain a very high efficiency of photosynthesis to support growth. The objectives for this research were (1) to understand how the marine diatom, *T. pseudonana*, allocates photosynthetic energy to metabolic process under a wide range of light limited growth rates and (2) to compare the results to existing data on photosynthetic energy allocation strategies under nutrient limitation. A long-term goal is to incorporate these measurements of photosynthetic energy allocation into the next generation of ecological models to estimate global primary production rates under varying environmental conditions.
Chapter 3 – Materials and Methods

Culture Conditions:

*Thalassiosira pseudonana* (Hustedt) Hasle et Heimdal CCMP 1355 was grown in 300 ml continuous culturing systems at 18°C using modified f/2+Si medium supplemented with Na$_2$SeO$_3$ at 10$^{-3}$M concentration from the recipe described by Guillard (1975). Data were collected from at least three independent continuous cultures grown at each growth irradiance. Growth rates that were defined by the growth irradiance was accomplished by growing batch cultures at the growth irradiance and establishing growth curves to determine growth rates at the light intensity they were grown. Then a peristaltic pump was set at a rate to match the growth rate predetermined by the light intensity so media was administered at the appropriate rate to maintain balanced growth. To maintain the continuous culture, fresh media input and outflow was set to match the light-limited growth rate according to the equation:

$$\mu = \frac{D}{V}$$

where $\mu$ is the specific growth rate (day$^{-1}$), D is dilution rate (ml day$^{-1}$), and V is culture volume (which was always 300 ml). Nitrate and phosphate concentrations in the media reservoir were 250 and 50 $\mu$M, respectively (Laws and Bannister, 1980). The growth irradiances used for this study were 200, 60, and 5 $\mu$mol photons m$^{-2}$ s$^{-1}$ supplied by cool-white fluorescent tubes termed ‘High’, ‘Medium’, and ‘Low’, respectively over the course of this paper. Light intensity was measured with a quantum meter (Biospherical Instruments QSL-100) with a 4π spherical quantum sensor. Cultures were continuously bubbled to keep cells in suspension to mimic vertical mixing, maintain a constant supply
of CO₂, and keep the cells exposed to the same average light intensity. All cultures were acclimated to their growth irradiances for at least 10 generations. Their steady-state statuses were verified by measurement of cell concentration that varied <5% over 3 days before performing experiments. Cell concentration, diameter, and volume were measured using a Multisizer 3 Coulter Counter equipped with a 100 µm aperture tube (Beckamn Coulter; Miami, FL).

Culture Characteristics:

Chlorophyll (Chl) concentrations (taken in triplicate) were determined from filtering 4-6 ml from all cultures onto a 25 mm glass fiber filter (Whatman GF/F) that was extracted overnight at -20°C in 90% acetone. A spectrophotometer was used to measure the absorptivity of the extract at wavelengths ranging from 400-700 nm and Chl was quantified according to the equation from Jeffrey and Humphrey (1975).

The spectrally averaged cross-sectional area (a*) for cells grown at each light level was measured by the filter pad method with corresponding path length corrections (Mitchell et al 2003). A 10 ml sample of culture was filtered onto a GF/F and then measured spectrophotometrically by directly exposing the filter to the spectral range (400-700 nm). We found no significant changes in a* determined for the different light sources used in collection of photosynthetic irradiance (PE) data (i.e., Eiko 2450 ELH 300W 120 V tungsten halogen bulbs were used in both the photosynthetron and for illumination of the culture chamber during O₂ production experiments, see below; data not shown). All production measurements were normalized to absorbed light (Falkowski
et al., 1985) to account for light source spectrum differences between instruments using the equation

\[ P^* = P \cdot (C_hl \cdot I_g \cdot \bar{a}^*)^{-1} \]

where \( P^* \) is the photosynthetic efficiency, \( P \) is the oxygen or carbon production rate, \( I_g \) is the growth irradiance, and \( \bar{a}^* \) is the spectrally averaged cross-sectional area.

Cellular carbon and nitrogen were measured using 2, 3, and 4 ml samples of culture filtered onto combusted GF/F filters and analyzed using an Exeter Analytical EA1 elemental analyzer (Coventry, England) generating a linear relationship between C and N and filtered volume. A filter “blank” was included using 4 ml of the culture filtrate that was re-filtered onto a precombusted GF/F filter. The “blank” values were subtracted from sample mass values.

\[ NPP = \frac{\mu \cdot C}{C_hl} \]

Production Measurements:

Photosynthesis-irradiance (PE) curves were generated from short-term (20 min) \(^{14}\text{C}\)-uptake experiments. 5 ml culture sample was diluted with 8 ml f/2+Si media then spiked with 5 \( \mu \text{Ci} \) NaH\(^{14}\text{CO}\). The \(^{14}\text{C}\)-spiked sample was aliquoted to 14-6 ml scintillation vials and exposed to 10 light intensities (0-1500 \( \mu \text{mol} \) photons \( m^{-2} \) \( s^{-1} \) from two tungsten projector bulbs; Lewis and Smith 1983) using a photosynthetron (CHPT Mfg Inc; Georgetown, DE) for 20 min at 18°C. Light intensities were measured using a 4\( \pi \) PAR sensor. After incubation, all samples were acidified with 10% HCl and allowed
to degas overnight. To measure total activity of the radioactive label added to the samples, two 50 µl subsamples of the inoculated sample were added to 50 µl phenethylamine and 900 µl H₂O. Five ml Ecoscint-A were added to all samples and ¹⁴C incorporation was measured using a scintillation counter. PE curves were modeled using a hyperbolic tangent model estimating Pₘₐₓ, α, and Iₙ (Jassby and Platt, 1976).

To quantify carbon excretion rates (DO¹⁴C), 30.2 ml culture sample was inoculated with 10 µCi NaH¹⁴CO, and aliquoted into six-7 ml scintillation vials. Three of the vials were wrapped with foil for “dark” and three were incubated at the growth irradiance. All samples were kept at 18°C for 24h. Two subsamples were collected and measured for total activity of radioactive label added using the procedure described above. After 24h, all samples were carefully filtered through GF/F filters. The filtrate was acidified with 10% HCl and degassed overnight. To limit light contamination, lights were dimmed during filtering. DO¹⁴C was quantified by scintillation counter.

¹⁴C pulse labeling experiments were done to measure incorporation of labeled C into different macromolecular pools. Culture samples (60.2 ml) were spiked with 15 µCi NaH¹⁴CO. This solution was aliquoted into three-20 ml scintillation vials and incubated at the growth irradiance at 18°C for 20 min. Following the 20 min incubation, each sample was filtered onto a GF/F filter, rinsed three times with 10 ml unlabeled f/2+Si media, then frozen at -20°C for later extraction, fractionation, and quantification following methods of (Smith and Geider, 1985). Two activity subsamples were collected and measured using the procedure described above. Filters were thawed under the flow hood overnight then 1.5 ml 2:1 v/v chloroform:methanol was added and the sample was extracted at -20°C for 12-24 h. The extracted sample was filtered through a GF/F then the
filter was rinsed twice with 2:1 chloroform:methanol. One ml dH$_2$O was added to the filtrate before centrifuging at 1400 rpm (Eppendorf 5418, USA Scientific) for 5 min. The top layer containing the lipid fraction was separated from the lower layer containing the aqueous/alcohol solution. The lipid layer was transferred to a scintillation vial and 10 ml Ecoscint-A was added then counted by scintillation counting. The filter along with the original filter from above were placed into a scintillation vial and extracted in 2 ml 5% trichloroacetic acid (TCA) at 80°C for 60 min. This hot TCA extract was filtered through another GF/F and rinsed 2x with 2 ml TCA leaving 3 filters in one vial containing the protein fraction. The filtrate was collected in a separate vial and allowed to evaporate to about 2 ml. This fraction contained the carbohydrate and some nucleic acids. Ecoscint-A was added to all fractions prior to scintillation counting.

Cellular protein and carbohydrate assays supplemented 20 min pulse-label experiments to determine biomass macromolecular composition. For protein assays, 8-10 ml culture was filtered in duplicate on a 25 mm Durapore PVDF membrane filter (Merck Millipore, Tullagreen, Ireland) then stored at -80°C for later extraction. Protein was extracted following the protocol of the Thermo Scientific Pierce Micro BCA (bicinchoninic acid) Protein Assay Kit (Thermo Fisher, Eugene, OR, USA). Standards were measured using bovine serum albumin.

For carbohydrate determination, 20-30 ml culture was filtered in duplicate onto a combusted GF/C filter and stored at -20°C for later extraction. Following the method of Granum, Kirkvold, and Myklestad 2002, filters were thawed then incubated in 5 ml 0.05M H$_2$SO$_4$ for 20 min at 60°C. Extracts were filtered onto a GF/C to collect cellular β-1,3-glucan and stored at 4°C. The original filter containing cell wall polysaccharide was
washed with H$_2$O and then dried at 60°C overnight. After drying, the polysaccharide was hydrolyzed using 0.5 ml 80% H$_2$SO$_4$ at 0-4°C for 20 h, 6 ml ice cold H$_2$O was added, and the solution was filtered onto another GF/C to collect cell wall hydrolyzate. Due to high cell concentration, both cellular and cell wall hydrolyzate samples were diluted by 4 fold so the starting solution was 500 µl sample and 1.5 ml H$_2$O for a total of 2 ml. To this solution, 500 µl 3% aqueous phenol was added then 5 ml concentrated sulfuric acid was added (Dubois et al. 1956). Each sample was stirred and allowed to stand for 30 min before measuring absorption. Carbohydrate samples were analyzed spectrophotometrically at a wavelength of 485 nm. Standards were measured using analytical grade D-(+)-glucose (Supelco, Bellefonte, PA, USA). To compare with 20 min $^{14}$C-labeled biofractionation data, we combined cell wall and cellular β-1,3-glucan fractions to yield total carbohydrate fractions.

We used membrane inlet mass spectrometry (MIMS) and $^{18}$O$_2$ to simultaneously quantify gross O$_2$ production, net O$_2$ production, and light-dependent respiration (LDR) (Halsey et al. 2010, 2013). 150-200 ml culture sample was concentrated to approximately 5 ml using a 0.1 µm polycarbonate membrane filter with a 47 mm GF/F filter underneath to minimize cell breakage. Cells were also kept suspended during the filtering to avoid cell damage. The concentrated sample and the $^{18}$O$_2$ tracer (final concentration of 40-70 uM) were added to a 5 ml Dubinsky chamber (Dubinsky et al. 1987) mounted to the membrane inlet system while continuously stirred at ~8 Hz using a magnetic stir bar and incubated at 20°C. The chamber was attached to a Prisma QMS-200 (Pfeiffer) quadrapole mass spectrometer with a closed ion source and electron multiplier detector for recording mass/charge (m/z) ratios of 32 ($^{16}$O$_2$), 36 ($^{18}$O$_2$), and 40 (Ar). All sample incubations
began with a 5 min dark period to confirm highly similar rates of $^{16}\text{O}_2$ and $^{18}\text{O}_2$ consumption in the dark. The suspension was exposed to a set range of target light levels (4 min per light level; attenuated by neutral density filters) that was selected for cultures grown under each of the three light intensities. The sets of light ranges were as follows: For cultures grown at the lowest light intensity the concentrated culture was exposed to approximately 3, 5, 7, 12, 20, 50, 100, and 200 $\mu$mol photons m$^{-2}$ s$^{-1}$. For cultures grown at the medium light intensity light exposures were approximately 5, 20, 50, 60, 70, 100, 250, and 650 $\mu$mol photons m$^{-2}$ s$^{-1}$, and for cultures grown at the highest light intensity light exposures were approximately 12, 20, 60, 150, 200, 250, 650, and 1500 $\mu$mol photons m$^{-2}$ s$^{-1}$. PE curves for gross and net $\text{O}_2$ were modeled using a hyperbolic tangent (Smith and Platt, 1984). At the completion of each experiment, the sample added to the chamber was collected for measurement of chlorophyll concentration. Gross $\text{O}_2$ measurements were determined from $^{16}\text{O}_2$ and $^{18}\text{O}_2$ signals in the light subtracting dark values. Net $\text{O}_2$ measurements were collected from $^{16}\text{O}_2$ signals, which were ascertained from the amount of oxygen being produced by the sample in the chamber when exposed to the various intensities of light. Light dependent respiration (LDR) measurements were collected from the difference in $^{18}\text{O}_2$ signals in the light and dark. To correct for changes in isotope dilution throughout the experiment, rates of oxygen production and consumption were calculated by dividing the observed rates by the fraction of $^{16}\text{O}_2$ or $^{18}\text{O}_2$ present during each light exposure.

Because the cell densities of samples added to the Dubinsky chamber varied between experiments, we conducted dilution experiments to establish relationships between chlorophyll concentrations and the light intensities that would be experienced by
the cells during the MIMS experiments. Furthermore, because Chl concentration per cell varied so dramatically depending on light limited growth rate, we carried out the dilution experiment for cells grown at each of the three growth irradiances. First, cultures were reduced in volume by filtration to a cell concentration that exceeded that used in MIMS experiments. After transfer to the chamber, light levels associated with each neutral density filter [selected for each light range set (High, Medium, and Low; see above)] were measured using a 4-π sensor inserted into the chamber. The concentrated cell suspension was subsequently diluted 1:2 and light levels associated with each neutral density filter were re-measured. This process was repeated four times and resulted in a set of equations for each neutral density filter and cells grown at high, medium, and low light intensities. These equations were used to determine the actual light exposures associated with final chlorophyll concentrations used in the MIMS experiments. For this reason, the light intensities for each neutral density filter used to expose cells grown at each light level, varied slightly between independent experiments.

The fraction of gross photosynthesis used directly for NO$_3^-$ and SO$_4^{2-}$ reduction ($DU_{NS}$) was calculated using cellular N from the CHN analysis and an assumed N:S ratio of 16:1.3 (Ho et al. 2003).

\[
DU_{NS} = \left[ \left(2 \ast \mu \ast (N + S) \right) + GP_{O2} \right] \ast (GP_{O2})^{-1}
\]

where 2 is a factor derived from the eight electrons required to reduce NO$_3^-$ or SO$_4^{2-}$ and four electrons released per O$_2$ molecule formed from the water-splitting reaction at PSII. $GP_{O2}$ is gross oxygen production as described above. $DU_{NS}$ values were used to calculate gross carbon fixation ($GP_c$) with the equation

\[
GP_c = (GP_{O2} - LDR) \ast (DU_{NS})^{-1}
\]
DU_{NS} values were also used to convert net oxygen production rates (NP_{O2}) to carbon units (NP_{O2/C}) to allow comparisons between GP_{C}, measures of net oxygen production and consumption (mitochondrial respiration, MR), and NPP_{C} following the equation

\[ NP_{O2/C} = (NP_{O2}) \times (DU_{NS})^{-1}. \]

Variable fluorescence \([F_v/F_m = (F_m - F_o)/F_m]\) was determined using fast-repetition-rate fluorometry [FRRf; (Kolber et al. 1998)] after samples were dark incubated for 5 min prior to initial fluorescence (F_o) and maximal fluorescence (F_m) measurements. F_o is the chlorophyll fluorescence when functional PSII reaction centers are fully oxidized and F_m is the chlorophyll fluorescence when the reaction centers are reduced. Sigma (\(\sigma_{PSII}\)) was also recorded to monitor the functional antennae size of PSII.
Chapter 4 – Results

Steady state continuous cultures of *Thalassiosira pseudonana* were grown at 5, 60, and 200 µmol photons m\(^{-2}\) s\(^{-1}\) generating specific growth rates of 0.2, 0.85, and 1.54 d\(^{-1}\), respectively (Table 1). Chl concentration increased 3.8 fold from 6.27 µg cell\(^{-1}\) in low light acclimated cultures to 1.64 µg cell\(^{-1}\) in high light acclimated cultures. This increase in cellular chlorophyll is a common characteristic of photoacclimation and was also reflected in the strong increases in Chl to carbon ratios with decreasing light limited growth rate. The near constancy of the C:N ratio across growth rates confirmed that nitrogen was not limiting, thus all behaviors measured could be assigned to physiological responses to light limitation.

Chlorophyll-specific net primary production (NPP\(^*\)) values increased with growth rate reflecting the strong influence of Chl at low growth rates. This physiological flexibility is also shown in the magnitude of change in NPP\(^*\) with light availability. NPP\(^*\) increased 12-fold between cells grown at the lowest light limited growth rate and cells grown at the fastest light limited growth rate. This increase in NPP\(^*\) mirrored the 12-fold increase in their growth irradiances. Furthermore, the 3-fold change in NPP\(^*\) between
Table 1: Steady-state physiological characteristics of *Thalassiosira pseudonana* grown at various growth irradiances (values in parentheses are SE for three independent continuous cultures)

<table>
<thead>
<tr>
<th>Growth Rate (day⁻¹)</th>
<th>Light Intensity (µmol photons m⁻² s⁻¹)</th>
<th>Cells ml⁻¹ (x 10⁶)</th>
<th>Cell Size (µm)</th>
<th>Chl a cell⁻¹ (µg cell⁻¹ x 10⁻⁷)</th>
<th>Chl C⁻¹ (µg µg⁻¹ x 10⁻²)</th>
<th>C cell⁻¹ (pg)</th>
<th>N cell⁻¹ (pg)</th>
<th>C:N</th>
<th>NPP⁺ (µmol C (mg Chl h⁻¹))</th>
<th>Fv/Fm</th>
<th>Sigma (σₚₛₚ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.20</td>
<td>5</td>
<td>1.43 (0.14)</td>
<td>4.04 (0.07)</td>
<td>6.27 (0.65)</td>
<td>8.25 (0.57)</td>
<td>8.34 (0.66)</td>
<td>1.18 (0.16)</td>
<td>7.07 (0.49)</td>
<td>8.86 (0.87)</td>
<td>0.58 (0.002)</td>
<td>567 (8.37)</td>
</tr>
<tr>
<td>0.85</td>
<td>60</td>
<td>2.21 (0.37)</td>
<td>4.24 (0.06)</td>
<td>1.92 (0.20)</td>
<td>2.71 (0.20)</td>
<td>7.90 (0.49)</td>
<td>1.02 (0.26)</td>
<td>7.73 (0.37)</td>
<td>115 (9.81)</td>
<td>0.56 (0.02)</td>
<td>545 (25.7)</td>
</tr>
<tr>
<td>1.54</td>
<td>200</td>
<td>1.56 (0.05)</td>
<td>4.59 (0.05)</td>
<td>1.64 (0.08)</td>
<td>1.76 (0.21)</td>
<td>8.51 (0.19)</td>
<td>1.21 (0.18)</td>
<td>7.02 (0.23)</td>
<td>279 (24.6)</td>
<td>0.56 (0.03)</td>
<td>497 (5.43)</td>
</tr>
</tbody>
</table>
cultures grown at 0.85 and 1.54 d⁻¹ matched the 3-fold increase in their growth irradiances. The strong decrease in NPP* with decreasing light limited growth rate was not associated with any change in variable fluorescence (Fv/FM). Invariant Fv/FM across light limited growth rates indicated that cells were fully acclimated to their growth irradiances, and photosynthetic efficiency was maintained at maximal rates at all light limited growth rates. Maintenance of Fv/FM was accompanied by σPSII that only decreased slightly with increasing growth irradiance.

Chl-specific gross oxygen production (GPP*O₂) was measured using membrane inlet mass spectrometry (MIMS) with ¹⁸O₂ as a tracer of respiration (see Methods). Figure 4 shows the PE curves for GPP*O₂ for cells grown at each light limited growth rate. Maximum rates of GPP*O₂ (P*max) strongly increased with light limited growth rate, but α* remained constant (Table 2). These behaviors in the PE parameters are characteristic of Eₖ-dependent variability. GPP*O₂ measured at the growth irradiance (P*Ig) also increased with growth rate (Fig 6 and Table 2).
Fig. 6 Gross O₂ production measurements for steady-state light limited *T. pseudonana* continuous cultures. High light cultures, 200 µE (filled circles); medium light cultures, 60 µE, (open circles); and low light cultures, 5 µE, (filled triangles). Data points are shown for triplicate independent continuous cultures grown at each light level. Slight differences in cell concentrations following concentration of cultures for measurements using MIMS cause variations in light exposures for each neutral density filter. These differences in cell concentration were accounted for as described in Methods. Model fits are shown as solid lines for each light limited growth rate. The dashed arrows highlight the oxygen production value at the growth irradiance (Pᵣ) for each light limited culture.
LDR increased from 1.71 µmol O\textsubscript{2} (mg Chl h\textsuperscript{-1}) in cells growing at 0.2 d\textsuperscript{-1} to 21.62 µmol O\textsubscript{2} (mg Chl h\textsuperscript{-1}) in cells growing 1.54 d\textsuperscript{-1}. These rates represent an increase in the fraction of GPP\textsuperscript{*}\textsubscript{O2} used for LDR from 5% at the fastest light limited growth rate to 12% at the slowest light limited growth rate.

Figure 7 shows the PE curves for Chl-specific net O\textsubscript{2} production (NP\textsuperscript{*}\textsubscript{O2/C}) for cells grown at each light limited growth rate. Similar to the results for GPP\textsuperscript{*}\textsubscript{O2}, maximal rates of NP\textsuperscript{*}\textsubscript{O2/C} strongly increased with light limited growth rate. Although the PE parameters for net O\textsubscript{2} production, P\textsuperscript{*}\textsubscript{max} and P\textsuperscript{*}\textsubscript{Ig}, increased strongly with growth rate, α\textsuperscript{*} slightly decreased at the lowest growth irradiance (Table 2). Interestingly, E\textsubscript{k} determined from the NP\textsuperscript{*}\textsubscript{O2/C} PE data was a relatively close match to the culture growth irradiances (Table 2). NP\textsuperscript{*}\textsubscript{O2/C} measured in the dark is mitochondrial respiration (MR). MR increased from 16 µmol C (mg Chl h\textsuperscript{-1}) in cells growing at 0.2 d\textsuperscript{-1} to 116 µmol C (mg Chl h\textsuperscript{-1}) in cells growing 1.54 d\textsuperscript{-1}. 
Fig. 7 Net O$_2$ production measurements, converted to C units (see Methods), for steady-state light limited *T. pseudonana* continuous cultures. High light cultures, 200 μE (*filled circles*); medium light cultures, 60 μE (*open circles*); and low light cultures, 5 μE (*filled triangles*). Slight differences in cell concentrations following concentration of cultures for measurements using MIMS cause variations in light exposures for each neutral density filter. These differences in cell concentration were accounted for as described in Methods. Model fits are shown as solid lines for each light limited growth rate.
Table 2: PE curve parameters from GPP*_{O2}, NP*_{O2/C}, and 20 min Chl-specific ^14C-uptake experiments obtained for *T. pseudonana* under three light limited steady state growth rates. (values in parentheses are SE for at least three independent cultures.)

<table>
<thead>
<tr>
<th>Growth Irradiance (μE)</th>
<th>GPP*_{O2}</th>
<th></th>
<th></th>
<th>NP*_{O2/C}</th>
<th></th>
<th></th>
<th>Chl-specific ^14C Uptake (20 min)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>200</td>
<td>60</td>
<td>5</td>
<td>200</td>
<td>60</td>
<td>5</td>
<td></td>
<td>200</td>
<td>60</td>
</tr>
<tr>
<td>P_max</td>
<td>751</td>
<td>281</td>
<td>111</td>
<td>692</td>
<td>247</td>
<td>82.3</td>
<td>610</td>
<td>245</td>
<td>67.5</td>
</tr>
<tr>
<td></td>
<td>(35.7)</td>
<td>(9.59)</td>
<td>(6.58)</td>
<td>(41.7)</td>
<td>(8.44)</td>
<td>(5.91)</td>
<td>(25.3)</td>
<td>(12.0)</td>
<td>(2.10)</td>
</tr>
<tr>
<td>α</td>
<td>3.21</td>
<td>3.39</td>
<td>2.94</td>
<td>2.78</td>
<td>2.94</td>
<td>2.45</td>
<td>2.33</td>
<td>1.77</td>
<td>1.30</td>
</tr>
<tr>
<td></td>
<td>(0.28)</td>
<td>(0.31)</td>
<td>(0.36)</td>
<td>(0.30)</td>
<td>(0.27)</td>
<td>(0.36)</td>
<td>(0.15)</td>
<td>(0.20)</td>
<td>(0.14)</td>
</tr>
<tr>
<td>E_K</td>
<td>234</td>
<td>82.7</td>
<td>37.8</td>
<td>249</td>
<td>83.8</td>
<td>33.6</td>
<td>262</td>
<td>138</td>
<td>52.1</td>
</tr>
<tr>
<td></td>
<td>(70.9)</td>
<td>(28.4)</td>
<td>(13.9)</td>
<td>(81.5)</td>
<td>(25.0)</td>
<td>(12.9)</td>
<td>(43.2)</td>
<td>(31.4)</td>
<td>(7.2)</td>
</tr>
<tr>
<td>r²</td>
<td>0.94</td>
<td>0.93</td>
<td>0.87</td>
<td>0.91</td>
<td>0.93</td>
<td>0.81</td>
<td>0.96</td>
<td>0.90</td>
<td>0.94</td>
</tr>
</tbody>
</table>
Figure 8 shows PE curves for short-term (20 min) Chl-specific $^{14}$C-uptake measurements. Once again, these results showed a strong increase in the maximal rate of Chl-specific $^{14}$C-uptake ($P_{\text{max}}^*$) with light limited growth. However, in contrast to the results for GPP$^*_\text{O}_2$ and NP$^*_\text{O}_2/C$, $^{14}$C-uptake $\alpha^*$ significantly increased with light limited growth rate. Despite the parallel trends in $P_{\text{max}}^*$ and $\alpha^*$, the shifts in these PE parameters were insufficient to overcome the $E_k$-dependent variation driven by photoacclimation. Nevertheless, the range of $E_k$ became increasingly constrained from GPP$^*_\text{O}_2$ to NP$^*_\text{O}_2/C$ to short-term $^{14}$C-uptake (Table 1).
Fig. 8 Short-term (20 min) carbon fixation measurements using $^{14}$C-labeled sodium bicarbonate for steady-state light limited *T. pseudonana* continuous cultures. High light cultures, 200 μE (filled circles); medium light cultures, 60 μE (open circles); and low light cultures, 5 μE (filled triangles). These PE curves show higher carbon fixation rates and $P_{\text{max}}$ with increasing growth irradiance while $a$ decreased slightly across all light limited growth rates. These properties are indicative of $E_{k}$-dependent variability (Behrenfeld et al. 2004).
Figure 9 shows the relationships between $P_{Ig}^*$ for $GPP_{O2}^*$, $GP_{C}^*$, $NP_{O2/C}^*$, and $NPP_{C}^*$ across all three light limited growth rates. These relationships were used to quantify energy allocation to major metabolic sinks. The dramatic increase in Chl-specific production with growth rate (Fig 9A) makes it difficult to compare energy allocation strategies between growth rates. Therefore, we normalized each of the four $P_{Ig}^*$ values to $GPP_{O2}^*$-$P_{Ig}^*$ for each of the light limited growth rates to show the fraction of $GPP_{O2}^*$ allocated to each of the major metabolic sinks (Fig 9B). The majority of energy derived through photosynthesis was retained as $NPP_{C}^*$ across all growth rates (purple areas). At the lowest growth rate 57% of the $GPP_{O2}^*$ was retained as $NPP_{C}^*$. The fraction of energy retained as biomass increased to ~65% in cells growing at the lowest and fastest growth rates and to 80% in cells growing at the medium growth rate. A constant 5% of $GPP_{O2}^*$ was used directly for the reduction of nitrate and sulfate (N+S; light blue areas) across all growth rates. The remaining energy was differentially allocated depending on the light limited growth rate. The fraction of energy used for LDR and MR was highest in cells growing at the lowest growth rate whereas the fraction of energy used for investment into reductant regeneration for biosynthesis was highest in cells growing at the fastest growth rate. MR in Fig 4 is the difference between $P_{Ig}^*$-$GP_{C}^*$ and $P_{Ig}^*$-$NP_{O2}^*$. These values were around 4-fold smaller than measured MR values in the dark for medium and fastest growth rates and about 14-fold smaller for slowest growth rates (Table 3).
Fig. 9 Summaries of energy allocation for at least three independent steady-state light limited *T. pseudonana* continuous cultures. (A) The portion of energy allocated to major metabolic sinks at light limited steady-state growth rates. The brackets to the right show which metabolic sinks correlate to the production measurements made during the experiment and help understand when products are in terms of O\textsubscript{2} or C units. GP\textsubscript{c} fraction encompasses MR, reductant, and NPP\textsuperscript{*}c. (B) Fraction of GPP\textsubscript{O2} allocated to major metabolic sinks in steady-state light limited *T. pseudonana* continuous cultures. For all growth rates, the majority of energy is retained as net growth (NPP\textsuperscript{*}c, purple bar). A constant 0.05 fraction of GPP\textsubscript{O2} is used directly for the reduction of nitrate and sulfate (N+S, light blue bar). The remaining energy is allocated to light dependent respiration (LDR, dark blue bar), mitochondrial respiration (MR, red bar), and reductant for biosynthesis (Reductant, green bar) in proportions that vary with light limited growth rate.
Table 3: Summary of energy allocation budget distribution of photosynthate into metabolic pools for *T. pseudonana* grown at different light limited steady-state growth rates. (values in parentheses are SE for at least three independent cultures.)

<table>
<thead>
<tr>
<th>Specific Growth Rate</th>
<th>GPP*&lt;sub&gt;O2&lt;/sub&gt;</th>
<th>LDR*</th>
<th>Chl-specific photosynthate used for D&lt;sub&gt;U&lt;/sub&gt;&lt;sub&gt;NS&lt;/sub&gt;</th>
<th>GP*&lt;sub&gt;C&lt;/sub&gt;</th>
<th>Chl-specific MR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Chl-specific C used for reductant for biosynthesis&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NPP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>day&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>µmol O&lt;sub&gt;2&lt;/sub&gt; (mg Chl h)&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>µmol C (mg Chl h)&lt;sup&gt;-1&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.20</td>
<td>13.8 (13.9)</td>
<td>1.71 (0.01)</td>
<td>1.06 (0.006)</td>
<td>11.1 (13.9)</td>
<td>1.24 (0.85)</td>
<td>1.34 (12.9)</td>
<td>8.86 (0.87)</td>
</tr>
<tr>
<td>0.85</td>
<td>145 (28.4)</td>
<td>5.57 (1.77)</td>
<td>1.06 (0.01)</td>
<td>132 (28.5)</td>
<td>19.5 (7.40)</td>
<td>2.89 (26.9)</td>
<td>115 (9.81)</td>
</tr>
<tr>
<td>1.54</td>
<td>431 (70.9)</td>
<td>21.62 (3.00)</td>
<td>1.05 (0.004)</td>
<td>390 (71.0)</td>
<td>31.1 (6.45)</td>
<td>79.5 (85.1)</td>
<td>279 (24.6)</td>
</tr>
</tbody>
</table>

GPP*<sub>O2</sub> is the sum of metabolic pools in columns 3, 4, and 5, and GP*<sub>C</sub> is the sum of metabolic pools in columns 6, 7, and 8. Values represent the means of measured rates from at least three independent steady-state continuous cultures. The high SE for low light measurements are due to the light levels before the growth irradiance at that intensity thus there are fewer measurements to account for any errors that may occur at such low light.

<sup>a</sup> Chl-specific MR = GP*<sub>C</sub> – NPP*<sub>O2/C</sub>

<sup>b</sup> Chl-specific C used for reductant for biosynthesis = NPP*<sub>O2/C</sub> – NPP*
Another potential metabolic pathway for photosynthetic energy use is carbon that is excreted from cells. We measured the rate of DO\(^{14}\)C accumulation in the filtrate of cultures incubated with NaH\(^{14}\)CO\(_3\) at the growth irradiance for 24 h. Across all growth rates, less than 2% of GPP\(^*\)\(_{O2}\) was lost as DO\(^{14}\)C (data not shown). Errors in the measurement of DO\(^{14}\)C could have arisen from some DO\(^{14}\)C getting trapped on the filter during filtering. However, rinsing the filter several times with unlabeled media and measuring the PO\(^{14}\)C minimized this potential source of error. The PO\(^{14}\)C values should match NPP\(_c\) but were lower across all light limiting growth rates suggesting that there was not the influence of trapped DO\(^{14}\)C skewing our results.
Fig. 10 Carbon allocated to macromolecular pools shifts dramatically over 24 h. (A) Light limited cells were incubated at their growth irradiances with NaH\(^{14}\)CO\(_3\) for 20 min and biofractionated into carbohydrate, protein, and lipid pools. (B) Total protein and carbohydrate (sum of cellular and cell wall fractions) were measured in cultures grown at each light limited growth rate. The lipid fraction was estimated by taking the difference in total cellular carbon (Table 1) and the sum of the biomass protein and carbohydrate fractions (blue and red bars in (B)). The values above each cluster of bars for both graphs represents the reduced C to oxidized C ratio (C\(_r\)/C\(_o\)) highlighting energy allocation trends to more reduced carbon forms in biomass especially for the medium and lower light limited growth rates.
Carbon allocation to different macromolecular pools varied dramatically depending on time-scale of the measurement. \(^{14}\)C-pulse labeling (20 min) was conducted to trace newly fixed carbon into carbohydrates, proteins, and lipid pools (Fig 10A). There was a decrease in the amount of energy allocated to protein synthesis with decreasing light. Carbohydrate synthesis was highest for the highest light limited growth rate and lowest for cells growing at the medium light intensity. Across all light limited growth rates very little energy was initially allocated to the biosynthesis of lipids (Fig 10A).

The macromolecular composition of the biomass of light limited cells changed dramatically when compared to the 20-min \(^{14}\)C-pulse label results. In cellular biomass, the fraction of carbon allocated to protein increased strongly with decreasing growth rate (Fig 8B). Furthermore, the amount of biomass carbon allocated to the lipid fraction was markedly higher in the two faster growing cultures than in cells grown at the slowest growth rate.

The fraction of more reduced C (protein and lipids) to more oxidized C (carbohydrates) was calculated across all light limited steady-state cultures (\(C_r:C_o\)). A higher portion of reduced C forms in bulk samples across all light limited growth rates. While there was only a slight increase observed in the high light limited growth rates, medium and low \(C_r:C_o\) ratios increased by approximately 2-fold in bulk samples (Fig 10A/B).
Chapter 5 – Discussion

Photosynthetic energy use was studied in the diatom, *T. pseudonana*, across a wide range of steady state light limited growth rates. A collection of production measurements, including GPP*O₂*, LDR*, NPP*O₂*, and NPP*C*, was combined with information about macromolecular composition of newly fixed carbon and biomass giving insights into mechanisms diatoms use to tune their photosynthetic metabolism in response to light limitation. Across all light limited growth rates *T. pseudonana* adjusts its photosynthetic machinery and cellular metabolism to maintain high growth efficiencies. Our results reveal new insights about light-dependent shifts in energy allocation that can be linked to the life history of *T. pseudonana* and require careful consideration when interpreting variability in photosynthesis-light relationships.

All our data was normalized to chlorophyll because it a common normalization parameter in oceanography for primary production studies as it is easily measured in the field and detectable from space through satellite imaging of ocean color. This allows our data to be compared to many other previous studies with greater ease. However, there are some problems associated with normalizing data to chlorophyll. Chlorophyll is not a direct measure of biomass and, as this study shows, varies drastically with resource limitation where lower light conditions yields greater chlorophyll concentrations and the same trend is seen in higher nutrient environments (Halsey et al., 2011, 2013). As this study and others show, this higher production of chlorophyll under such regimes does not necessarily directly correlate to higher biomass. This confounds production measurements received from satellite data as the conditions of the environment these cells are measured is not taken into consideration. Regardless of these limitations
associated with normalizing data to chlorophyll, it is still common practice in the scientific community. A potentially better normalization parameter is carbon as this is directly correlated to biomass. Unfortunately measuring carbon is not as simple in the field as chlorophyll and cannot be measured by satellites. While normalizing our data to chlorophyll may have masked other behaviors we may see in energy allocation, the data was also normalized to carbon (data not shown) and revealed similar trends between light limited growth rates. What changed was the strong $E_k$-dependent variability in the PE curves indicative of photoacclimation and the maximal rate of photosynthesis ($P_{\text{max}}$) was less variable between light limiting growth rates.

Photoacclimation is a physiological response involving fine-tuning of cellular light harvesting apparatus to match the growth irradiance. Several commonly observed characteristics demonstrate that our light-limited continuous cultures were fully acclimated to their environment. The strong increase in Chl:C with decreasing growth rate and constant $F_{\text{V}}/F_{\text{M}}$ measured in our cultures are quantitatively similar to values observed by others (Laws and Bannister, 1980; Geider, 1997; Suggett et al., 2009; Bittar et al., 2013; Vandenhecke et al., 2015). Whereas Chl:C increased 4.75-fold, $\sigma_{\text{PSII}}$ only increased slightly with decreasing light-limited growth rate. Thus, to maintain maximal photosynthetic efficiency at low light-limited growth rates, cells increase the number of reaction centers per unit chlorophyll, but only minimally alter $\sigma_{\text{PSII}}$. Thus, at the level of the initial light harvesting reactions, the overall photoacclimation response involves major adjustments of Chl:C and reaction center numbers, but only minor changes in $\sigma_{\text{PSII}}$ resulting in maximal $F_{\text{V}}/F_{\text{M}}$. Behaviors in photosynthesis-irradiance (PE) relationships that are characteristic of photoacclimation [namely, constant light limited slopes ($\alpha^*$) and
variable maximal rates of photosynthesis ($P_{\text{max}}^*$) were observed at the level of PSII but not when PE curves were generated from short-term $^{14}\text{C}$-uptake rates. $E_k$-dependent variability is commonly associated with photoacclimation where constant $\alpha^*$ and variable $P_{\text{max}}^*$ causes major changes in $E_k$ (the light saturation index). $E_k$-dependent variability was observed in the PE behaviors when measuring $O_2$ production (Table 2). However, when PE curves were generated using short-term $^{14}\text{C}$-uptake, $\alpha^*$ increased significantly with growth rate, albeit to a lesser extent than increases in $P_{\text{max}}^*$. Parallel changes in $\alpha^*$ and $P_{\text{max}}^*$ that result in no shifts in $E_k$ ($E_k$-independent variability) are now known to be associated with changes in carbon metabolism across a diel cycle and during nutrient-limited growth (Halsey et al. 2010, 2013). Growth rate dependent shifts in carbon metabolism were similarly observed in this study (see below), however the details of carbon metabolism across the cell cycle during light limitation have not yet been resolved. Nevertheless, the unexpected growth rate-dependent shifts in $\alpha^*$ measured in this study during light limited growth suggests that changes in carbon metabolism can contaminate PE relationships. Thus, variability in $E_k$ can be significantly dampened, especially when $^{14}\text{C}$-uptake is used to measure photosynthetic rates [compare ranges of $E_k$ for PE curves generated using oxygen production ($\text{GPP}^*_{\text{O}_2}$) and $^{14}\text{C}$-uptake, Table 2]. These results further complicate interpretations of PE curves that are commonly used for describing phytoplankton responses to environmental variables.

$T.\ \text{pseudonana}$ is highly efficient at converting light energy to biomass under light limiting conditions. Across all growth rates, $\text{GPP}^*_{\text{O}_2}:\text{NPP}^*_C$ was <2, compared to $\text{GPP}^*_{\text{O}_2}:\text{NPP}^*_C$ values ~3 where nitrogen was the limiting resource in diatoms and green algae (Halsey et al., 2013, 2014, Jakob et al., 2007). $\text{GPP}^*_{\text{O}_2}:\text{NPP}^*_C$ in light limited $T.$
pseudonana was 1.54, 1.26, and 1.56 in cells growing at 0.2, 0.85, and 1.54 d\(^{-1}\), respectively. Interestingly, a similarly low ratio was also measured in Phaeodactylum tricornutum when grown under light limited conditions, but the ratio increased to 2.82 when light became saturating (Wagner et al. 2006). GPP\(^*\)\(_{O2}\):NPP\(^*\)\(_C\) appears to be higher in phytoplankton from other taxonomic groups. GPP\(^*\)\(_{O2}\):NPP\(^*\)\(_C\) was \(\sim 3.0\) in a light limited green alga and cyanobacterium (Wagner et al. 2006,). Higher values >6 have been measured in a light limited cryptophyte with the decreased efficiency attributed to higher energy allocation to LDR processes (Kunath et al. 2012). Taken together these data suggest that diatoms have evolved a growth strategy that allows for a highly efficient light energy to biomass conversion when light is the limiting resource. This high-energy use efficiency may be a property that may reflect its adaptive life history. Diatoms are known to be particularly dominant during deeply mixing events (Leventer, 1991) and in upwelling coastal waters where light availability is saturating (MacIntyre et al., 2002; Gameiro et al., 2011).

These results also suggest that cells have different energy use efficiencies that operate depending on the limiting resource. Carefully controlled laboratory culture studies have revealed patterns of energy use that may be linked to adaptive physiology and growth conditions. For example, low GPP\(^*\)\(_{O2}\):NPP\(^*\)\(_C\) values appear to be associated with light limited diatoms (this study, Wagner et al., 2006). Similarly, low values were observed in a motile prasinophyte and were attributed to physiological trade offs in energy investment strategies where high pigment content was maintained to achieve high photosynthetic rates even under low background nutrient availability (Halsey et al. 2014). GPP\(^*\)\(_{O2}\):NPP\(^*\)\(_C\) appears to converge around values \(\sim 3\) under conditions of nutrient
limitation but more work needs to be done to understand the continued effects of light and nitrogen limitation (Jakob et al. 2007). Nevertheless, these studies demonstrate a potential framework for predicting physiological responses to environmental conditions given knowledge of the phytoplankton community.

Relationships between other measures of productivity are useful for understanding strategies phytoplankton use to optimize energy allocation. For example, $\frac{NP^*_{O_2}:NPP^*_C}{NP^*_{O_2}:NPP^*_C}$ quantifies energy used for biosynthesis of macromolecules that are more biochemically reduced than the initial product of carbon fixation; glyceraldehyde 3-phosphate (i.e. lipids, proteins). We found $\frac{NP^*_{O_2}:NPP^*_C}{NP^*_{O_2}:NPP^*_C}$ ranged from 0.98 to 1.29 across all light limited growth rates. These data were calculated using $NP_{O_2}$ rates converted to $C$ units. To compare with other studies that do not convert from $O_2$ to $C$ units, our $\frac{NP^*_{O_2}:NPP^*_C}{NP^*_{O_2}:NPP^*_C}$ ratios were 1.04 to 1.35. These values are remarkably similar to $\frac{NP^*_{O_2}:NPP^*_C}{NP^*_{O_2}:NPP^*_C}$ values that ranged from 0.95 to 1.4 in $P. tricornutum$ and $Chlorella vulgaris$, a green algae (Wagner et al. 2006 and Jakob et al 2007). Higher ratios, 1.75 and 2.21, were observed in a cyanobacterium and a cyrtophyte, respectively, (Kunath et al. 2012) suggesting species differences in the reduction state of biomass. For $T. pseudonana$, there was very little change in $\frac{NP^*_{O_2}:NPP^*_C}{NP^*_{O_2}:NPP^*_C}$ across all growth rates. This result is surprising because it is counter to our initial hypothesis that photoacclimation would increase demand for thylakoid membrane synthesis, and hence, increase energy allocated to biosynthesis of these energy-rich macromolecules at very low light-limited growth rates (Raven, 1984).

Although $\frac{NP^*_{O_2}:NPP^*_C}{NP^*_{O_2}:NPP^*_C}$ was constant across light limited growth rates, carbon metabolism was markedly different. Metabolic processing of carbon from our
biofractionation experiments that traced newly fixed carbon into carbohydrates, proteins, and lipids, together with biomass biofractionation experiments, allowed insight into how energy is allocated to different end products in *T. pseudonana* under light limited growth rates. From low to high light acclimated growth rates, protein trends completely reversed from increasing in short term biofractionation experiments to decreasing in biomass biofractionation experiments. Interestingly, it was originally hypothesized that under low light acclimation, cells would have invested more energy in the synthesis of higher energy carbon forms such as lipids (Raven et al., 1984). However, the results from this study show that under low light, *T. pseudonana* invested the least amount of energy into lipid biosynthesis compared to higher light acclimated cells, but instead slow growing cells allocated the majority of their long-term energy into protein synthesis (Fig. 8B). It was hypothesized by Richardson et al. (1983) that “a more efficient net conversion of CO₂, H₂O, NO₃⁻, and SO₄²⁻ into protein is unlikely to characterize a low light adapted unicell.” However, our results showed that low light acclimated cells allocated the majority of harvested energy to protein synthesis, and these cells also devoted a higher fraction of energy to protein when compared to cells grown at higher light limited growth rates.

The increasing proportion of energy allocated to protein with decreasing light limiting growth rates was also observed for nutrient limited cultures (Halsey et al., 2011). However, in contrast to nutrient limited cells in which Cᵢ:Cₒ increased 6.5 fold with growth rate (Halsey et al., 2011), our results showed Cᵢ:Cₒ decreased ~2 fold with light limited growth rate. Together these results suggest markedly different energy allocation
strategies depending on the limiting resource (light vs. nutrients) and also support the idea that $\alpha^*$ is not entirely insensitive to light limitation (see above).

It is often assumed that MR rates are equivalent in the dark and the light. However, our results that compare calculated values of MR at the growth irradiance (i.e., $GP^*_{C} - NP^*_{O2:C}$) and measured or modeled values of MR from the net oxygen loss rates in the dark during MIMS experiments (i.e., Fig 4, $O_2$ production at zero light) show that this assumption is not valid. Calculated MR values were consistently ~4 to 14-fold lower than measured values. This finding is consistent with some previous studies that observed enhanced post illumination respiration, although the basis for the difference in MR in the dark and light is not understood (Bate et al., 1988; Geider et al., 1985; Weger et al., 1989). In *P. tricornutum*, MR in the dark was about ten times larger than MR calculated from $NP^*_{O2}$ production derived at the compensation irradiance (Geider et al., 1985). As with that study, we also found that MR increased linearly with decreasing light limited growth rate (Fig 7), a result that reflects the increasing requirements for maintenance energy (ATP) at low growth rates. At high light limited growth rates, most of the energy generated from respiration is allocated for energy generation for cellular growth (Klipfus et al., 2012).

LDR may decrease reliance on carbon stores for ATP production. Similar to MR, our results showed that LDR also increased with decreasing growth rates (Fig 7). These data can help explain at least part of the previously unresolved differences between rates of mitochondrial respiration in the dark and light. Both MR and LDR serve to produce ATP for cell processes. Thus, by switching to LDR pathways for ATP production in the light, cells reduce their reliance on MR for their total ATP requirements.
Our results suggest that pathway gating between LDR and MR is especially important for cells such as diatoms with life histories that include deep mixing. A primary difference between LDR and MR is the source of electrons used to drive ATP synthesis. For MR, carbon oxidation produces NADH that is fed into the MR electron transport chain. In contrast, LDR occurs prior to carbon fixation by reduction of O\textsubscript{2} to H\textsubscript{2}O on the stromal side of the thylakoid membrane in the chloroplast to enhance the proton motive force for ATP generation (Asada, 1999). The sensitivity of LDR to light-limited growth rate suggests that LDR pathways are highly tuned to light availability, and LDR pathways supplement ATP production derived from MR. Switching from 100% MR in the dark to ~50% MR plus ~35% LDR in the light, when averaged among all light limited growth rates, is a form of pathway gating that we suggest is important to conserve carbon stores when light availability is scarce. Significant carbon stores are required to persist over prolonged periods of darkness (Geider et al. 2009; Talmy et al. 2013) such as seasonal deep mixing events. Switching to LDR preserves carbon resources for use during those prolonged dark periods.
Chapter 6 – Conclusion

This research is fundamental in adding to our knowledge on diatom physiology, in particular, how diatoms respond to a wide range of light limited growth rates. This work also provides a compelling comparison to similar studies that have measured photosynthetic energy allocation strategies of algae in response to nutrient limitation. We found that the majority of photosynthetic energy across all light limited acclimated growth rates was allocated to NPP$^*_c$ and a consistent fraction of 5% of total energy was used for nitrate and sulfate reduction. Differences that were observed between light acclimated growth rates included (1) a higher portion of energy that was allocated for LDR and MR at low light acclimated growth rates, (2) a higher fraction of energy that was invested into the biosynthesis of macromolecules at high light acclimated growth rates, and (3) $C_r/C_o$ of biomass was over two-fold higher in low light acclimated cells but remained nearly unchanged for higher light acclimated cells. The higher biochemical reduction state of low light acclimated cells was due to conservation of protein in these cells. Predictable photoacclimation behaviors were observed in Chl:C and some, but not all, PE parameters. It is worth highlighting that $\alpha^*$ increased with light limiting growth rate when photosynthesis was measured using $^{14}$C-uptake. Although the shifts in $\alpha^*$ were not sufficient to overcome $E_k$-dependent variability, the changes did significantly narrow the range of $E_k$ observed when compared with PE curves generated from $O_2$ production rates. Our results show that low light acclimated cells are highly efficient at converting harvested light energy into biomass. Understanding photosynthetic energy allocation strategies of different phytoplankton taxa and in response to different environmental
conditions is needed to link physiological traits with ecology to create more accurate ecological models estimating global primary production.

The next steps following this research are many but there are a few of particular interest that come to mind. The mechanisms driving substantial changes in mitochondrial respiration in the light and dark requires further investigation. Greater attention needs to be paid to tease out the contributions of MR and LDR in the light and dark. Such differences were not previously observed in comparable studies on phytoplankton during nutrient limitation. Another area of debate that was not thoroughly investigated in this research is the impact of pigment packaging. There was some evidence of pigment packaging (data not shown) in cells grown at the lowest light limited growth rate, but it did not appear to effect photosynthetic efficiency, so the question remains, “what is the consequence of pigment packaging?” It is a concept discussed repeatedly in the literature as a potential explanation for lower efficiencies seen at lower irradiances, but it remains a phenomenon whose significance is not well understood by the scientific community.

Applying this research to the field is another major future step. Research expeditions to the mid-Atlantic measuring primary production of natural assemblages across latitudinal and longitudinal transects over diel cycles will allow invaluable comparison to the laboratory data collection through this research. Field data that would compliment this research could include measurements of chlorophyll, profiling of the water column over the course of the day using \(^{14}\)C for various incubation periods, biofractionation of macromolecular pools over a diel cycle using \(^{14}\)C, and flow cytometry to retrieve community composition.
This study contributed new information about diatom ecophysiology. These results will help better understand how phytoplankton are able to respond, acclimate, and grow under environmental stresses such as light limitation. Still, there is much about phytoplankton physiology that remains unknown, a fact that makes this a fascinating field.
Chapter 7 – Bibliography


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