The Report Committee for Brian Christopher Byrwa
Certifies that this is the approved version of the following report:

Lipid Analysis of *Phaeodactylum tricornutum* in Response to Decadienal Stress

APPROVED BY
SUPERVISING COMMITTEE:

Supervisor: _____
Mona Mehdy

_____
K. Sathasivan

Lipid Analysis of *Phaeodactylum tricornutum* in Response to Trans Trans 2,4 Decadienal Stress

by

Brian Christopher Byrwa, B.S.

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Dedication

This work is dedicated to the memory of my great aunt, Dorothy Carol Shanfal. Without her support and love, none of this would have been possible. I would also like to dedicate this to my wife and beloved daughter for their unending support and patience while growing through this process.

Acknowledgements

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Abstract

Lipid Analysis of *Phaeodactylum tricornutum* in Response to Trans Trans 2,4 Decadienal Stress

Brian Christopher Byrwa M.A.

The University of Texas at Austin, 2011

Supervisor: Mona Mehdy

Considering the nature of increasing global temperatures associated with elevated atmospheric carbon dioxide levels as a result of increased demand for energy, it is notable to consider viable options to reduce the strain that these increased carbon dioxide emissions are having on the overall impact of the global climate. *Phaeodactylum tricornutum*, a marine phytoplankton may be utilized to this end. Its unique ability to increase lipid production under environmental stress conditions, in particular those lipids that can easily be converted into biodiesel, make it an ideal candidate for this use. Here, we examine the effects of trans trans 2,4 decadienal (or DD for short), an aldehyde that is known to induce cell death in the diatom at high concentrations, as they relate to changes in the lipid biosynthesis pathway. 100 ml Axenic cultures of the diatom *P. tricornutum* were grown to exponential stage, harvested and treated with decadienal at a concentration of 5µg/ml to determine effects on lipid production after 24 hours. Qualitative analysis

undertaken using Nile red staining of treated and untreated cells indicated increased fluorescence of treated cells compared to unstained water controls, however this increase may not be attributable to increased lipid production due to the fact that cells were unfixed and must be verified through other means. Initial attempts to verify this finding through thin layer chromatography and qPCR were inconclusive.

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Chapter 1: Introduction

In considering the exponential growth of the human population, and that the consequential energy demands needed to sustain this growth are supplied primarily by the burning of fossil fuels, it makes sense to consider approaches that reduce the additional carbon dioxide burden these fuels place on our atmosphere. Anthropogenic induced climate change poses a significant challenge to sustaining life on our planet, and new approaches to meet our energy demands must be focused on efficiency and reduction of greenhouse gasses that are implicated in exacerbating the current climate conditions. Many new sources of energy may be a part of the solution; however, fossil fuels cannot continue to be considered the mainstay because of their consequent production of carbon dioxide gas. Novel approaches to energy reduction and efficiency are an important first step in tackling this problem. Oil created from sources other than fossil fuels is a promising alternative to current energy demands, moreover, combined with the power of photosynthesis, biofuels, or oils created from growing living plant matter, make practical sense, as their ability to carry out photosynthesis provides a common sense approach to removing the tons of carbon dioxide that are emitted into our atmosphere as a matter of current routine energy production. (Chisti, 2007) A number of candidate organisms are already being utilized to this end, each with their benefits and detractors. Currently, ethanol from corn is being utilized to supplement gasoline stocks in the United States and elsewhere, however, it currently requires significantly more energy to produce ethanol from corn than the energy contained in it. Energy from ethanol requires approximately 29% more energy to produce than what can be obtained in a single gallon of ethanol (Pimentel, 2002). In addition, corn used for fuel production increases the cost of food commodities as a result of reducing the supply of available corn stocks typically used for edible consumption (Trostle, 2008). A common sense approach to this dilemma would be to create biofuels out of materials that don't affect the world food supply. Photosynthetic microalgae are an excellent example of how we might best solve this problem as

they don't compete with the global food markets as corn does, and there are a wide variety of species that may be well suited to this task. Additionally, because of their ability to exploit both marine and freshwater environments, they may have the ability to impact this problem in a meaningful way through rapid development of techniques suited to a wide range of conditions. Of the many species of algae under consideration for this task, *Phaeodactylum tricornutum*, a marine diatom, provides promising results to this end. There are a number of reasons for this organism to be considered for this purpose, chief among them is its ability to increase lipid production while being subjected to a variety of environmental stresses. There is much still to be learned about how this organism may be utilized for biofuel production, however, the fact that its genome has been fully sequenced by the United States Department of Energy's Joint Genome Institute is a first step in being able to learn the intimate details of how it responds to environmental changes that make it such an ideal choice for this purpose.

The purpose of this study is to investigate a particular stress on this alga to determine its impact on lipid production. Trans, trans 2,4 decadienal, has been identified as a signal transducer that affects the genetic mechanisms of *P. tricornutum* (Vardi et al, 2008). Further investigation of what impacts these genetic changes entail are needed in order to evaluate the best way to utilize it for biofuel production. Initially, it was proposed to investigate genes integral to the lipid bio-synthesis pathway utilizing qRT-PCR for fold expression changes in order to determine what impact treatment with decadienal had on up-regulating or down-regulating said genes. However, standardization of DNA primers (an essential step needed in this undertaking) was not achieved. Lipid analysis instead was carried out using qualitative means as an alternative. Fluorescent microscopy utilizing Nile Red staining was used to compare treated vs untreated cells. Additionally, thin layer chromatography was carried out to determine changes in lipid classes, notably those most desirable for use as feed stocks for biofuel production, triacylglyceride and diacylglyceride.

The general proceeding of this report format will be first to discuss the literature supporting this current research effort, followed by a discussion the materials and methodologies utilized to conduct the research. After summarizing results, some thoughts as how to best apply the knowledge gained from this effort to practice in a high school science classroom setting is included as a conclusion to the report.

Chapter 2: Review of the Literature

Preface

There has been significant interest in the use of micro-algae as a potential for bio-fuel development. The diatom *Phaeodactylum tricornutum* may be a potential study organism for these purposes because of its sequenced genome and highly adaptive nature in exploiting habitat under a variety of conditions. This particular species of diatom exists as a small microscopic phytoplankton and responsible for 20% of the total worldwide organic carbon fixation annually (DeMartino et al., 2009) It has a porous outer silica composed shell called a frustule and it lives in marine environments. *P. tricornutum* has been utilized as a model over several decades to explore diatom physiology. These organisms are photosynthetic and have the ability to interconvert between three different morphotypes: fusiform, oval, or triradiate. (DeMartino et al., 2011). There is a good deal of the expressed proteome of this organism that is not widely understood, therefore, there is great potential for determining novel gene function and expression in relation to this organism's interaction within its environment. In a study published in the journal Genome Biology, (Maheswari et al., 2010) have produced a rich library of expressed sequenced tags (over 130,00 ESTs) of *P. tricornutum* in an attempt to further connect our understanding of how the organism's genomic expression is related to other eukaryotes. The study created 15 unique cDNA libraries after culturing *P. tricornutum* under 15 different environmental conditions. These results were then analyzed for gene expression and compared to known libraries sequenced for various other genomes including plant, animal, and diatoms in order to determine the top 20 most highly expressed cDNAs from the 15 different culturing conditions. The overarching idea of this work was that there are a great number ESTs that have not yet been correlated with a particular protein, however, a significant number of the ESTs do represent bona fide genes as was supported by gene ontology analysis, Interpro domain analysis, and expression analysis of diatom orthologous genes. In this study, we plan to look at various

genes related to lipid synthesis under the specific environmental stress of trans, trans 2,4 decadienal in order to determine what role this stress plays in the biosynthesis of lipids in hopes that it may be better utilized as a possible feedstock in the creation of bio-fuel.

Diatoms play an important role in regulating the carbon cycle as well as other nutrient cycles. Because of the increase of atmospheric carbon and its impact on global climate change, there is a need for energy sources to supplement or even supplant traditional fossil fuel based forms of energy whose continued use has contributed to the excess of atmospheric carbon dioxide, a known green house gas. In a recent article in the journal Current Opinion in Plant Biology, it was cited that diatoms are responsible for around 40% of the net primary productivity in the ocean. (Bowler et al, 2010) The use of *P. tricornutum* as a potential source for bio-fuel/biodiesel has yet to be recognized and may serve as an attractive alternative to fossil fuels because of their capacity to remove atmospheric carbon through photosynthesis. In addition our dependence of fossil fuels for electrical energy has increased this atmospheric constituent at an exponential rate consistent with global population increase. The need to replace fossil fuels with clean energy alternatives is pressing, and a variety of sources must be considered if we are to mitigate the damaging effects of pollution from combustion. Solar and wind are alternatives, however biofuel from diatoms/algae represents an attractive option because of its high lipid yield compared other plant sources of fuel (in some cases 10-20 times greater) (Gouveia and Oliveira, 2009), and it has little impact of the food supply as other sources of bio-fuels such as ethanol, from corn or soybeans do. Additionally, because of their ability to exploit a variety of conditions, they are worthy of consideration for this particular task because of this ability. Also, there are already fully sequenced genomes in the diatom family available for study currently (*Thalassiorira pseudonana and Phaeodactylum tricornutum*). These organisms have a rapid growth cycle which makes them ideal for producing results on a short timetable as well.

Stresses and Potential Effects

Current research indicates that there are a variety of stress conditions that affect the growth and development of microalgae. These conditions range from the effects of potential pollutants, micronutrient load, inter and intra-specific interactions, as well as a variety of environmental conditions. The following represent a brief synopsis of what is understood about how these conditions impact diatoms.

Allelopathy

Allelopathic effects may need to be clarified before we can explain how different chemicals produced by species growing together impact each other. It has been proposed that compounds associated with three specific macroalgae may have an impact on growth patterns of the microalga *Prorocentru donghaiense* under laboratory conditions. After evaluating for Allelopathic affects of varying concentrations, some caused an inhibition of growth with those of a non polar nature, "Both aqueous and methanol extracts of the macroalgae had strong growth inhibitory effects on *P. donghaiense*, while the other three organic solvent extracts (acetone, ether and chloroform) had no apparent effect on its growth" (Wang et al, 2007). In addition to the compounds specified by Wang et al, compounds thought previously to be toxic now, are being re-classified as those that act allelopathically, stimulating and inhibiting mirco algal growth in the environment, thus causing a re-examination of the nature of toxins and consider reclassifying them along with a whole host of allelopathic compounds. As a result of the complex nature of the trophic interactions that occur between many species living in the same niche, evidence of allelopathy in the field may be hard to attain (LaFlaive and Ten-Hagge, 2007). Instead, researchers often have to point to the production of secondary metabolites that are produced amongst those species and sort out which may be having an effect on the other. If

large scale cultivation is to be undertaken, these relationships need to be better defined so that yield can be maximized.

Trans Trans 2,4 Decadienal

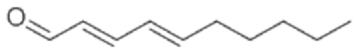

Figure 1: Trans Trans 2,4 Decadienal (or DD) (www.chemicalbook.com)

Poly unsaturated aldehydes (PUAs) such as trans trans 2,4 decadienal play a significant role in the complex interactions between various species of diatoms and their environment, notably *P. tricornutum*. PUA's have been reported to have a wide range of affects on herbivorous predators such as copepods and have been demonstrated to reduce the reproductive ability of these organisms as a response to herbivorous stress on diatoms. The enzymatic cascade that creates diatomic PUA's from membrane phospholipids is initiated by a direct result of grazing or loss of membrane integrity (Ribalet et al, 2007). They have also been demonstrated to cause programmed cell death of diatoms via up-regulation of a gene identified as PtNOA1 (nitric oxide associated protein) (Vardi et al,2008). In a recent study, it was shown that this particular gene is involved in regulation of a complex signal pathway that induces programmed cell death through increased NO production and suppression of plastid localized superoxide dismutase, an enzyme that plays an important role in oxidative stress responses in diatoms (Wolfe-Simon et al, 2006) (Brownlee, 2008). High doses of DD led to diatom cell death, however, pre-treatment with lower concentrations primed resistance to the compound and its effects. What was not described very well in the literature is how lipid production in *P tricornutum* is affected by changes that may occur as a result of varying DD concentration. High concentrations of decadienal elicit an apoptic response, whereas lower concentrations seemingly prime resistance. Because this

aldehyde acts as a stressor at certain concentrations, it may be that the diatom will react in a way that causes it to produce more triacylglycerols as a means of conserving energy as other environmental stresses such as nitrogen limitation cause it to do (Yu et al., 2009).

While not all diatoms produce DD (specifically *P. tricornutum*), it is one of several aldehydes produced by diatoms that reduce the reproductive capacity of its crustacean predators (Pohner et al,2002). However, their research has shown these interactions to be species dependent. They also report that *P. tricornutum* produces a variety of similar aldehyde compounds, notably, polar oxo-acids 9-ONDE and 12-ODTE. This may account for this species's inhibitory affect on predation, even though it produces no decadienal. It may be possible to see how lipid content and growth in *P tricornutum* may be affected by varying rates of decadienal. Several diatom specific cyclin genes related to the cell cycle may be turned on as a result. In addition to this, it has been reported that the high decadienal library displayed gene ontology matched to steroid metabolism as well as proteins involved in responses to biotic stimuli (Mahesawari et al, 2010). Differential expression of several cyclin genes, (dsCYC3, dsCYC7 and dsCYC10) in response to nitrate starved, nitrate repleted and iron limited cultures has also been reported (Huysman et al, 2010). These genes are important because they play a role in cell cycle regulation and may also be differentially expressed as a result of exposure to Decadienal.

Diatom Morphology/Life History

Diatoms reproduce for the most part asexually, and exhibit transformation between four distinct morphotypes in *P. tricornutum* (Bowler, et al,2010). These transformations are thought to be linked to environmental stresses as the organism converts between the oval/round form to the fusiform and triradiate form. The triradiate and fusiform morphs tend to have a lower bloom density and are able to behave more planktonically, while the round/oval form have a higher

density and tend to sink when they are stressed (Bowler et al, 2010). This may be related to their ability to clump or cluster together more easily in the round oval form than in the other morphs. This may allow them to escape predation possibly or even to exploit niche habitats where resources are plentiful. These organisms have a very high tolerance to stress and can exist under a variety of conditions. It may be possible in a future proposal to look at variation in lipid content between the three forms to determine ideal harvest conditions as well as specific genes that function as a result of the stresses that interconvert them. The triradiate, fusiform and oval morphs all form long chains in culture while the round cells produce a mucilage that causes them to adhere strongly to culture vessels.

Lipid Biosynthesis In Microalgae

Understanding the unique nature of how microalgae create lipids is an important piece of the biofuel puzzle. Knowing specifically what lipid precursors are created and where in the cell they are formed leads us to better design experiments aimed at increasing specific lipid class production. Chief among lipid classes for biofuel production are the triacylglycerides or TAGs for short. These non polar lipids are highly sought after as a precursor to biofuels because they are easily created into biodiesel through a process known as transesterification. This typically involves reacting TAGs with an alcohol, either methanol or ethanol used as a catalyst to create ethyl or methyl esters and glycerol. These esters are utilizable as biofuel or biodiesel because their combustion properties are close enough to that of commercially produced diesel gasoline and often provide better results than conventionally produced diesel. These qualities include better lubricative properties, more favorable emission signatures, as well as being more environmentally benign (Liu and Zhao, 2007). A number of important factors affecting the production of biofuels created from microalgae need to be taken into account when designing practical research experiments aimed at improvement of knowledge base supporting emerging

technological advances of this renewable resource. The following sections delineate their importance and significance with respect to efforts to increase TAG accumulation.

Resource Limitation and Growth

The role that limiting nutrients have on TAG production is fairly straight forward, typically, limiting nutrients tends to increase TAG production inversely with growth. Several conditions where limiting Nitrogen, Silicon and Phosphorus have been examined and have demonstrated a variety of results. In the case of *P.tricornutum*, phosphorus limitation was found to increase TAG percentage (Reitan et al., 1994). Additionally, Nitrogen starvation has been shown to have a similar effect on the species. It has been proclaimed to be the most significant limiting nutrient to have an increasing effect on TAG production (Hu et al., 2008). Of significant note considering resource limitation, it has been proposed by other researchers that different resource limiting conditions may have very different impacts on TAG production. (Yu et al., 2009). Because the metabolic production of TAG is dependent of a variety of resource conditions, it would be expected that each variety and combination of limiting resources could lead to variable TAG accumulation signatures that are species and condition dependent. Growth conditions also have been demonstrated to have variable effects on total lipid production, and present a sort of chicken or the egg scenario with regard to decreasing resources. The general trend observed is that cultures harvested at late exponential phases have increased TAG levels, however this may or may not be attributable to the limiting resource conditions present as the cultures are approaching late stage growth (Hu et al., 2008). Again in this case, *P. tricornutum* has been demonstrated to show no appreciable increase in total lipid production as a result of stage of harvest, however late stage tends to show increased TAG and decreased polar lipids as well (Alonso et al., 2000).

Mechanisms of TAG Production in Microalgae

The physical locality of production of TAGs within the cell and the metabolic resources utilized in production may vary from species to species, yet several prominent features of this process may be consistently attributable to the majority of microalgae. TAGs are typically produced in the chloroplast under stress conditions. Microalgae utilize polar and non polar lipids in production of TAGs as a means to store energy in lipid bodies both in the chloroplast and also in the cytosol as well. Under stress conditions, cells undergo degradation of the chloroplast membrane and these lipids are converted by Acetyl CoA caroboxylase (ACCase) and Diacylglyerol transferases to TAGs as a generalized mechanism. The committed step to TAG production is the transfer of an acetyl group to Acetyl CoA to form Malonyl CoA by an ACCase. These metabolites proceed through a chain of enzymatic reactions that ultimately ends in a Diacylglycerol transferase converting diacylglycerol to triacylglycerides (Ohlrogge and Browse, 1995). It has been suggested that stress causes an accumulation of electrons in the photosynthetic electron transport chain. This may be a result of oxidized components set in motion from apoptosis triggered by high Decadienal concentrations. This in turn causes an increase in reactive oxygen species that damage the membrane making its degraded components available for secondary metabolism into TAGs by the previously mentioned enzymatic pathway (Hu et al., 2008). This provides rationale for the increase in TAGs and decrease in polar lipids previously noted as a result of resource limiting stresses. An additional interesting impact observed concomitant with this process is the production of carotenoid lipid bodies that are produced similarly (Zhekisheva et al., 2002). These act as a protective sunscreen, distributing light away from the photosynthetic membrane. Hu et al, also speculate that because this mechanism is able to utilize a variety of degraded phospholipid components (Phosphatidylcholine, Phosphatidylethanolamine, galactolipids or toxic fatty acids) as acyl donors, that it serves as a detoxifying mechanism to cope with membrane degradation. However,

if de novo synthesis enzymes in the TAG biosynthesis pathway are found to be up-regulated through qPCR, some other mechanisms must be at work affecting the balance of lipid production. The enzymes involved in these pathways deserve thorough examination by more precise methods of gene expression determination. This analysis may be provided through qPCR which shall be discussed in more detail later in the methods section of this report.

General Overview

The overall experimental plan proposed for this research is twofold. First, a preparation of total RNA for qPCR analysis of gene expression of 50 ml cultures *P. tricornutum* was undertaken in order to determine the impact of stress induced by trans trans 2,4 decadienal after a 6 hour time period. Included in this experimental set were a methanol solvent control and a water control as well. A whole complement of genes to be examined related to the TAG lipid synthesis pathway were initially identified, however time and resources permitted only one of these genes to be examined, Diacyglycerol transferase. Housekeeping genes for qPCR were selected based on the previous work done by Magali Siaut and colleagues detailed in a publication in Gene (Siaut et al., 2001). They suggest a series of primers to be used as constitutively expressed reference genes for qPCR expression changes utilizing the $2^{-\Delta\Delta Ct}$ comparative Ct method of calculation. The second set of experiments was designed to demonstrate changes in lipid content and classes 24 hours after inducing the same stress conditions with trans trans 2,4 decadienal. Lipid changes were qualitatively examined using Nile Red staining in preparation for fluorescent microscopy. Thin layer chromatography was also utilized in an effort to determine changes in lipid classes as a result of this stress being induced.

Chapter 3: Methods

Algal Growth Conditions

Algal culture samples were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton of the *Phaeodactylum tricornutum* clone 2561. Axenic cultures were inoculated in 100 ml batches from 20 ml of starter culture that had achieved lag growth stage and cell density of approximately ~5.0 x 10^6 cells/ml and 80 ml of F/2 artificial seawater medium and subjected to a 20^{o}C constant temperature incubation and 12:12 hour diel cycle. Approximate cell concentrations of starting cultures was calculated to be ~1.25x10^6 cells/ml. Cultures were monitored daily and gently swirled to suspend cells and allow them to grow planktonicaly in suspension. Cell counts were taken during the incubation process using a Haemocytometer and culture absorbance values were taken of one ml samples at 680nm using a standard lab spectrophotometer.

Decadienal Dose Conditions

For experimental considerations, three 100 ml triplicate cultures were grown as previously described. After triplicates reached an average density of ~5.00 x 10^6 cells/ml, cultures were pooled prior to total RNA isolation to make a uniform cell population, then redistributed into 12 100ml flasks containing 50ml of cell suspension each. Trans, Trans 2,4 Decadienal (food grade) was obtained from Sigma Aldrich and prepared to a 10mM stock concentration in methanol solvent. Three of the 50 ml cultures were treated with the low and high concentrations of Decadienal (.5µg/ml and 5 µg/ml), three were used as a methanol solvent control, and three were used as a water control using an equivalent volume. After 6 hours of treatment, ~40 ml of each experimental set and controls were removed for centrifugation at 10,000 rpm for 10 minutes at 4^0C in preparation for total RNA isolation.

Total RNA Isolation

A number of different techniques were attempted in order to isolate total RNA from cell cultures in advance of settling of a final protocol that worked best. Initially, Qiagen's RNeasy isolation kit was utilized with liquid nitrogen grinding with mortar and pestle as a means of providing powdered cell extracts. Bead beating was also utilized as means of cell disruption prior to RNA isolation using the Qiagen kit as was sonication. We were unable to obtain RNA of sufficient quality as evaluated by formaldehyde gel analysis for cDNA preparation utilizing these methods of cell disruption. Ultimately Invitrogen's Trizol™ Plant reagent method was used according to manufacturer's protocol for total RNA isolation. Cells were harvested using a Beckman/Coulter Allegra x-22R high speed centrifuge @ 4^{o}C for 10 minutes at 10,000 rpm and subjected to liquid nitrogen grinding with mortar and pestle before proceeding with the Invitrogen protocol.

Total RNA was evaluated using a Nanodrop spectrophotometer to determine 260nm/280nm and 260nm/230nm ratios in order to assess RNA quality and provide an estimate of the concentration of RNA obtained.

Formaldehyde Gel Electrophoresis of Total RNA Extracted from *P. tricornutum*.

RNA samples obtained were evaluated using formaldehyde agarose gel electrophoresis using Ambion's NorthernMax Gly 10x gel Prep/Running buffer #AM8678 and NorthernMax 10x Denaturing Gel Buffer#AM8676 and were prepared to manufacturer's specifications with the following exception: gels were scaled down to a 50 ml recipe to accommodate the use of Bio Rad's mini Gel rig apparatus. A Fermentas High Range riboruler #SM1823 was used with a range of 6000-200 bases. Ambion Loading dye AM8552 was added to each RNA sample run on the gel and ethidium bromide was added to each gel prior to cooling. Gel electrophoresis was carried out under a fume hood utilizing a Bio-RAd powersupply @70V until the loading dye

transversed approximately ¾ of the gel length. Gels were imaged by illumination at 300nm UV light and taking a photo.

cDNA Synthesis From Experimental RNA

Much as was the case with RNA isolation, a number of techniques were needed in order to best determine how to proceed with cDNA synthesis. Initially, cDNA was prepared from total RNA extracts from both practice RNA isolations and experimental runs, the initial being done using AB Bio's High Capacity cDNA Reverse Transcriptase kit (Part#4368814) according to manufacturer's recommendations. This kit yielded unreliable results when consequent cDNA was used to standardize DNA primers for the qPCR portion of this experiment. It was determined that was because it was out of date (expired 6-11). Other methods of cDNA preparation were explored. An additional method of cDNA synthesis was carried out using Invitrogen's Superscript first strand synthesis system for RT-PCR Cat #11904-018 as an additional means of creating cDNA as an alternative using random hexamers provided with the kit along with superscriptIII enzyme provided with the kit. cDNA was prepared from total RNA isolated from the high decadienal concentration experimental set after DNase treatment per instructions in the Invitrogen kit.

As an additional step to remove possible contaminants from first strand cDNA produced using the later method, a washing procedure was carried out using a Phenol Chloroform Isoamyl alcohol (PCI) treatment. This following protocol was modified from work done by Suslov and Steindler published in Nucleic Acids Research, 2007. 1.37µl of glycogen (20ng/µl) was added to 27.6µl of DNase treated cDNA reaction created from the Invitrogen kit (#11904-018). 710µl of TE buffer was added in error instead of 71.0µl. 100µl of PCI was added and the sample was vortexed for one minute. Sample was allowed to stand 5 min at room temperature, then centrifuged @ 7000 x g for 5 minutes at rm temp. The aqueous phase (now in excess due to

pipetting error) was transferred evenly to 4 new tubes. 1/10 volume of ammonium acetate was added to each tube and vortexed. 2.6 volume of 100% EtOH was added to each tube and transferred to -20^{o}C for precipitation over 48 hours. Tubes were centrifuged @ 4^{o}C at 15000x g for 40 minutes. Pellet was washed with 70% EtOH (1ml-- .25 ml each tube) at room temperature. Pellet was detached from the bottom of the tube and centrifuged again at 15,000 x g for 20 minutes more at rm. temperature. The supernatant was then removed through aspiration with a pipette and the tubes were then spun down. Remaining ethanol was aspirated using smaller P20 pipette tips without disturbing the pellet. The tubes were air dried and subsequent volumes of RNAse free water totaling 30µl were utilized to recombine and collect the samples into a single tube.

cDNA Evaluation using Standard PCR conditions

Practice cDNA was created using the first method listed in the previous section from RNA that was isolated from *P. tricornutum* and was tested under standard PCR conditions using Actin and Tata box binding protein developed by Magali Siaut and colleagues at the Laboratory of Cell Signaling, Stazione Zoologica Anton Dohrn, Villa Comunale in Naples, Italy. Actin primer sequence was: Q-Act12 fw: 5'- TCGCCTGAGTCGAGAACACA-3' and rv: Q-Act-rv 5'-GCCCATCCAGTCCTGTTGAC-3', and also Tata box Binding protein (TTB) forward: Q-TBP-fw 5'-ACCGGAGTCAAGAGCACACAC- 3', and reverse: Q TBP-rv 5' CGGAATGCGCGTATACCAGT-3' (Siaut et al., 2007). The Actin primer set was compared to a no template control on a 50 ml 1% agarose minigel using NewEngland Biolabs N3236S 50 bp DNA ladder.

qPCR Primer Design

Primer design was carried out utilizing both Perl Primer and Primer3 primer design software. The following conditions were observed when constructing primers utilizing these programs: 58^{o}C-60^{o}C acceptable annealing temperature range, G/C content of no more than 50-55%, Product size >50 bp <300 bp. Blast analysis was conducted on all primer sets created to ensure that primer sets would not anneal to differential genes not intended for research. Usage of the KEGG data base linked to the Joint Genome Institute web site was carried out in order to accurately match selected enzymes for research to their appropriate genes in the *P.tricornutum* database on the JGI website. ΔG values were selected that were no smaller than -8 for each primer set. Primers were ordered from Integrated DNA Technologies and were delivered in variable mmol masses as a dry powder which were then dissolved in TE buffer to create a 100 mM working stock solution for qPCR assay. The forward sequence for the Diacyclyglycerol transferase primer used was: AATGGGCAGTATGCTGGAAC, the reverse sequence is: TGTCGATCGTTGCATTCATT with a product size of 184 bp.

qPCR Primer Standardization

Serial dilutions of the following concentrations were made from existing cDNA created using both the ABI kit and Invitrogen kit for separate qPCR standardization runs. The following template concentrations were used: 10ng/µl, 5 ng/µl, 1 ng/µl, .2 ng/µl, .04 ng/µl, .008 ng/µl, and .0016 ng/µl. These dilutions were prepared on a 96 well qPCR plate in triplicate and using both forward and reverse primers for Tata binding box protein and Diacylglyceride transferase primers created using primer 3 and perl primer. Master mix was prepared using the following recipe for each well carried out in triplicate: .5 µl of forward primer + .5 µl of reverse primer + 12.5 µl of Power Cyber green Mix + 6.5 µl of RNase free water + 5 µl of template for each concentration in the serial dilution. The reaction volume in each well equaled 25 µl (5 µl of

template + 20 µl of master mix). Samples were run on the Applied Biosystems 7900A RT-qPCR machine in the MBB building of the UT ICMB core facilities. Samples were run for approximately 2 hrs and the data was analyzed using MS excel and SDS 2.0 software packages.

Lipid Extraction

Total lipids were extracted using a modified Bligh and Dyer procedure involving methanol and chloroform (Bligh and Dyer, 1959). 1 ml of algal suspension was centrifuged at 10,000 rpm for 3 min at room temperature. The supernatant was removed. The pellet was re-suspended in 1 ml of distilled water. One ml of chloroform was added followed by a 2 ml addition of methanol and left undisturbed for 10 minutes. One more ml of Chloroform was added, mixed and left standing for ten more minutes. One ml of distilled water was added and mixed. Solution was allowed to stand until a clear separation of phases appeared. The bottom lipid phase was removed via Pasteur pipette while gently bubbling though the protein disc, stored in a new vial. The solvent was blown off using a steady stream of nitrogen gas and the oil was re-suspended in a 1 ml of 6:1 chloroform/methanol mixture and stored @ -20^0C for TLC processing. Lipid samples were extracted from decadienal treated cultures (and all controls) of *P. tricornutum* using the same 5µg/ml concentration after 24 hours of incubation. The cell concentrations prior to extraction were approximately ~5.0x 10^6 cells/ml as measured by haematocytometer.

Thin Layer Chromatography

Thin layer chromatography was carried out using 20x20 silica-gel-G plates manufactured by Analtech (cat#01001) that had been activated by being heated at 50-60^{o}C for 15-20 minutes and then stored in an air tight acrylic box. 1 cm lanes were scored on each plate. Triolein standard obtained from Mpbio was prepared to a concentration of 1 mg/ml and 5 µl was

spotted in the first lane of the plate. Diolein standard obtained from Supelco was prepared to the same concentration of 1 mg/ml and an amount of 5 µl was spotted on the second lane of the plate. The third lane was left blank. In lanes 4- 6 increasing volumes of 10, 20 and 30µl of oil extracted from the Decadienal treated sample were applied in 5 µl aliquots and allowed to dry between spotting. The 7^{th} lane was left intentionally blank. Similarly, extracted oil from the solvent control and water control were applied in the same manner in lanes 8-10 and 12-14 respectively leaving a blank lane between each group of three increasing concentrations. The last two lanes on the right the diolein and triolein standards were repeated but using a 10µl volume of the same 1mg/ml concentration instead. A running solvent of 70:30:1 petroleum ether/diethyl ether/acetic acid was prepared and a small volume (~30 ml) was added to the development chamber to process the plate. The solvent was allowed to run up a paper towel prior to running the TLC gel in order to saturate the chamber with solvent. The plate was the placed in the chamber and allowed to run until the solvent had reached one cm from the top. The TLC plate was then transferred to a developing chamber where iodine crystals were allowed to sublime, reacting with the standards for approximately 30 minutes until dark orange spots appeared on the plate indicating the location of standards that had been spotted. A digital photograph was taken of the plate after processing with iodine to preserve the results for analysis.

Nile Red Staining of Lipids and Fluorescent Microscopy

Nile red staining was carried out on each of the three experimental groups, (DD treated, water and solvent controls). Nile red was prepared to a concentration of 250µg/ml in acetone solvent. 100 µl of a $5x10^6$ cells/ml suspension were stained using ~100 µl of Nile red dye for 5 minutes. Cell cover slips were sealed with Revlon™ clear acrylic nail polish. For each Nile red stained slide, an unstained water control was included as a comparison for auto-fluorescence

detection. Slides were imaged using a Zeis Axiovert 200M fluorescent microscope using 100x oil immersion and a 10x ocular for a total magnification of 1000x at the University of Texas at Austin Institute of Cell and Molecular Biology's core facilities. A "Texas Red" filter package was used to image the diatoms with an excitation wavelength of 540 nm. Nile red staining protocol was adapted from work done by P. Geenspan and colleagues (Greenspan et al., 1985).

Chapter 4: Results and Analysis

Total RNA Isolation and Formaldehyde Gel Electrophoresis

In consideration of the data presented here, it's important to note that several practice attempts were necessary to improve RNA isolation techniques and gel electrophoresis before achieving worthwhile results. The initial attempts at this analysis can be seen as a progression of improvement as can be seen in figures 2 and 3 below. Figure one depicts the first gel electrophoresis run shows the RNA ladder on the leftmost lane and in the second adjacent lane, that the quality of RNA obtained is minimal at best, not producing the sharp double bands of 28S and 18S rRNA as expected, but rather a smear in the 4000 base range of the ladder. The bright band seen just above the highest MW band visible on the MW ladder most likely represents genomic DNA obtained during total RNA isolation. Figure 3 demonstrates RNA obtained from the decadienal experimental groups and controls. Several of the total RNA extractions were not run because the RNA concentrations were too low and what RNA was produced was intended to be saved for the creation of cDNA to be used in qPCR analysis.

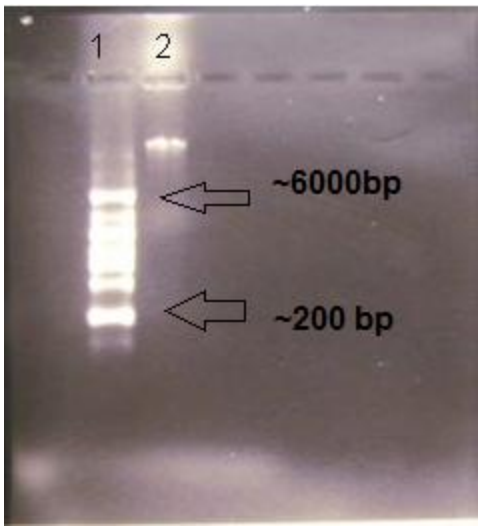

Figure 2: First practice RNA isolation

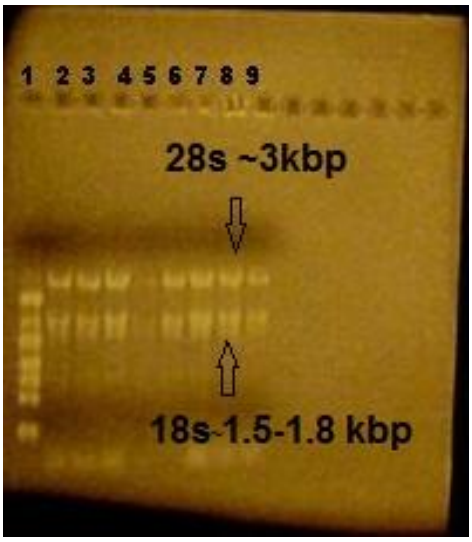

Figure 3: RNA isolation of experimental time points with improved RNA quality

In Figure 3, lane 1 depicts the RNA Fermentas ladder described in chapter 2. Bands from the top down represent the following numbers of nucleotide base pair amounts: 6000b, 4000b, 3000b, 2000b, 1500b, 1000b, 500b, 200b. Note the improvement of the quality of RNA showing the 28s and 18s bands of rRNA with higher intensity of the 28s band for most samples run compared to the RNA sample run in figure 2. Estimated concentrations in ng/µl ranged from 505.6ng/µl to 1035ng/µl with an average of 770.3 ng/µl. These concentrations were estimated by nanodrop spectroscopy. 260/280 ratios for all samples were outside the acceptable range of 1.8-2 with all values for this ratio ranging from 2.09-2.24. In addition, 260/230 ratios were also all less than the acceptable threshold of 2.3 (ranged from .89-1.7). These values suggest the presence of a salt or other contaminant and therefore concentration values obtained from the nanodrop are therefore not reliable and cannot be accurately used to estimate the amount RNA obtained per cell. For reasons unknown, the RNA ladder in both figures 2 and 3 ran further down the gel than expected. This could be due potentially to an error in the gel casting, possibly not being level upon formation or degradation of the of some component of the dye or ladder. Alternatively, it could be that something in the RNA preparation was hindering the progress of

the RNA in the gel as well. For all samples run, Numbering from left to right with the ladder being lane 1, lanes 2-3 correlate with the first two sets of the triplicate run of the low dose decadienal RNA extraction and 2 µg of each sample was loaded . The third was not run due to low estimated concentration and was preserved for cDNA synthesis. An estimated 2 µg of RNA was loaded in lanes 4-6. These lanes correlate with the solvent control triplicate. Lanes 7-9 correlate with the high decadienal dosed RNA extraction. An estimated 2 µg of RNA sample was loaded in lane 7, while lane 8 and 9 contained 1 µg each. The low intensity observed in lane 5 may be attibutable to uneven loading of the sample, due to inaccurate pipetting.

DNA Electrophoresis Under Standard PCR Conditions

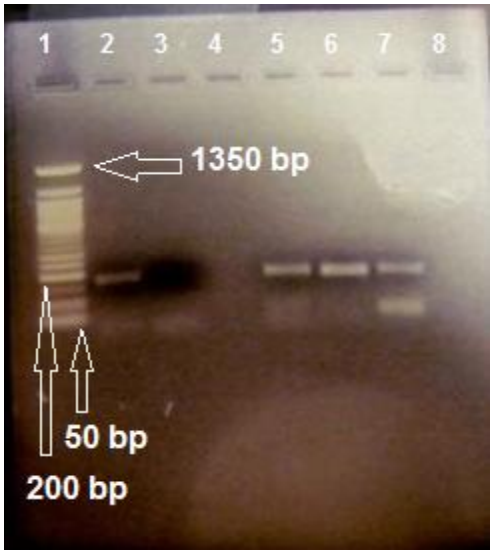

Figure 4: DNA gel showing PCR product formation after cDNA synthesis

In figure 4 we see a DNA gel that was run using cDNA created from 2µg total RNA isolated from *P. tricornutum* as a practice run to improve technique. The cDNA was run under standard PCR conditions with the Actin primers specified previously in order to determine if the sample contained only the PCR product specified by those primers. In lane 1, a 50 bp mw ladder from New England Biolabs was run, the largest and smalled bands denoted by arrows on the gel. The band in between the high and low MW corresponds to 200 bp. The product specified by the Actin primers is supposed to be about 176 bp and shows up just under the 200bp band of the ladder indicating accurate annealing of the primers. All lanes to the right of the 3rd were shared on the gel to analyze data from different experiement and those results are not applicable or related to the first three lanes in any way and only serve as a qualitative reference point for the ladder. The only common point of reference is that the PCR products created were made from primers designed to yield roughly the same size amplicon as those tested in the far left lanes (~ < 300bp).

qPCR Standardization

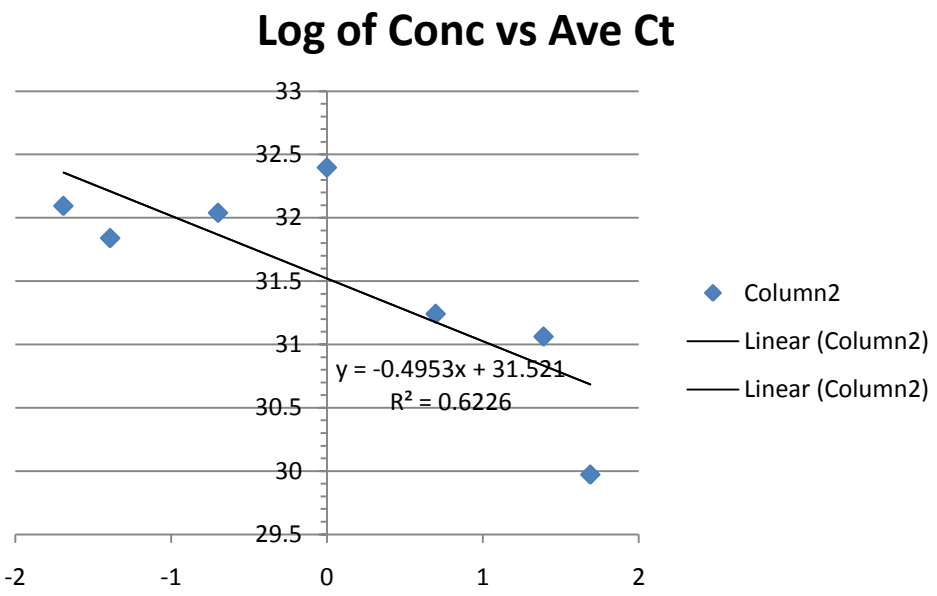

Figure 5: qPCR standardization curve using Diacylglyceride transferase primers.

The graph represented in Figure 5 depicts the log of the concentrations of cDNA template in the serial dilutions from the qPCR run vs the average Ct values for each concentration assayed. This was carried out using the Diacylglycerceride transferase primers created and referenced earlier. cDNA was created from a 1µg of the total RNA isolated from the High decadienal control. This cDNA was also further purified using the PIC/ethanol precipitation method referenced in the methods section. Each concentration assayed was aliquoted in triplicate. Those values which differed by more than one Ct value were removed when averaged in order to improve the results, however, they are still far from acceptable range needed to progress with additional qPCR quantitation of gene expression. A regression line of best fit with R^2 analysis was carried out using MS Excel statistics features built into the program.

Expected results for such a calibration are a slope that approximates -3.33 (acceptable slope range is -3.1 to -3.6) and R^2 values of .999 (accepted range for R^2 should be no less than .990). The R^2 values and subsequent slope presented in this figure demonstrate that the data from the standardization is far from acceptable for proceding with qPCR quantitition as the R^2 values obtained (.6226) demonstrate that Y values are not a very good predictor of X. A number of explanations for these results have been considered. The most consitent being poor primer design or probable contamination of the cDNA sample. Upon examiniation of the zero template controls conducted with this assay, melting curve data showed melting peaks at 81^{o}C and a 2 had peaks at 73^{o}C indicating possible DNA contamination or primer dimer formation. It may also be that not enough cDNA template was generated during cDNA formation to achieve the desired results. An inability to accurately determine starting concentration of cDNA makes achieving an accurate dillution series extremely difficult.

Thin Layer Chromatography Analysis of Lipids Extracted from *P. tricornutum*

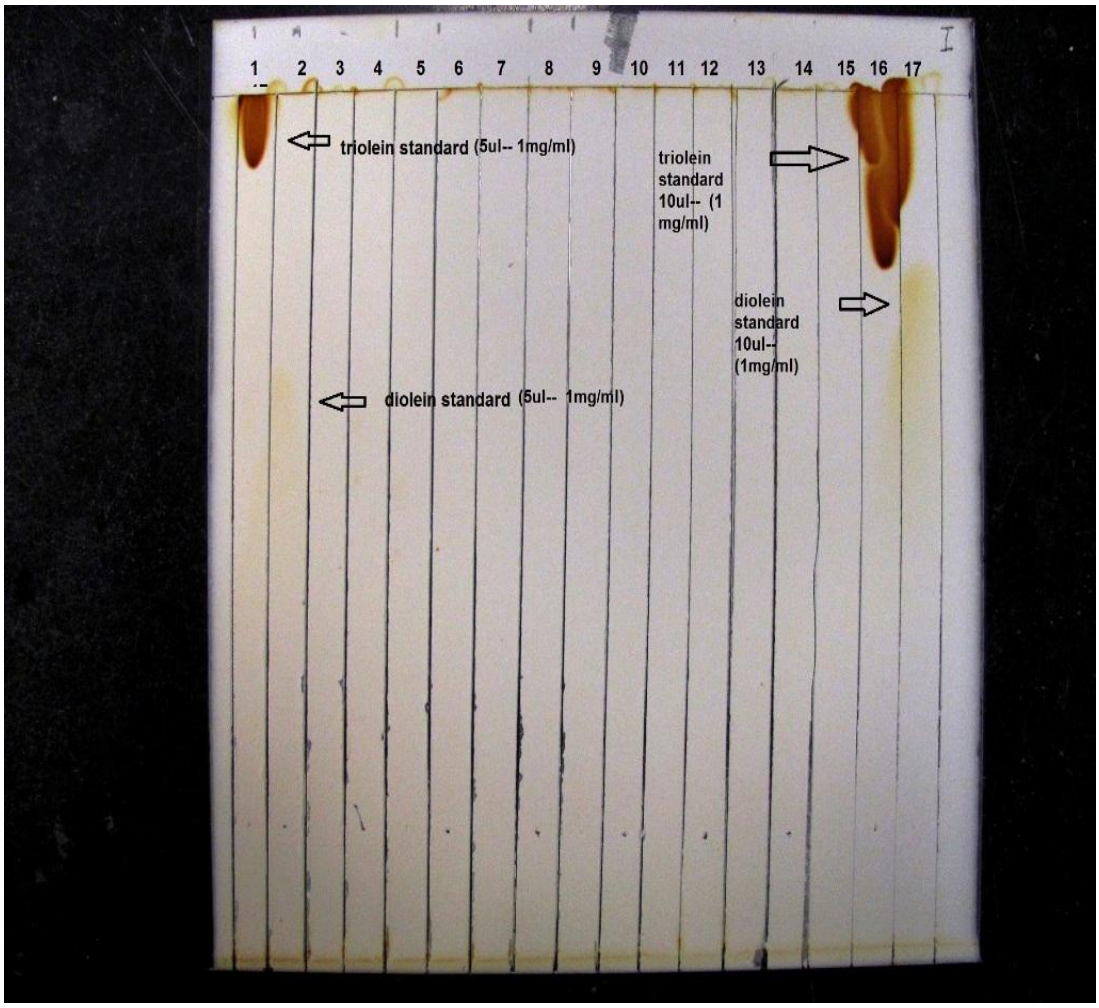

Figure 6: TLC analysis of extracted oil from *P. tricornutum* after 24 hr DD treatment

The information presented in Figure 6 demonstrates efforts to look at what concentration of oil was extracted from the *P. tricornutum* under experimental conditions. While a variety of increasing concentrations of samples were assayed, the only result that showed up clearly enough to be seen are the diolein and triolein standards. The same standards are repeated on the left at twice the initial volumes of 10µl. This accounts for the darker stained regions of standard

on the left side of the plate as opposed to the right side of the plate. The experimental samples were dissolved in 1 ml of 6:1 chloroform/methanol mixture. This volume dillution is far to high to achieve any visible results on the plate as shown. None of the aliquots of extracted oil were able to be visualized clearly as a result of possibly low oil content, or their being extremely diluted. It may be that the darkened areas at the top of plate are matched to the triolein standards, however, because of the bleedover from the standards, this analysis cannot be considered to be conclusive.

Nile Red Fluorescent Microscopy of Lipid Bodies in *P. tricornutum*

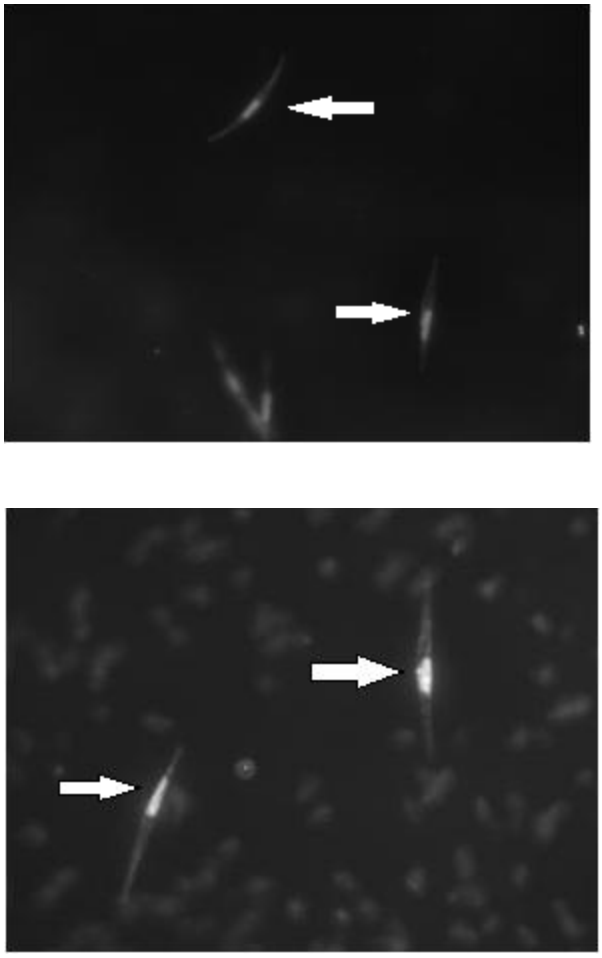

Figure 7: DD treated unstained (top) compared withNile Red Stained (bottom)

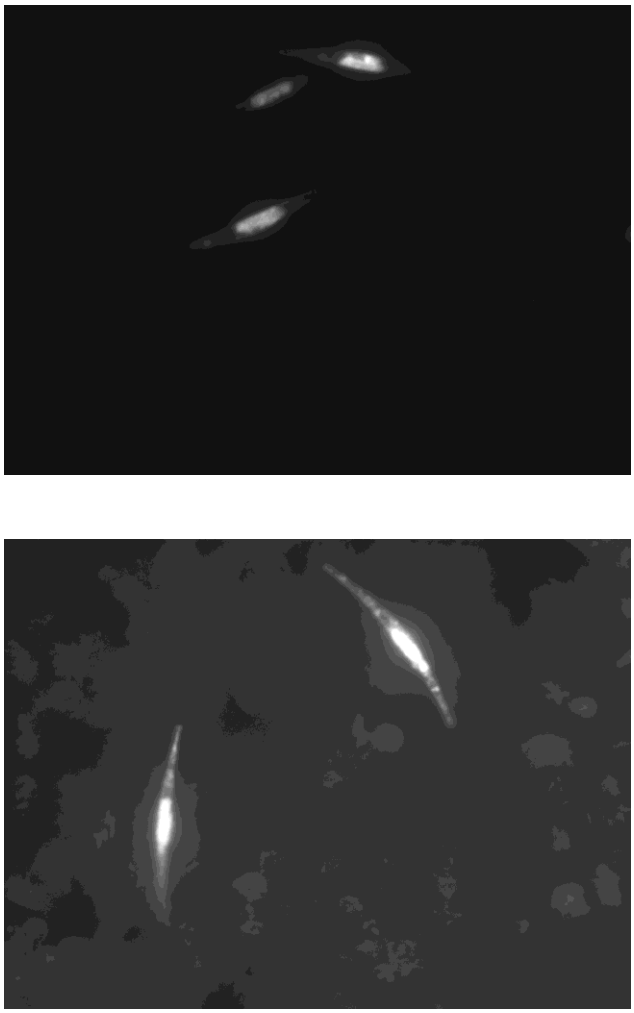

Figure 8: Methanol solvent control (top unstained), Nile Red Stained (bottom)

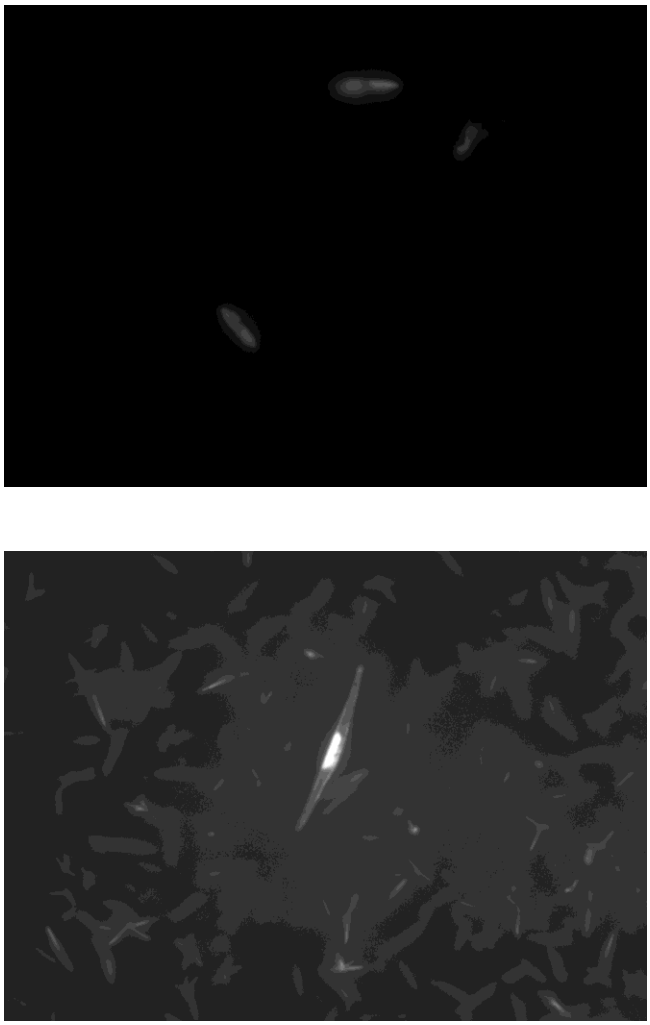

Figure 9: Untreated water ctrl. no stain (top) compared withNile Red Stained (bottom)

In each of the preceeding figures, 7,8 and 9, There is a noticble difference in brightness between the top unstained images and the bottom Nile red stained images with the bottom image being brighter than the top ones. All slides were magnified at 1000x using oil immersion. It may be that this is due to increased lipid production, however it is difficult to discern if this increase is due to the effect of treatment or due to the stress placed on the cells from staining or being harvested at late exponential stage. The difference in fluorescence between the stained groups shows that the methanol and water controls are fluorescing more than the DD treated

cells, however determining a qualitative difference being a result of the treatment with aldehyde is difficult to say at best. It may be that the reason the DD treated stained slides are flourescing less than the controls is because of impacts of the staining. Even though all cells were unfixed, cells were viewed approximately no later than 2 hours post treatment with stain compared with 24 hours of treatment, so any changes in lipid characteristics between the groups would be speculative. The silica frustule is porous and could very well be subject to osmolarity changes as a result of the acetone component of the Nile Red dye. A suitable viability assay was not able to be found to complement this study to verify the data. Fluoroscein diacetate (FDA), one such dye used in flow cytometry experiments was proven to be unreliable in a 2007 study published in the Marine Ecology Progress Series when working with this species of algae, citing three separate studies where it failed to produce consistent results(Garvey et al.,2007).

Chapter 5: Conclusions

Preliminary Conclusions

After careful consideration of the materials and methods, and data analysis, more needs to be done to further our understanding of how trans trans 2,4 Decadienal impacts lipid synthesis of the marine alga *Phaeodactylum tricornutum*. From the initial observation of the fluorescent microscopy slide series, it does appear that lipid production was increased, however it is difficult to know which genes are responsible for these changes and more importantly whether or not they are direct result of treatment with decadienal. The original basis for the hypothesis that DD may increase lipids was supported by (Maheswari et al.,2010) as they identified a whole series of estimated sequence tags of cDNA that showed differential expression when exposed to lethal and non lethal dose of the aldehyde. Without the necessary qPCR analysis of identified genes as support, an experimental plan that examines these conditions more closely is needed to make this evaluation.

Oil samples extracted from one ml of cell cultures produces very small amounts of oil that must be solved in very small volume (.1 ml or less) in order to be visualized with thin layer chromatography. Additionally, more precise means of lipid analysis, either by mass spectroscopy or gas chromatography need to be examined as a means to further clarify lipid characteristics of this algae under these experimental conditions.

Further experimentation as mentioned previously takes time. A number of considerations for improving the current experimental efforts have already been suggested, however, looking to future experimentation with these topics makes sense as mentioned in the introduction, there is still much to be learned about how the lipid production characteristics may vary based on stimuli from its environment. One aspect that was completely unexplored regarding this is the role of other organisms in the environment and how they impact these diatoms through trophic interactions. Predation, mutualism, and other symbiotic conditions are highly variable and may

be difficult to simulate in a laboratory environment, but the role of predator prey interactions cannot be overlooked as they provide new insights as to how *P. tricornutum* responds to pressures from grazing, potential pollutants and other environmental stresses. Because the state of the coastal environments are changing as a result of natural processes such as changing tides, drought, flood, seasonality etc.. each condition has potential to allow us to demonstrate how these organisms respond to such stress.

Additional Considerations With Regard to Error

While efforts undertaken in this experimentation were wide ranging in their scope, a narrower focus for the experimental aim may have been better suited for the time constraints needed to complete the work. Measuring gene expression changes with qPCR techniques involves several intricate steps that must be practiced multiple times in order to acquire a proficiency level sufficient for obtaining repeatable and reliable results. Taking the time to practice the techniques helps to reduce human error though out the experimental process. As is the case with the majority of molecular biological techniques, it's often difficult to determine at what point in a protocol mistakes may have been made. As a general rule, utilizing materials that are freshly prepared and within the manufacturer's expiration date are good rules of thumb to avoid the costly impacts on time, effort, and resources needed to successfully re-complete these kinds of experiments. Making certain precision measuring equipment is accurately calibrated to standard is a significant way to reduce potential sources of error as well. Micro-pipettors are extremely sensitive and precise measuring devices who's calibration must be checked regularly, if not at the outset of any experimental procedure. With regard to specific sources of error in this experimentation, the difficulty in attempting to standardize qPCR primers could have been minimized if the cDNA kit that was used was not out of date. Making sure that all reagents are kept at their proper temperatures is another source of possible error. RNA can

degrade rapidly under unfavorable temperature conditions without the protection of some sort of buffer, so it is important not to leave samples on ice for indefinite periods of time. Another additional source of error that could have contributed to the difficulties of the qPCR process could have stemmed from not changing gloves frequenly enough, or making the assumption that work areas are free and clean of contaminants because they appear to be. Regular sanitization of workspace and lab benches with 70% ethanol is essential to avoid contaminations that can dismantle an entire experimental effort. In addition to these guidelines, multiple practice attempts with each procedure is a must if they are to achieved to the skill level needed for reproducible results. Only then can you begin to look for sources of error that may be extraneous to improper lab bench technique.

Chapter 6: Applications to Practice

The Role of Practice

As a secondary high school science teacher with over a decade of practical teaching experience, this journey has led me to some important conclusions about how my experiences with direct scientific research may inform and shape my teaching practice. Of significant note, is the notion of what skills students will need to acquire in their high school tenure that will best prepare them for work in a scientific discipline or collegiate degree plan. The idea that effort leads to ability is not lost on me as my experiences in the lab during this time have led me to understand what I believe to be the single most important factor necessary to acquire new skills: Practice. As an educator, my students come to me with a wide and variable skill set pertaining to their ability to read, write and carry out arithmetic operations. I am amazed at the deficiencies I see sometimes in student ability, however, in my experiences this summer, I recognized that I too suffered from disuse of many of the mathematical concepts needed to complete my research. While I certainly am not devoid of mathematical ability, certain skills such as knowing when and how to apply specific proportional logic operation to create serial dilutions for qPCR, (for example $C_1V_1 = C_2V_2$) were more difficult for me to utilize because they had been presented within a different frame of reference or context. I have utilized that same exact proportionality constant in performing and teaching gas law calculations for my students in class, however, because it was presented in a new context with specific use to calculating a very different set of data, it took some time for me to come to terms with how to use it to prepare these dilutions. Another example that comes to mind is the use of dimensional analysis. While this skill is one that I used on a regular basis, both in the lab and also in teaching, some conversions are more easier carried out because of more familiarity with them. While centimeters, meters and kilometers are units I easily have practiced inter-converting many many times in my teaching practice, microliter, nanoliters and picoliters I have not. While I am able to do dimensional

analysis with these prefixes, it took me a while before I had them practiced well enough to make them second nature. This brings me back to the idea of practice. Novice learners need time to practice skills until they have them down well enough to make good use of them. Each student in my classroom has varying skill level with many different academic skill sets. If I am to prepare them for the eventuality of a possible career in science, or post secondary science educational path, I must assess and evaluate their ability with these skill sets that accompany learning in science. Students must be given multiple opportunities to practice these skills without being made to feel as if they should already know how to do them. This is the biggest challenge for me as a teacher because I'm often surprised at what skills they are lacking in, however they must be given the chance to practice until they have improved their ability to the point at which their deficit is no considered to be a liability to their learning. A number of skills seem to follow naturally from a hierarchy of importance. Standard order of mathematic operations, dimensional analysis, fractional math and proportionality, algebraic manipulation are all skills at some point or another have been the lynch pin to attaining deeper understanding of how to do science. The best way that I can think to do that is have students utilize these skills in the context of an experiment. By being able to put them into context with actual data collection and analysis, students may better come to understand the limitations and practical nature of their use.

Mentoring

Some final thoughts with regard to additional insight gained from the entirety of this experience. There was an intentional effort by the UTeach program to imbed within its curricular structure the role of mentorship. I have been very fortunate during this experience to have had several more experienced mentors guide me through this experience, letting me know what to expect, how to deal with challenges and problems, and primarily laying the groundwork

for helping me come to terms with navigating my own outcomes and educational experiences. I believe that good mentoring too, is an essential partner in developing the knowledge and skill base through guided and independent practice with new skills. The approaches I take in attempting to improve my student's ability are not to be taken lightly, and a positive demeanor insistent upon their ability to achieve through practice, and that they can and will improve if they continue to put forth the effort is essential, if not absolutely necessary. If not for the mentors I have had encouraging me throughout this process, I may never have come to complete it. I benefited greatly from speaking with others in the field that had more knowledge than myself and were only happy to help guide and direct me in my quest to better understand what I was trying to learn. They took pleasure in being able to direct me in a way just because I was interested and asked them for guidance. This natural spark of discovery inspires us and when others see it reflected in a potential mentee, it only serves to help them better relate to a time when they too needed assistance in achieving the expertise that they may now possess.

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