

Title of Document:

CHARACTERIZATION AND ANALYSIS OF  
THE EXOGENOUS APPLICATION OF  
SELECTED PHYTOHORMONES ON  
*C. REINHARDTII* METABOLISM

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ABSTRACT

Introduction of algal biofuel on an industrial scale will only be possible if production cost can be lowered, either by speeding algal growth, increasing lipid production per cell, or both. Our approach examined the application of phytohormones to algae to reach this goal. Bioinformatics and literature led us to four phytohormones: Indole-3-acetic acid, Epibrassinolide, Trans-zeatin, and Trans-zeatin-riboside. These were systematically introduced to algae at a range of concentrations. IAA, Epibrassinolide, and Trans-zeatin-riboside increased algae growth rates at concentrations of  $10^{-8}$  M to  $10^{-12}$  M. A treatment Trans-zeatin-riboside yielded an increase in growth rate of  $31.1 \pm 9.4\%$  ( $p < 0.05$ ,  $n = 8$ ), an increase in algal dry mass by  $38.9 \pm 11.9\%$  ( $p < 0.05$ ,  $n = 8$ ), a negligible change in specific lipid mass ( $p > 0.05$ ,  $n = 8$ ), and, most notably, a lipid concentration increase of  $44.6 \pm 42.1\%$  ( $p < 0.05$ ,  $n = 8$ ). A treatment of Trans-zeatin-riboside at  $10^{-11}$  M yields practical benefits to biodiesel production.

CHARACTERIZATION AND ANALYSIS OF THE EXOGENOUS  
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By

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## **Dedication**

We dedicate this thesis to the University of Maryland for helping us to learn and grow over the past four years.

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# 1. Introduction

Today's society relies heavily on fossil fuels to heat and power homes, run factories, transport goods, and drive cars. Despite the fact that Earth's supply of fossil fuels is limited, consumption is expected to rise by about 60% in the next 25 years (Rittmann, 2008). The rate of expansion of worldwide energy demands coupled with the non-renewable nature of fossil fuels is creating an unsustainable future.

Alternative energy sources, like solar, nuclear, and wind energy, have been tested and implemented to various extents, but cannot provide an efficiently transportable fuel to power engines in the same manner as liquid fuels.

In order to move towards a more sustainable future, we must explore alternative energy sources. While some alternatives, such as hydroelectric power, nuclear power, solar power, and wind power have been used for some time, they are still relatively niche sources and are either uneconomical or unable to fill the role of fossil fuels. Hydroelectric energy production depends on large facilities that must be near water sources. This limitation severely curtails the practicality of this technology for powering the current transportation sector. Although nuclear power has been explored for several decades as a potential alternative energy source, the projected levelized cost (lifetime cost converted to equal annual payment) of electricity from nuclear power is high. More importantly, nuclear power plants produce mass quantities of waste that are harmful to both the environment and to human health. Furthermore, the widespread implementation of nuclear power introduces the

potential for this energy source to be used as a weapon. While solar power has recently seen a rise in efficiency, it still cannot match the low cost or ease of implementation of fossil fuels. Wind power, though clean, is inefficient because of its reliance on optimal weather conditions to generate power. In order for a fuel source to be seamlessly integrated, its functionality and effectiveness must be independent of uncontrollable conditions. Moreover, it must be able to fulfill the diverse roles of fossil fuels.

Biofuels, which include bio-ethanol and biodiesel, have the potential to meet the requirements that the other alternative energies cannot. Similar to diesel, these biofuels can be used directly in current engines. The production of first generation biofuels, however, has many problems, including a need for an immense amount of farmland and water for the growth of crops. In addition, fossil fuels are required for both the harvest and transportation of crops and fuels. Today, crops grown specifically for biofuel production, such as corn, sugarcane, and soybeans, are competing directly with crops grown for human consumption. New sources of biofuels must be explored to avoid the issue of limited farmland. To this end, we have conducted research in the realm of algal biofuels.

Algae naturally produce long hydrocarbon lipids, which act as membrane components, sources of energy, and signaling molecules in algal cells. These lipids can be readily transformed into diesel fuel substitutes through a carefully controlled chemical reaction known as transesterification. Biodiesel from algae is renewable, similar to first-generation biofuels, but has the potential to surpass first-generation

biofuels in practicality. Additionally, because algae are aquatic organisms, they can be cultivated without the occupation of farmland for food crops. Finally, algae can produce more fuel per area of farmed land than crops like corn and soybeans. However, current production of algal biofuel is costly and renders algal biofuel economically unfeasible and consequently, an unrealistic alternative to fossil fuels. Therefore, the primary roadblock to successfully introducing algal biofuel as a replacement for fossil fuels is not determining if it will work as a substitute, but instead making its production more economically viable.

Current research on algal biodiesel is widespread and varied. Research experiments have included, but are not limited to, maximizing the efficiency of photobioreactors, optimizing algal growth conditions, determining the algal species most suited for large-scale biofuel production, and improving the cultivation of algae in outdoor ponds. Many companies and research groups are seizing the opportunity to make advances in this promising field. For example, the Department of Energy founded the Aquatic Species Program to focus on algal biofuel research. Although the Aquatic Species Program was concluded in 1996, its research laid important foundations for subsequent biofuel research around the nation, including companies such as Green Fuel Technologies. In 2006, Solix Biofuels began research on developing algal reactor technology that could be used with existing power stations. Today, Solix Biofuels is improving the efficiency of this technology and is conducting research on different species of algae (Gross, 2008). In 2007, the Department of Energy's National Renewable Energy Laboratory (NREL) and the

Chevron Corporation announced their collaboration on a research project to examine the production of transport fuels from algae (Gross, 2008). In July 2010, Exxon Mobil and Synthetic Genomics, Inc. (SGI) announced the opening of a new greenhouse facility in La Jolla, California, where researchers from both companies would evaluate different growth conditions, growth systems, natural and engineered algal strains, and the sustainability of each step in biodiesel processing (ExxonMobil 2010). Additionally, Dr. Yusuf Chisti, notable algae scientist of Massey University, New Zealand, has investigated the viability of algae grown in seawater, as it would then not compete with food or water supplies (Extance 2010). The highly diverse nature of research currently being conducted in the field of algal biofuels is a testament to the potential algae hold as a viable alternative energy source. Despite these efforts, current biofuel production practices are not economically viable. Therefore, additional research must be conducted in order to explore new ways of maximizing the efficiency of algal biofuel production.

Currently, one of the most promising avenues in improving algal biofuel production is nutrient deprivation. This method involves starving the algae of essential nutrients, which triggers a stress response that leads to an increase in lipid production. This stress response, however, also slows down the growth of the algal cells. In order to avoid this compromise between lipid yield and growth rate, the actual signaling pathways being affected by nutrient deprivation must be directly examined and manipulated. We have therefore chosen a novel way that could potentially target these algal growth and metabolic pathways directly and without

compromise. This approach involves the exogenous application of phytohormones, or plant hormones, to algal cultures. The algae *Chlamydomonas reinhardtii* was chosen for experimentation, due to its position as a model organism: the entire genome has been sequenced, procedures for growth and maintenance of cultures are well-established, and its comprehensive research base forms a strong foundation for groundbreaking research. *C. reinhardtii* does not naturally produce the greatest lipid yields when compared with other algal species, which also makes this species an ideal candidate for the exploration of the effects of phytohormones on algae. We concluded that if there were a significant increase in *C. reinhardtii* lipid yields due to the exogenous application of phytohormones, the principles of phytohormone application could be applied to other algal strains currently used in biofuel production, thus increasing the overall cost efficiency of biofuel production.

Why and how could phytohormones potentially produce effects in *C. reinhardtii*? We first selected the model plant, *Arabidopsis thaliana* to understand the role of phytohormones in plants and to explore the possible role of phytohormones in *C. reinhardtii*. We specifically chose *A. thaliana* for these purposes because it is not only a model organism, but also evolutionarily related to *C. reinhardtii*. Green algae and land plants can both be traced back to the green plant lineage, *Viridiplantae* (Merchant et al., 2007). We postulated that the common ancestral lineages of *C. reinhardtii* and *A. thaliana* would be supported through genetic homologies between the two species. We conducted an extensive comparative genomic analysis of the two organisms to identify some of these genetic homologies. Specifically, we focused on

proteomic homologies that could suggest the reception and functionality of specific phytohormones in *C. reinhardtii*. Based on the results of this genomic analysis, we identified three classes of phytohormones that show the greatest potential for augmenting effects on *C. reinhardtii*: auxins, brassinosteroids, and cytokinins. From these three classes, we chose one auxin, one brassinosteroid and two cytokinins. These classes of phytohormones regulate growth and metabolic processes in *A. thaliana*. Based on the roles of these phytohormones in *A. thaliana* and the proteomic homologies identified between *A. thaliana* and *C. reinhardtii*, we hypothesized that the application of these phytohormones to *C. reinhardtii* could potentially increase the growth efficiency and/or lipid yields of *C. reinhardtii*.

Lipid yields are dependent on both the total amount of algae produced, as well as the amount of lipids produced per cell of algae. An increased algal growth rate would result in a greater total amount of algae, which may produce more bulk lipids. Also, higher lipid productions per cell would increase total lipid yield. Augmenting algal growth and lipid yields, either singly or in conjunction with each other, would contribute to the maximization of algal biofuel production efficiency.

In the future, our findings regarding the effects of phytohormones on algal lipid production could be applied to the field of genetic engineering for the purpose of biofuel production. Our current method involves exogenously applying phytohormones to algal cultures, but genetic engineering allows for the possibility of manipulating algal species to auto-produce phytohormones. Auto-production of

phytohormones would significantly increase both the cost-effectiveness and time efficiency of algal biofuel production.

We adopted a low-technology, low-cost approach to growth and extraction procedures, which allowed us to explore the possibility of increasing the efficiency of small-scale, low-energy biofuel production. After we established the optimal growth conditions for *C. reinhardtii*, we introduced various concentrations of the different phytohormones to the algal cultures by direct supplementation to the culture media. We first tested phytohormones individually and later introduced combinations of these phytohormones to the algal cultures to determine the potential for synergistic effects between phytohormones. We determined which pairs of phytohormones to test by consulting literature on combinatorial effects of phytohormones. We also closely monitored the growth of the cultures supplemented with phytohormones by spectrophotometry. At the end of a growing cycle, we centrifuged the algae and then dried the algae by lyophilization in preparation for Soxhlet extraction. We then identified and quantified the lipids obtained from these processes using electrospray ionization - liquid chromatography - mass spectrometry (ESI-LC-MS). Finally, we conducted statistical analyses to determine the significance of the obtained results.

Our novel approach to increasing algal biofuel viability has great implications for understanding and directly manipulating algal growth and metabolic pathways. Much of the current research on algal biofuel is focused on varying factors involved in algae cultivation. While improving algal growth conditions and biofuel production efficiency is important in making algal biofuel economically viable, it is also practical

to research how to manipulate the algae to produce lipids in yields that would meet today's energy demands. Phytohormone supplementation taps into the potential for algae to grow faster and to produce lipids in yields that are not naturally possible.

## 1.1 Research Questions

Our research was directed by the following questions:

- 1) Do phytohormones affect the growth mechanisms of *C. reinhardtii* similarly to how they affect the growth mechanisms of plants, and if so, can they induce an increase in growth rate of *C. reinhardtii*?
- 2) Do phytohormones affect the metabolic pathways of lipid production in *C. reinhardtii* in a similar manner to how they affect the metabolic pathways of lipid production in plants, and if so, can they induce greater lipid yields in *C. reinhardtii*?

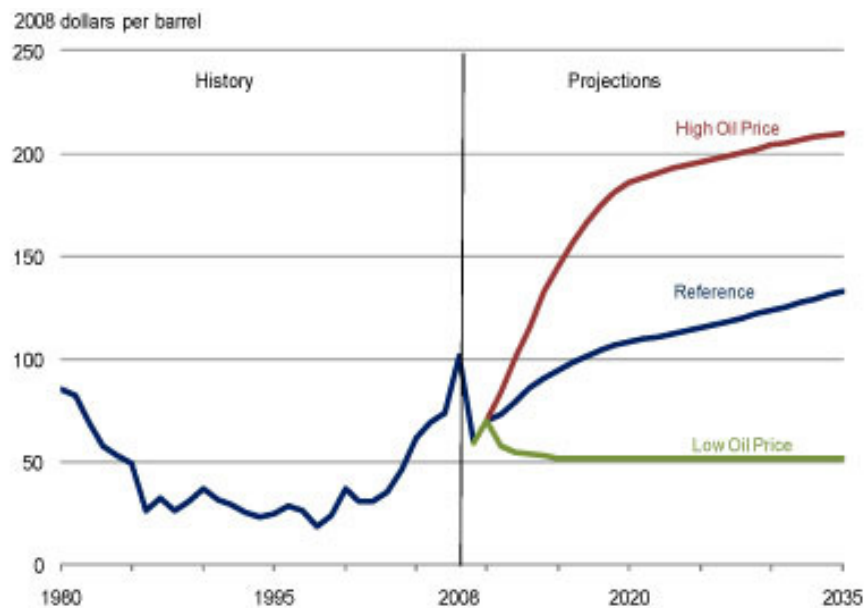
## **2. Literature Review**

### **2.1 The Current State of Alternative Energy**

#### **2.1.1 Background**

Researchers today are exploring a great number of alternative energy systems to find a viable replacement for fossil fuels, which include coal, natural gas, and petroleum. A major problem associated with these traditional energy sources is their non-renewability. Currently world energy needs are met largely by fossil fuels, especially for transportation purposes, but this trend cannot be sustained due to increased demand and limited supply. A recent statistical model of oil reserves and recoverable oil suggests that world production of oil will peak in 2014 (Nashawi, Malallah, & Al-Bisharah, 2010). This model, called the Hubbert Model, accurately predicted the 1970 oil bubble and has been recommended by experts for modeling the forecast of oil production. According to data compiled from 47 oil producing countries, the current crude oil reserves total 2140 billion stock-tank barrels (BSTB), and the remaining recoverable oil totals to 1161 BSTB (Nashawi, et al., 2010). The uncertainty in estimates for current oil production capacities is reflected in the supply-demand of this commodity worldwide. The \$50 fluctuation in the price of a barrel of crude oil between 2008 and 2009 also reflects this uncertainty (Department, 2010). Projections from the US Energy Information Administration (EIA), a data and statistical analysis agency within the US Department of Energy, suggests future

increases in the gap between highs and lows of oil prices leading up to the year 2035, as demonstrated in Figure 1. Based on these predictions, the market for fossil fuels is becoming increasingly volatile. Without the ability to produce more oil than is naturally occurring, there is no future in complete reliability on fossil fuel power.



**Figure 1: Projected gaps in worldwide crude oil prices based on variable supply-demand conditions reflecting 2008 and 2009 periods (Source: EIA 2010)**

An ideal alternative to fossil fuels must therefore be renewable and versatile. Renewable energy sources are defined as those which are not exhausted by use over time and are capable of being regenerated in a relatively short period of time. Such sources of energy include hydroelectric energy, nuclear power, solar power, wind power, and biomass-derived fuels. Analysis of the electricity market reveals the discrepancy between fossil fuels and alternative energy sources. Fossil fuel-based power plants, the primary source of electricity generation today, produce 85% of the current supply (Verbruggen, 2008). The remainder of the electricity supply is derived

from alternative energy sources. Direct comparison of these alternative energy sources can be performed by comparing the electricity generation occurring in the United States under these technologies. Estimates from the EIA, completed in 2010, place total renewable energy sources at 10.5% of the total electricity grid (Administration, 2011). Hydroelectric energy contributes 61% of this total renewable energy, up from 57% in 2007, while solar power, wind power, biomass, and geothermal contribute the remaining 39%, an increase from 32% of 2007 (Administration, 2011). Nuclear power contributes approximately 19.2% of the energy for electricity (Department, 2010). Fossil fuels, however, still provide the majority of electricity to the US grid, with coal-based electricity contributing 45.3%, natural gas-based electricity at 23.9%, and petroleum-based electricity providing 1.0% of total electricity (see Figure 2).

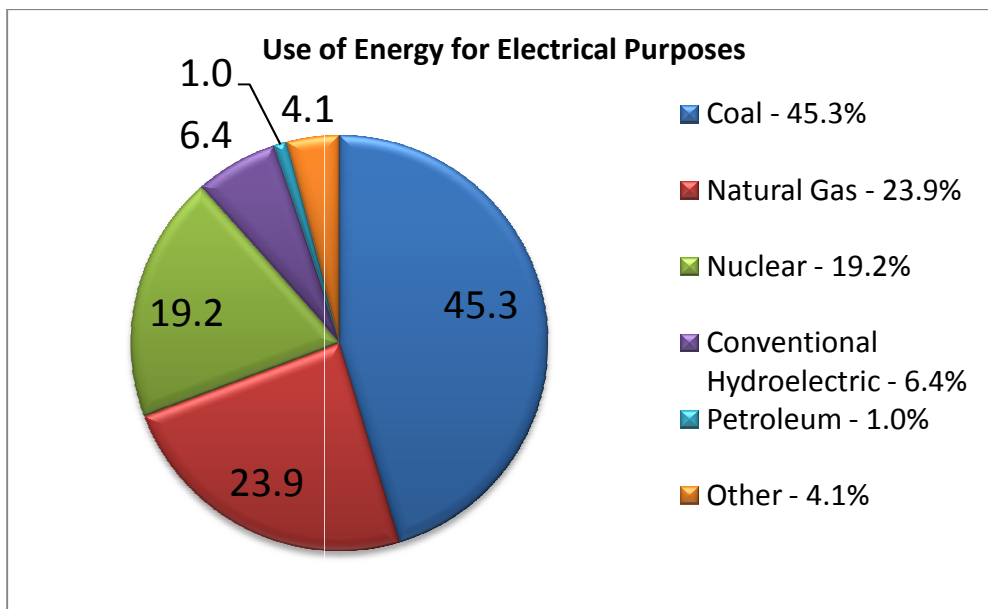


Figure 2: Conventional use of energy for electrical purposes (Note: values do not total to 100%)

While hydroelectric, nuclear, solar, and wind technologies are renewable, none fulfill all the necessary criteria to compete with fossil fuels as the primary source of energy. In order to compete with petroleum derived from fossil fuels, the optimal energy source must be competitive in costs and offer a strong case for utilization in the extant mobile transportation infrastructure. The often steep production costs of these alternative energy technologies, however, lead to higher end-user costs than those of petroleum. Additionally, new sources of energy must power motor vehicles, which represent 46% of petroleum use (Administration, 2011; Conti). Adapting to the current fuel distribution and transportation infrastructure is critical to reducing transitional costs of implementing new alternative energy technologies.

### **2.1.2 The Economics of Alternative Energy**

A major roadblock to the widespread implementation of these alternatives is that, currently, they are not cost-effective. A US Department of Energy report calculated that these energy systems retain a higher cost than fossil fuels, which produce electricity at a cost of \$0.03 per kWh (Conti). On the other hand, nuclear and solar power both cost four times as much, and wind energy is five to ten times more expensive depending on the location of the turbines. Hydroelectric energy is just two to four times more expensive than conventional petroleum, given availability of appropriate water sources (Wagner, 2007). The costs of these technologies can be

realistically compared by analyzing their levelized costs of production (see Table 1). This calculation of cost factors in the capital, operating, and maintenance costs of power plants and equates them to the total revenue that can be generated over a period of time (i.e. 40 years). This resulting cost can then be compared across technologies by adjusting for inflation in currency (see Table 2).

**Table 1: Costs of traditional and alternative sources of energy (2007) (Conti; Wagner, 2007)**

<b>Energy Source</b>	<b>Estimated Levelized Cost of Energy Generation (USD/kWh)</b>
<b>Fossil Fuels</b>	\$ 0.03
<b>Nuclear Power</b>	\$ 0.12
<b>Solar Power</b>	\$ 0.12
<b>Wind Power</b>	\$ 0.15 - 0.30
<b>Hydroelectric Power</b>	\$ 0.06 - 0.12

**Table 2: Estimated levelized costs of new generation resources (2016 projection) (Department, 2010)**

Plant Type	Capacity Factor (%)	U.S. Average Levelized Costs (2008 \$/megawatthour) for Plants Entering Service in 2016				
		Levelized Capital Cost	Fixed O&M	Variable O&M (including fuel)	Transmission Investment	Total System Levelized Cost
Conventional Coal	85	69.2	3.8	23.9	3.6	100.4
Advanced Coal	85	81.2	5.3	20.4	3.6	110.5
Advanced Coal with CCS	85	92.6	6.3	26.4	3.9	129.3
Natural Gas-fired						
Conventional Combined Cycle	87	22.9	1.7	54.9	3.6	83.1
Advanced Combined Cycle	87	22.4	1.6	51.7	3.6	79.3
Advanced CC with CCS	87	43.8	2.7	63.0	3.8	113.3
Conventional Combustion Turbine	30	41.1	4.7	82.9	10.8	139.5
Advanced Combustion Turbine	30	38.5	4.1	70.0	10.8	123.5
Advanced Nuclear	90	94.9	11.7	9.4	3.0	119.0
Wind	34.4	130.5	10.4	0.0	8.4	149.3
Wind – Offshore	39.3	159.9	23.8	0.0	7.4	191.1
Solar PV	21.7	376.8	6.4	0.0	13.0	396.1
Solar Thermal	31.2	224.4	21.8	0.0	10.4	256.6
Geothermal	90	88.0	22.9	0.0	4.8	115.7
Biomass	83	73.3	9.1	24.9	3.8	111.0
Hydro	51.4	103.7	3.5	7.1	5.7	119.9

## **2.1.3 Traditional Energy Alternatives**

### **2.1.3.1 Hydroelectric Energy**

Taking a more in-depth look at alternative energy sources, hydroelectric energy generation from rivers contributes the biggest load in supplying electricity to the United States. It currently provides 6.4% of the total electricity consumed (Department, 2010). Globally, however, hydroelectric energy provides 20% of the total electricity supply (Bakis, 2007). As an older, better-established technology, hydroelectric energy generation from rivers is more pervasive than the other alternative energy sources. Despite this fact, it still remains expensive. Building hydroelectric plants requires immense capital resources averaging \$1900 per kWh in 2007 USD. This is much greater than the initial capital costs of conventional fossil fuel-reliant power plants, which cost \$500 per kWh (Sovacool & Watts, 2009). However, this amount of money is lower than that needed for biofuel combustion plants, nuclear power plants, and solar power based plants. For the end-user, new hydroelectric plant construction by 2016 would provide electricity at the levelized cost of \$0.119 per kWh in 2008 USD (Department, 2010). This cost is higher than the levelized cost of electricity generation from coal or natural gas fired power plants.

Recently, waves and tides have been explored as a form of hydroelectricity generation. The energy in waves of large bodies of water can be harnessed by translating wave motion to mechanical motion of turbines in a similar manner as river-based hydroelectric energy production (Meyer, 2009). The motion of tides can turn a turbine as does flowing water in a river. While tides can provide usable

electricity, their availability and output is variable due to changing natural climatological patterns. Still, tidal cycles are more easily forecasted when compared to wind, another variable energy source (Denny, 2009). Though this may appear as an advantage of this system, the operation of such power plants on a cyclical schedule increases the mechanical stress on moving parts of the turbines, resulting in increased rate of component failure and higher maintenance costs (Denny & O'Malley, 2009). As a result, given high initial capital costs, electricity production from tidal energy will remain very expensive for the near future (Denny & O'Malley, 2009; Meyer, 2009).

#### **2.1.3.2 Nuclear Power**

Nuclear power offers the next best current alternative following hydroelectric power. This technology generates electricity through its release of heat from nuclear reactions. This technology has been explored for several decades now and has applied in many practical instances. Nuclear plant operating costs, however, have been declining very slowly in comparison to development of technology and other alternative energy sources. This slow rate of cost reduction is exacerbated by the expected increases in waste management costs for radioactive material (Du, Parsons, & Massachusetts Institute of Technology. Nuclear Fuel Cycle Technology and Policy, 2009). Currently, it is estimated that the levelized cost for nuclear-derived electricity is \$0.084 per kWh in 2007 USD, while moderate coal and gas prices are \$0.062 per kWh and \$0.065 per kWh, respectively ((Du, et al., 2009; JOSKOW, #160, L.,

PARSONS, & E., 2009). If the initial capital costs could be reduced to be equivalent to other power plants, the levelized cost of nuclear power would be reduced to \$0.066/kWh in 2007 USD, according to a study conducted at the MIT Center for Energy and Environmental Policy Research (Du, et al., 2009).

A second study by the EIA provides similarly disappointing levelized cost estimates for electricity from nuclear power. According to this study, the levelized cost of power from plants that would begin service in 2016 is high; it would cost \$0.119 per kWh in 2008 USD (Department, 2010). These high costs demonstrate that nuclear power does not provide the best energy solution nor will it achieve viability in the coming decade. Given this possibility, investors on Wall Street are currently hesitant to sink capital into the future of nuclear power (Saier & Trevors, 2010).

Their apprehension stems from the dramatic advances required in plant designs and construction that would be required in order to overcome the cost and concerns for nuclear reactors. Corroborated by economic evidence of its large waste management costs, further investment is unlikely to make this alternative energy a viable competitor to fossil fuels in the near future.

### **2.1.3.3 Solar Power**

Unlike nuclear power, solar power has the potential to provide waste-free and safe renewable energy. Sunlight is an abundant and essentially limitless form of energy which has been harnessed to make electricity through the use of solar photovoltaic (SPV) systems. This technology offers flexibility in increasing

production volume at low capital costs and negligible maintenance costs, excluding replacement costs due to finite solar panel lifetimes (Tiwari & Dubey, 2010). In order for SPV electricity to be economically competitive, however, it must reach grid parity, the point at which the cost to consumers is equal to or lower than that of grid electricity. Currently, the United States consumes about 98.6 quads [1 quad =  $2.9 \times 10^{11}$  kWh; 98.6 quads =  $2.9 \times 10^{13}$  kWh] of solar power each year, which is only a small fraction of the 46,700 quads [ $1.4 \times 10^{16}$  kWh] of solar energy that fall on the contiguous 48 states annually. Of the 98.6 quads consumed annually, only 0.08 quads [ $2.3 \times 10^{11}$  kWh] are actually converted to electrical energy (Dresselhaus & Thomas, 2001). This extremely poor ratio of solar intensity to energy production indicates the inefficiency of current solar power systems.

In addition to an inefficient conversion of available solar energy, installation of SPV systems require a large initial capital investment. For a typical residential system, the total installed cost is comprised of the PV module retail price, the balance of the system, installation, design, permits and other miscellaneous costs (Sista et al., 2010). The current installed cost of a standard 3.8 kW residential SPV system is \$8730 per kW in 2009 USD, while the grid parity price is \$2025 per kW (Yang, 2010). After taking into account operating cost, capital recovery, capacity and energy generated, the estimated levelized cost of SPV electricity is \$0.492 per kWh (Sista, et al., 2010). Compared to a grid parity cost of \$ 0.116 per kWh in 2009 USD, the cost of SPV electricity is, thus, far from competitive (Sista, et al., 2010). The EIA also reports a similarly high levelized cost of electricity production from SPV of \$0.396

per kWh in 2008 USD (Department, 2010). Although some recent reports suggest that solar grid parity may be reached by the year 2012, historical trends indicate that the necessary reduction in installed PV cost to reach grid parity is unlikely (Sista, et al., 2010).

Reducing the cost of SPV requires improvements to several facets of this technology. The problems associated with solar energy include the variability of sunlight with the time of day, weather conditions, and seasons (Dresselhaus & Thomas, 2001). Current efforts are aimed at increasing efficiency of photovoltaic modules to make the most of available sunlight. Additionally, research is focused on optimizing materials flows and processes to decrease manufacturing costs and increasing the manufacturing scale of single photovoltaic manufacturing plants (Fthenakis, 2009). Overall, based on the current state of SPV technology, it is an unlikely successor to fossil fuels.

#### **2.1.3.4 Wind Power**

Just as solar power does, wind power offers a waste-free and safe energy. Wind energy in air currents is transformed into electrical energy through the use of turbines, similarly to hydroelectric power. The energy output from a turbine depends on both the size of the turbine blades and the speed of the wind as it passes through the rotor. As turbines have increased in size and been concentrated in more turbulent areas, the cost of harnessing energy from wind power has declined significantly over the past 20 years (Association, 2009). In the 1980's, when large-scale wind turbines

were first put to use, wind-generated electricity cost \$0.30 per kWh in levelized 1980 USD. In 2009, the EIA reported that levelized cost of electricity from wind energy was approximately \$0.149 per kWh in 2008 USD (Department, 2010). The cost of wind-generated electricity is expected to continue to fall as technology improves and larger wind plants are built.

To make the further implementation of wind technology possible, reductions in the costs must be continued. The production of wind energy is a very capital-intensive process; in addition to the actual turbine, many other items must be purchased in order for the turbine to function properly, such as the foundation, necessary electrical equipment, and grid connection accessories. Approximately 75% of the total cost of producing electricity from wind is derived from these initial capital costs (Krohn, Morthorst, & Awerbuch, 2009). However, the government has stepped in to push adaption of wind power. The government currently subsidizes wind power farms by offering production tax credits. Including these tax credits, the best turbines operating under ideal wind conditions can produce electricity at a levelized cost of less than \$0.05 per kWh (Association, 2009). Although the production tax credit (\$0.015 per kWh) helps mitigate the costs of establishing a wind energy production site, the very high initial investment that is required remains a barrier to the expansion of this technology (Association, 2009). Wind power thus does not provide cost competitive energy compared to that from fossil fuels. Significant reductions to the initial costs of building a wind farm are necessary to increase viability.

## **2.1.4 Biofuels**

### **2.1.4.1 The Problem of Portability**

Aside from their failure to be economically competitive with fossil fuels, another issue faced by the above alternative energy sources is the issue of portability. An ideal fuel source would be easily transportable in existing infrastructure and compatible with the internal combustion engines used in most existing automobiles. Hydroelectric, nuclear, solar, and wind technologies are at a major disadvantage compared to liquid fuels such as petroleum because of their lack of portability (Ozaktas, Cigizoglu, & Karaosmanoglu, 1997). Currently, petroleum-based fossil fuels are transported throughout the United States through a network of pipelines. For this reason, an energy source must be able to flow through the existing pipelines if it is to be competitive with petroleum. While non-liquid forms of energy show promise on a smaller scale, the substitution of any of these alternatives for liquid fuel would essentially uproot the pipeline infrastructure and require a complete renovation of the nation's fuel distribution system. Progress has been made in the development of electrical vehicles, but most of the electric cars that are currently being produced are hybrid electrics, which still require gasoline. Therefore a liquid substitute to traditional gasoline is essential.

### **2.1.4.2 Biofuel Background**

Energy sources that fulfill the portability requirement are limited. However, one alternative source that is readily portable is biofuel. Biofuels encompass all liquid

fuels that are derived from biological matter. They are chemically similar to current liquid fuels, and have the advantage of compatibility with current internal combustion engines (Demirbas, 2006). Currently they are the most promising alternative to petroleum fuel. An estimate by the EIA from 2005 suggests that an electric power plant running on biofuel would produce energy at \$0.051 per kWh. This is \$0.01-0.03 per kWh greater than that of energy produced by burning fossil fuels. While this difference is smaller than that of most other alternative energy systems, costs need to be further reduced for biofuel to be economically viable. However, there is promise in the advancement of two major types of biofuel: ethanol and biodiesel. These can be obtained from a variety of different sources which have yet to be fully explored and optimized.

#### **2.1.4.3 Ethanol Background and Economics**

Ethanol is an environmentally-friendly and easily manageable liquid substitute for fossil fuels. Many view it as a promising alternative that will aid the United States in reducing dependency on foreign crude oil imports (Searchinger et al., 2008). However, its higher cost and a lower energy density compared to gasoline explain its slow assimilation into the American fuel economy. In current application, ethanol is combined with traditional gasoline in a fixed ratio, between 70-85% (e.g. E85 gasoline blend contains 85% ethanol). Ethanol can be derived from several different sources, including microorganisms. However, the prevalent sources of ethanol are corn and other crops such as sugarcane. Use of these sources imposes the opportunity

costs of using the same land that would otherwise be used for food production and storage. Since the United States and other countries began growing significant amounts of corn and soybeans specifically for biofuel production, world prices for these and other food commodities have increased drastically. Between January 2005 and June 2008 corn prices nearly tripled; wheat prices rose by 127%, and rice prices increased by 170% (Rubin, 2008). According to research conducted by the International Monetary Fund, these price increases were primarily caused by the growing demand for biofuels. The effects of these price increases have been devastating, particularly to low-income individuals residing in developing countries, and expanding the use of corn-based ethanol will lead to increased stress on land resources and could potentially cause worldwide food shortages (Rosen, Shapouri, Quanbeck, & Meade, 2007). In addition, increased or mandated ethanol production from feedstocks is likely to stimulate an increase in the prices of other commonly-used sources of energy. For example, studies have shown that higher ethanol fuel production leads to an increase in the cost of natural gas (Whistance & Thompson, 2010).

Another factor is that ethanol is still not as energy or cost efficient as fossil fuels. In 2005, 14.3% of US corn crops were used to produce ethanol fuel; however, the energy yield was only 1.72% of fossil fuels energy output (Hill, Nelson, Tilman, Polasky, & Tiffany, 2006). The ethanol net production cost was \$0.46 per energy-equivalent-liter in 2005, while traditional gasoline production prices averaged \$0.44 per liter (Hill, et al., 2006). While US gasoline prices averaged \$1.90 per gallon in

2007, US ethanol prices were approximately \$2.50 per gallon that same year (Goldemberg, 2007). The combustion of ethanol is simply less efficient than that of traditional fossil fuels.

#### **2.1.4.4 Ethanol Portability and Other Considerations**

Ethanol is a liquid fuel, similar to gasoline in that it can be pumped into automobile tanks in order to fuel vehicles. For this reason, it may seem that decreasing the costs of ethanol will allow it to replace gasoline as the primary energy source for transportation. However, in addition to the higher cost and lower energy output of ethanol, it is incompatible with the current American fuel distribution infrastructure. It is unsuited for transportation through extant pipelines because of its corrosive properties (Yacobucci, Schnepf, & Library of Congress. Congressional Research, 2007). Its hygroscopic nature will attract moisture in pipeline which results in phase separation of ethanol-gasoline blends and reduced engine performance in a vehicle. Further, ethanol is a better solvent than gasoline. Shipments of ethanol in existing pipeline would pick up impurities that had accumulated in the pipeline. Many manufacturers, thus, are wary of using pipelines for distribution and instead rely on trucks to deliver ethanol-gasoline blends (Whims, 2002). Additionally, most corn farms are concentrated in the Midwestern United States, but gasoline consumption is highest along the East and West Coasts. Therefore existing pipelines would not suffice for ethanol transport as they are not situated in Midwestern US (Whims, 2002). Pipelines, however, are not completely out of the picture. Efforts are being

made to enable pipeline transportation of ethanol or ethanol-blended gasoline by coating pipeline interiors with corrosion-resistant materials or utilizing dewatering solutions (Whims, 2002; Yacobucci, et al., 2007). However, such projects, even if successful, are likely to be expensive.

While most fuel ethanol has been derived from crops, other potential sources have been exploited as well. In Brazil for example, the price per gallon of sugarcane ethanol has declined so that it is below that of gallon of gasoline (Goldemberg, 2007). Another potential option is cellulosic ethanol. Cellulosic ethanol is created from the conversion of non-edible parts of crops, trees such as hybrid poplar and willow, wood residues such as chips and sawdust, municipal residues, paper and sewage sludge, and grasses such as switchgrass, sorghum, and miscanthus (Solomon, Barnes, & Halvorsen, 2007). This cellulose is converted into sugars, which are then fermented into ethanol (Solomon, et al., 2007). One advantage of cellulosic ethanol is that it is unlikely to compete with human food supplies because most of its sources are inedible or waste products. Other advantages include a reduction in net CO<sub>2</sub> emissions, competitive projected costs, and a greater energy output to input ratio than grain ethanol (Solomon, et al., 2007). Still, the relatively small amount of energy available from ethanol leaves many skeptical of its incorporation as a major source of fuel and its potential economic impact.

#### **2.1.4.5 Biodiesel Background**

Although crop-derived biodiesels are involved in this "food vs. fuel" debate as well, they are far more energy efficient than ethanol. Soybean biodiesel provides 93% more energy than its starting materials during processing, while corn ethanol has only a 25% net energy gain (Hill, et al., 2006). While biodiesel has traditionally been derived from vegetable oils or animal fats, recent research has shown promise in obtaining biodiesel from algal species. The vast majority of previous research has been focused on the production of biodiesel from plant lipids, including soybean oil, sunflower oil, and coconut oil (Ma & Hanna, 1999). However, any lipids can theoretically be converted to biodiesel, even those of algae. Algal biodiesel's properties are in line with the global movement toward clean and renewable fuel sources. It is non-toxic, biodegradable, and produces comparatively low levels of atmospheric emissions making for an environmentally friendly alternative to fossil fuels (Ma & Hanna, 1999). The most common and straightforward method for this conversion process is transesterification, a reaction of lipids and alcohol that produces fatty acid esters from the lipids. The transesterification of algal oils has been successfully performed in industrial scale bioreactors (X. Li, Xu, & Wu, 2007; Xiong, Li, Xiang, & Wu, 2008). Factors influencing the effectiveness of this reaction include the purity of the harvested lipids and experimental parameters such as temperature, alcohol content, and the presence of reaction-accelerating catalysts. Application of biodiesel produced from algae has already been tested in several applications, including a highly successful two-hour test flight on a Continental Airlines Boeing 737-800 in which a 50%-50% mixture of biodiesel and conventional jet fuel was used

(Biello, 2009). Despite its renewability and promise, the commercial introduction of biodiesel has been slowed by the high cost and low yield of the transesterification process (Fukuda, Kondo, & Noda, 2001).

#### **2.1.4.6 Algal Biofuels and Biodiesel**

Although crop-derived fuels have been viewed as a positive step towards renewable energy, research indicates that algae may be the best source for biodiesel because of its high lipid content (Hossain, Salleh, Boyce, Chowdhury, & Naquiuddin, 2008). In recent years, the push from the "food vs. fuel" conflict has stimulated increased interest in biodiesel obtained from sources other than food crops.

Calculations have demonstrated that biodiesel from food crops would only meet 50% of transportation fuel demands, even if 24% of the total US cropland were used for biodiesel production. Contrary to crop-based fuel, biodiesel produced from algae could meet as much as 50% of the fuel demand while utilizing only 3% of the total cropland (Chisti, 2007). Since research has demonstrated that the oil content in algae can exceed 80% of the biomass dry weight, algae have the potential for high lipid productivities (Chisti, 2007). Though algal biodiesel has a promising future, the limitations of current growth rates and lipid yields must be carefully weighed. In selecting an algal species for biodiesel production, there are several factors to consider. The algal species must provide a fast growth rate and high lipid yield. On average, green algae cells double in 24h. This translates to a mean specific growth rate, mean number of doubling cycles, of  $0.69 \text{ day}^{-1}$  (Griffiths & Harrison, 2009). See

Table 3 for the doubling time of some common algal species. Note that these doubling times are for growth in the algae's optimal conditions, the maximum growth rate obtainable. Therefore, it represents the maximum growth rate obtainable under standard growth conditions. Lipid content is the second important factor to consider. It indicates the volume of biodiesel that can be extracted for a given mass of algae. See Table 4 for a summary of the lipid contents by weight percent. These values, even at the lower end, greatly exceed the oil productivity of the best producing oil crops (Carioca, Hiluy Filho, Leal, & Macambira, 2009; Chisti, 2007). An additional factor to consider is the chemical structures of the lipids produced. Lipids with fewer functional groups, in particular neutral lipids such as triacylglycerols, are more easily converted to usable fuels than other lipid types as they require minimal post-transesterification refinement (Hu et al., 2008).

Currently, production of biodiesel from unaltered, natural algae does not provide production rates to make this fuel cost-effective in comparison to fossil fuels. The yields of lipids and the growth rates of the algae must be increased to make biodiesel a logical option to fossil fuels.

**Table 3: Doubling time of some common algal species**

<b>Algal Species</b>	<b>Growth Rate (day<sup>-1</sup>)</b>	<b>Growth Conditions</b>
<i>C. reinhardtii</i>	1.5	Ideal
<i>C. vulgaris</i>	2.6	Ideal
<i>B. braunii</i>	2.0	Ideal
<i>C. protothecoides</i>	1.1	Ideal, Heterotrophic

**Table 4: Lipid contents (by weight percent) of some common algal species (Source: Lee et al. 1998; Sialve et al. 2009; Xiong et al. 2008)**

<b>Algal Species</b>	<b>Lipid Content</b>
<i>C. reinhardtii</i>	<b>21%</b>
<i>C. vulgaris</i>	14-22%
<i>B. braunii</i>	26.8%
<i>C. protothecoides</i>	50-57%

#### **2.1.4.7 Economics of Algal Biodiesel**

A reduction in the cost of algal biofuel production can be brought about in three ways. These are increasing the growth rates, increasing lipid yields of the algae (resulting in increased lipid production per unit of biomass), and decreasing the energy requirements of production, particularly the harvesting and extraction processes. Several methods of cost reduction via increased growth rates and lipid yields have been targeted for current research. Genetic engineering of algal strains is one of the most promising fields of research to increase the metabolic production of lipids. Alternative research has focused on improving nutrient supplementation and oxidative stress in order to increase lipid yields.

Costs can be cut in other facets of algal biodiesel production as well. In its current state, the production process remains too expensive to be implemented on a large scale. While growing algae is relatively inexpensive due to the low costs of many of the inputs (i.e. sunlight, water, etc.), the post-growth procedures are extremely costly. The biomass must be harvested, the lipids extracted, and the

remaining cell components recovered. These post-growth steps, necessary for preparation of lipids for conversion to biofuel, contribute the majority of the costs associated with the process (Wijffels & Barbosa, 2010). The preferred method of harvesting the algal biomass is centrifugation, a very expensive process due to its large capital costs and energy requirements that translate to additional expenses. Currently, the harvesting step contributes 20-30% of the total cost of algal cultivation (Demirbas, 2006). If improvements can be made in the design of photobioreactors, biomass harvesting techniques, and downstream processing technology, costs can be reduced considerably.

Extraction of lipids from the algal cells is also another costly step. This requires the use of expensive solvents, which add significantly to production costs. Using the current production technologies available, the current cost of one liter of algal biodiesel is \$8.80 (Fortman et al., 2008). This cost translates to a per-gallon cost of \$33.31, or \$1399.08 per barrel. Crude oil currently sells for under \$80 per barrel. Based on current analyst estimates, it is not expected to surpass \$200 per barrel anytime soon (Department, 2010). Thus, at this point algal biodiesel is far too expensive to be sold commercially. Its cost must be reduced by at least a factor of ten for it to become a competitive alternative to current options. Though the suggested improvements in production processes will reduce cost and make production more efficient, making gains in growth rates and lipid yields remains the primary priority to improve the efficiency of this biofuel. (Q. Li, Du, & Liu, 2008). By being able to produce more lipids, at a faster rate per area, cost-effectiveness of this alternative

energy will be significantly improved. To compare algal biodiesel to some of the other popular alternative energy options, Table 5 includes a summary of the important factors in alternative energy that have been discussed so far.

**Table 5: Checklist of ideal criteria for alternative energy**

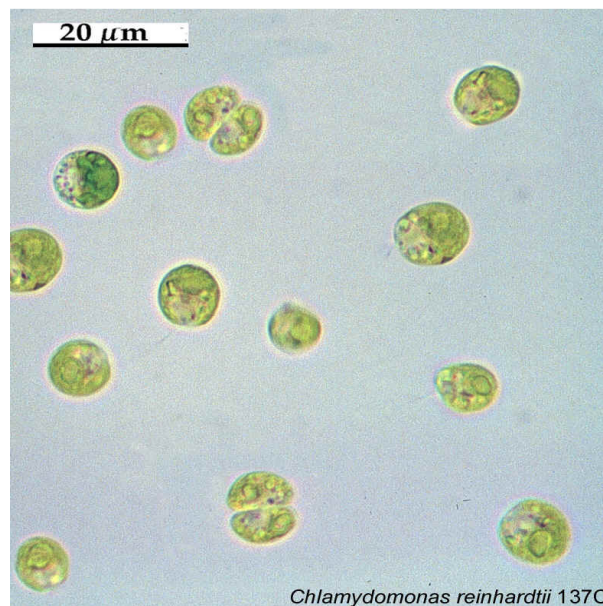
	<b>Portable through pipelines</b>	<b>Compatible with existing engines</b>	<b>Renewable</b>	<b>Does not compete with food supply</b>
<b>Petroleum</b>	Yes	Yes	No	Yes
<b>Hydroelectric</b>	No	No	Yes	Yes
<b>Nuclear</b>	No	No	Yes	Yes
<b>Solar</b>	No	No	Yes	Yes
<b>Wind</b>	No	No	Yes	Yes
<b>Ethanol</b>	No	Yes	Yes	No
<b>Crop biodiesel</b>	Yes	Yes	Yes	No
<b>Algal biodiesel</b>	<b>Yes</b>	<b>Yes</b>	<b>Yes</b>	<b>Yes</b>

## 2.2 Chlamydomonas reinhardtii

### 2.2.1 Chlamydomonas reinhardtii for Biodiesel

We have selected *Chlamydomonas reinhardtii* for our investigation of biofuel production. *C. reinhardtii* has been extensively studied and is considered a model organism whose physiology can be applied across different algal species. Its well characterized growth and metabolism make it especially suitable for examining changes to these processes. Effects on growth and lipid metabolism due to influences of environmental and nutrient conditions are readily observable in this alga. This

makes *C. reinhardtii* practical and effective for experimentation and measuring consequential changes in growth rates and lipid yields (Guschina & Harwood, 2006). Additionally, when compared to other algal species, *C. reinhardtii* allows easy growth and cheap maintenance of cultures. This is due to its well documented and fast reproductive rates, measured as specific growth rate ( $\mu$ ) or number of doublings in a day, and photosynthetic capacities, the quantified flux of photons across a cell that can be utilized for photosynthesis and results in the production of O<sub>2</sub>. These properties make simpler and frequent analysis of its growth and lipid production possible, thereby reducing costs of experiments. Further, as a model organism, *C. reinhardtii* also has a fully sequenced genome which allows for accessibility in uncovering genetic features that lend to its phenotypes, and it allows comparison of genomes to other organisms. A thorough analysis of this algal species' genetic relevance to this study will be conducted in subsequent sections.



**Figure 3: Light microscopy images of *C. reinhardtii*** (Source: <http://www.uniprot.org/taxonomy/3055>)

Additionally, *C. reinhardtii*, like other algae, can produce lipids which can be converted to biodiesel through a transesterification reaction.(Chisti, 2007; Thompson, 1996). This reaction uses a nucleophile, such as methanol, to attack the carboxylate group of the fatty acid esters of the lipid. This proceeds to the separation of the fatty acid methyl esters from the glycerol backbone. This reaction possibility makes *C. reinhardtii* particularly relevant to studying the impact of changes in growth and metabolism on biodiesel productivity. Although other algae can potentially produce biofuels at higher productivity levels as seen Table 3. *C. reinhardtii* is a more favorable organism for study due to its greater simplicity.

Other organisms such as *Botryococcus braunii* have very high lipid yields. However these lipids come in the form of triterpenes, which cannot undergo transesterification (Metzger & Largeau, 2005). Instead, they must undergo a more complicated hydrocracking procedure to transform the triterpenes into octane, kerosene, or diesel (Hillen, Pollard, Wake, & White, 2004). Additionally, the fact that *C. reinhardtii* has a fully sequenced genome allows the prospect for exploration of empirical differences in the different types of lipids produced due to changes at the genomic level. Therefore, *C. reinhardtii* was the logical and suitable choice for investigating changes in growth rates and lipid yields due to effects of phytohormones. As a model organism, physiological responses found in this alga would be more likely to translate to other algal species. This could lend to further

improvements in lipids yields from algae that already show higher productivity than *C. reinhardtii*.

## **2.2.2 Biological Characteristics**

### **2.2.2.1 Metabolism**

To optimize the lipid production of *C. reinhardtii*, it is important to understand the photosynthetic and metabolic processes that take place within the cells (Boyle & Morgan, 2009). *C. reinhardtii* is a facultative heterotroph, meaning that while it performs photosynthesis to produce energy, it can also grow by fixing carbon in the absence of light (El-Sheekh, Kotkat, & Hammouda, 1994). Normally, *C. reinhardtii* grows photoautotrophically using its photosynthetic apparatus to harvest light to transfer electrons and eventually produce NADPH and ATP. These molecules allow the cell to function and fix carbon via the Calvin Cycle (Rochaix, 1995). *C. reinhardtii* grown heterotrophically on only acetate, however, has been shown to produce a lower biomass yield, stressing the importance of light for growth. *C. reinhardtii* has also been demonstrated to grow mixotrophically, which utilizes photosynthesis and carbon molecules in culture medium. Refer to the subsequent section titled Photosynthetic Capacity for discussion of the light utilization efficiency of *C. reinhardtii*.

Genomic and biochemical information has enabled the reconstruction of the *C. reinhardtii* metabolic network. Results from this model indicate biomass yields of 28.9 g algae per mole carbon for autotrophic growth, but only 15 g algae per mole

carbon for heterotrophic growth (Boyle & Morgan, 2009). Additional genomic techniques applied to *C. reinhardtii* have delineated several lipid biosynthesis pathways. Further, comparison of lipid biosynthesis pathways between *C. reinhardtii* and *A. thaliana* have revealed strong similarity. Known and hypothesized pathways for biosynthesis of lipids in *C. reinhardtii* are illustrated in Figure 4. These pathways in *C. reinhardtii* show slight deviations that make them simpler than those in flowering plants (Riekhof, Sears, & Benning, 2005).

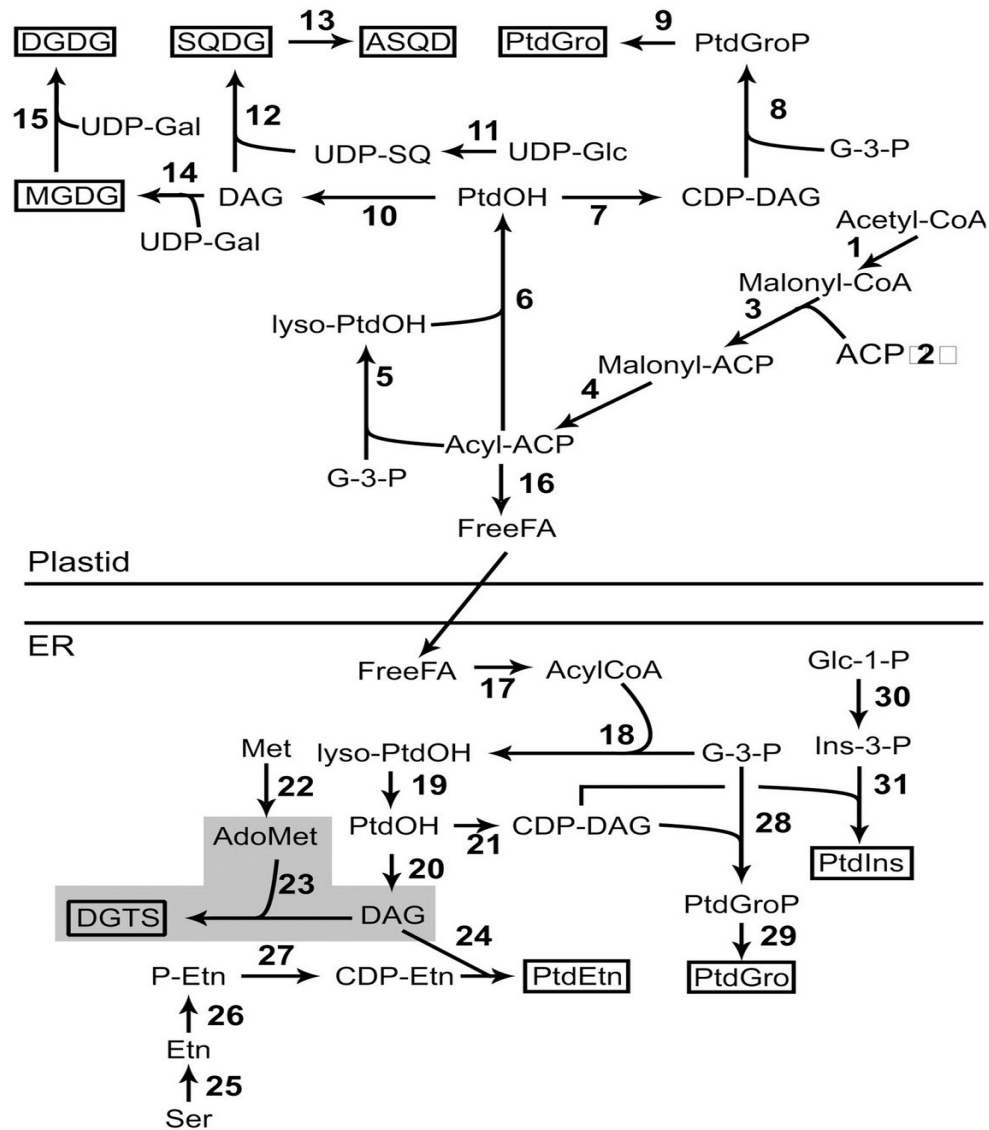


Figure 4: Known and hypothesized pathways for biosynthesis of lipids in *C. reinhardtii*. The lipids of interest in algae are DATA, diacylglycerol; DGDG, digalactosyldiacylglycerol; DGTS, diacylglyceryl-N,N,N-trimethylhomoserine; FA, fatty acid; MGDG, MGDG, mono-galactosyldiacylglycerol; PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol; PtdIns, phosphatidylinositol; and SQDG, sulfoquinovosyldiacylglycerol. (Source: Rheikof et al 2005, Eukaryotic Cell 2005)

#### 2.2.2.2 Reproductive Capacity

One of the reasons *C. reinhardtii* is the algal organism of choice is its short life cycle and generation time (Levine & Ebersold, 1960). *C. reinhardtii* can grow

under a variety of conditions contingent upon autotrophic, heterotrophic, or mixotrophic means of growth. At the bare minimum in Minimal media, containing only a few inorganic salts, trace elements, and paired to a light source emitting light between 2700 to 5400 lux can result in doubling rates between 6-25 hours (Donnan, Carvill, Gilliland, & John, 1985; Levine & Ebersold, 1960). This wide range of specific growth results in the past is due to the ability of *C. reinhardtii* to undergo both sexual and asexual reproduction (Levine & Ebersold, 1960). Sexual reproduction generally takes longer because it takes 2 to 4 hours for *C. reinhardtii* to prepare for meiosis, 12 hours to finish meiosis and create gametes, and then 3.5 hours to finish fusion of gamete cells (Levine & Ebersold, 1960). Asexual reproduction is much faster because *C. reinhardtii* will undergo multiple fission cycles within the doubling period. The actual number of daughter cells generated depends on the cell volume of the paternal cell. This variable is directly affected by changes in light cycles, nutrition, and cell size (Oldenhof, Zachleder, & Van den Ende, 2007; Spudich & Sager, 1980).

In addition to these factors, doubling time can be further hastened to 5 hours by increasing mineral salt concentration 10 fold, aerating the culture with 5% CO<sub>2</sub>, and agitating cultures over 1 L in total volume (Levine & Ebersold, 1960). Under normal photosynthetic growth, *C. reinhardtii* will reproduce to a final cell concentration of  $2 \times 10^7$  cells/mL (Levine & Ebersold, 1960). Depending on the focus of a particular study, a variety of growth conditions can be selected from those

described here in order to regulate the *C. reinhardtii* cell cycle and growth rate (Donnan, et al., 1985).

### **2.2.2.3 Photosynthetic Capacity**

The PSI and PSII photosystems of *C. reinhardtii* absorb light of wavelengths 705 nm and 680 nm, respectively (Rodolfi et al., 2008). The minimum photosynthetic capacity of *C. reinhardtii* is 0.103 O<sub>2</sub> evolved per photon absorbed at an 84% photon utilization of absorbed light. Under normal conditions, however, several biological and ecological issues of growth in algal cultures influence efficiency of the photosynthetic processes. More than 80% of light absorbed over a normal day may be wasted by large algal clumps scattering incoming light. Larger algal clumps block light from reaching the smaller clumps, leading to loss of available light and therefore decreasing the maximum efficiency of the algal culture. Thus, increasing intensities of light may not provide positive benefits to growth due to scattering of incoming light by the naturally forming clumps of algae (Melis, 2009).

Further, there is a large discrepancy between the total amount of available energy and the amount of energy actually utilized in photosynthesis. Photon saturation levels, the maximum number of photons that the photosynthetic apparatus of the algae can utilize at a given instant, for algal cultures reach approximately 400  $\mu\text{mol photons per m}^2/\text{s}$ , whereas total light levels can reach 2500  $\mu\text{mol photons per m}^2/\text{s}$  at midday (Melis, 2009). A majority of the energy in available light is thus wasted. Most of this light also happens to fall on to cells closest to the surface of a

culture flask. One method for correcting for this problem is to decrease the size of the light harvesting antennae complexes in the algae (Beckmann et al., 2009). This can increase the photosynthetic efficiency of algae by reducing the available amount of light per cell. The absorption of fewer photons per cell translates to increased efficiency for the entire algal culture by reduction of total light dissipation. Thus, cells in the center of the culture flask would receive more incoming photons. Due to all of these factors affecting photosynthetic efficiency of algae, the reproduction capacity and, thus, growth rate of *C. reinhardtii* can be different among algal cultures.

#### **2.2.2.4 Biomass productivity of *C. reinhardtii***

The biomass productivity of algae is a product of the growth rate and the period of growth. The conditions under which *C. reinhardtii* is grown can have significant influence on both of these variables. The light and temperature set for cultures in our study will closely match those used by Akimoto et al. (Akimoto, Yamada, Ohtaguchi, & Koide, 1997). Optimally, in room temperature and indirect sunlight conditions, *C. reinhardtii* has a specific growth rate of  $1.50 \text{ day}^{-1}$  (Akimoto, et al., 1997). Growing algae under these conditions does not provide the fastest growth rate; however, it is most cost-effective as it reduces maintenance costs for cultures. At the mentioned specific growth rate, *C. reinhardtii* can be expected to accumulate to a maximum yield of 1.11g/L of growth media in dry weight (Akimoto, et al., 1997). A higher cell yield would also imply increase of total lipid yield from a

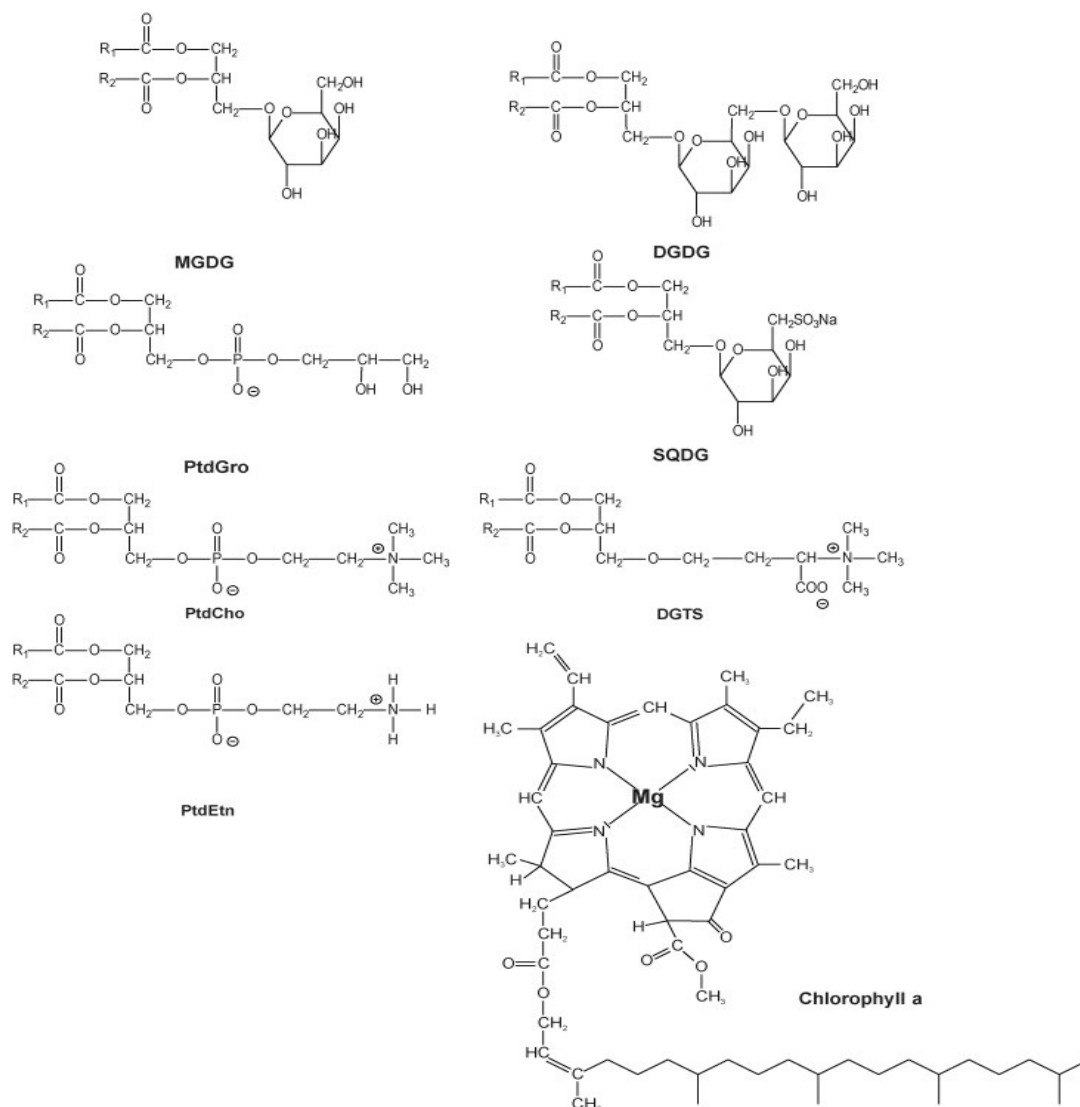
culture.

### 2.2.3 Lipids of *C. reinhardtii*

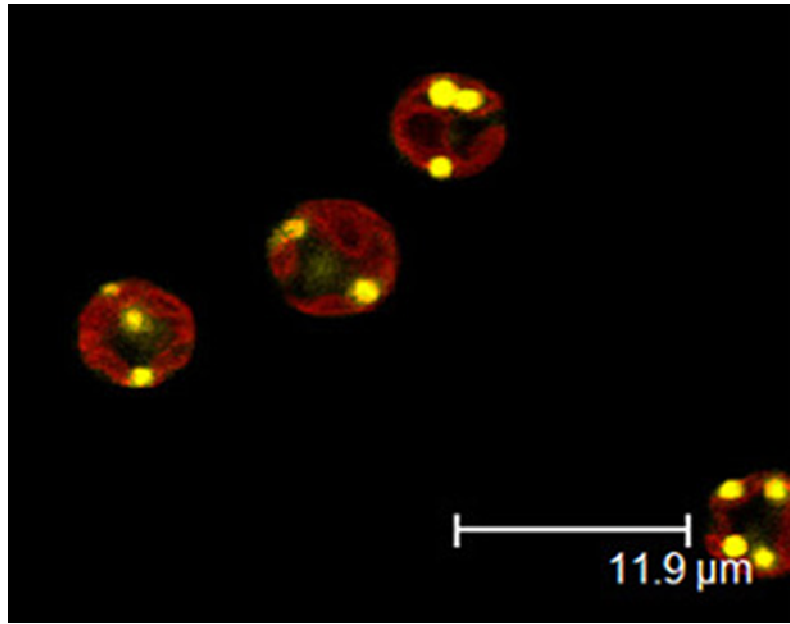
Biodiesel from algae can be generated by transesterification of any type of ester lipids found in the algal cell. These lipids fall into three primary types: neutral lipids, glycolipids, and polar (i.e. acidic) lipids (Cooksey, Guckert, Williams, & Callis, 1987). Of the three, neutral lipids, including triacylglycerides, are most favorable for conversion to biodiesel due to their higher degree of saturation (Hu, et al., 2008). A higher degree of saturation, or fewer double bonds in the lipid molecule, results in biodiesel that requires minimal post-production refinement (Meher, Vidya Sagar, & Naik, 2006).

*C. reinhardtii* contains four major glycerolipids: Monogalactosyl-diacylglycerol (MGDG), digalactosyl-diacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), and glycerophospholipids (GP) (Sato, Hagio, Wada, & Tsuzuki, 2000; Vieler, Wilhelm, Goss, Süß, & Schiller, 2007a; Z. T. Wang, Ullrich, Joo, Waffenschmidt, & Goodenough, 2009). The structures of these lipids are illustrated in Figure 5. The percentages for MGDG, DGDG, and SQDG, and GP in *C. reinhardtii* are 42%, 12%, 13%, and 9% respectively. The GP group accounts for phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), and phosphatidylglycerol (PtdGro). Another major source of lipids is lipid bodies. In *C. reinhardtii*, lipid bodies may form as seen in Figure 6. Lipid bodies are composed of

90% triacylglycerol and 10% free fatty acids. Of the fatty acids associated with triacylglycerol, 50% are C16 to C18 saturated fatty acids, 25% is oleic acid, and the remaining 25% are other unsaturated fatty acids (Wang et al., 2009). Besides these lipids, Chlorophyll a is a major constituent of *C. reinhardtii*. This pigment is vital for the photosynthetic apparatus. It also contains a single fatty acid chain that can be used in biodiesel production. (Vieler, et al., 2007).



**Figure 5: Structures of the most important lipids in *C. reinhardtii*.** The abbreviations are as follows – MGDG: Monogalactosyl-diacylglycerol, DGDG: digalactosyl-diacylglycerol, SQDG: sulfoquinovosyldiacylglycerol, PtdGro: phosphatidylglycerol, PtdCho: phosphatidylcholine, PtdEtn: phosphatidylethanolamine, and DGTS: 1,2-diacylglycerol-3-O-4'-(N,N,N-trimethyl)-homoserine. *Source: Vieler et al. 2007.*



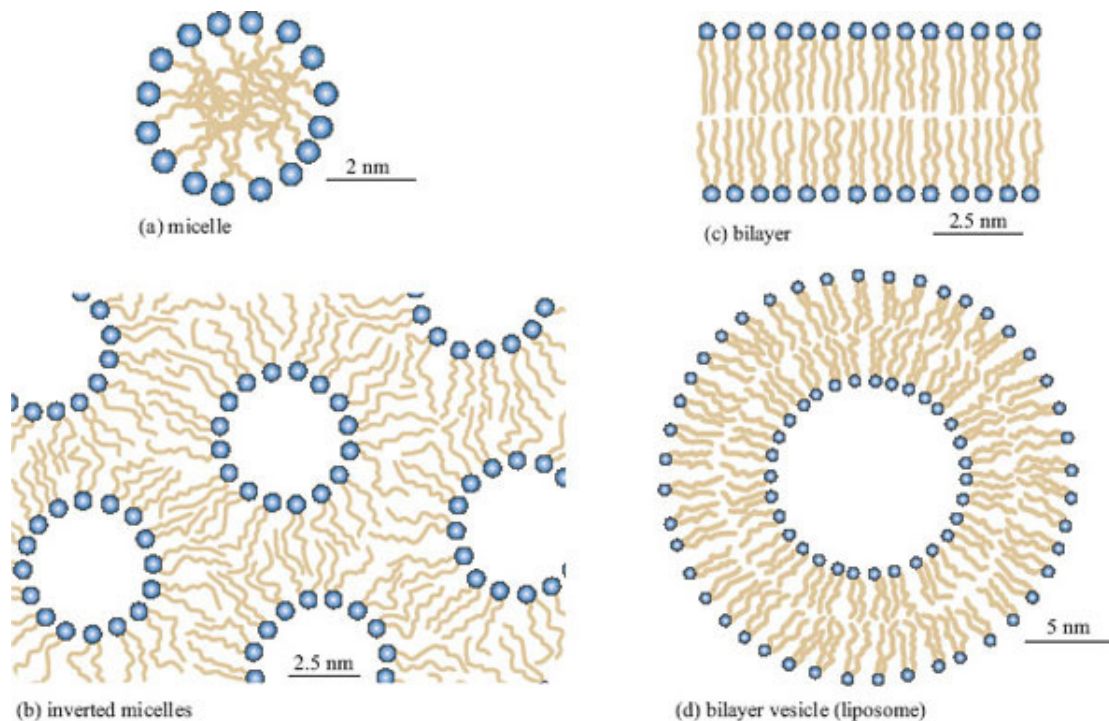
**Figure 6: Confocal microscopy survey of lipid bodies in *C. reinhardtii*.** Red is the autofluorescence of chlorophyll and yellow is fluorescence of lipid bodies stained with Nile Red dye. These algae represent wild-type *C. reinhardtii*. (*Source: Wang et al 2009, © Eukaryotic cell, 2009.*)

### 2.2.3.1 Role in Membranes

The specific lipids for biodiesel derived from algae come primarily from their plasma membranes, endomembranes, chloroplast membranes, and lipid bodies. In *C. reinhardtii*, plasma membranes are mostly composed of polar lipids, glyceroglycolipids and GP. These serve in maintaining structural integrity of cell membranes and participating in cellular signaling (Vieler, et al., 2007). Membranes aggregate in four different organizational structures, illustrated in Figure 7: micelles,

a lipid bilayer, inverted micelles, and lipid bilayer vesicles. Each of these structures maximizes structural integrity of the membrane based on the constituent lipids involved. A membrane, thus, separates the external and internal cell environments, allowing selectivity in exchange of necessary molecules with the surrounding environment.

Additionally, the properties of the lipids in a membrane contribute to flexibility and permeability of the membrane. This flexibility allows for rapid incorporation of proteins involved in cellular and chemical processes of the cell into the membrane. Membrane flexibility also aids in survival of cells under environmental changes in temperature. By altering the membrane lipids types and structures, the membrane will maintain functionality of the cell. At lower temperatures, the lipids in membranes become crystalline instead of gel-like by reducing the number of double bonds of lipid fatty acid chains (Karp & Shield, 2008). Under crystalline conditions, proteins that may negatively affect cell function are “squeezed out” using lipid rafts. This creates protein free patches, which allow protein rich parts of the cell membrane to continue functioning while adjusting to the change in fluidity of the membrane structure (Harwood and Russell 2006). Thus, the variety of lipids present in the membranes play roles in maintaining shape, flexibility, and functionality of the plasma membrane.



**Figure 7: The organization structures for lipid aggregation occurs as (a) monolayer micelles, (b) inverted micelles, (c) lipid bilayers, and (d) bilayer fused vesicles. The lipid bilayers are most prominent in cell membranes and bilayer vesicles are features of cellular organelles. Source: <http://openlearn.open.ac.uk>**

### 2.2.3.2 Role in Photosynthesis

The most abundant lipids in plant and algae photosynthetic tissues are MGDG, DGDG, and SQDG. It is suspected that these lipids aid in the movement of hydronium ions along the membrane surface through charge interactions, thereby assisting in the function of the enzyme ATPase. These acidic lipids, thus, are vital in stabilizing the thylakoid membranes for light-dependent reactions (Chisti, 2007). They function in preserving the structural integrity of the membrane, particularly the chloroplast membranes and help to maintain normal functioning of photosystem II (PSII) complex. Defects in the production of SQDG have been shown to produce

significant decreases in the activity of the PSII (Sato, et al., 2000). The total level of these acidic lipids is carefully balanced in *C. reinhardtii* in order to ensure survival under varying conditions of light (Sato, et al., 2000). Under phosphorous deprivation, SQDG is produced in greater quantity, and under sulfur deprivation, GP is more abundant. These lipids can be utilized for biodiesel, though they may not be optimal if their polar functional groups drastically affect transesterification reaction rates. These properties will be discussed in a subsequent section.

### **2.2.3.3 Role in Energy Reserves**

Lipids, with their long saturated carbon chains, are energy rich molecules. Algae, however, store energy primarily as starch and other carbohydrates. Under conditions of nutrient deprivation, lipids may also contribute as energy reserves by being stored as lipid bodies in the form of neutral triacylglycerides (Hu, et al., 2008). *Neochloris oleoabundans*, for example, shows a 34-50% increase in lipid content under nitrogen deprivation. *Chlorella vulgaris* and *Scenedesmus obliquus* also show similar increases in lipid content when grown under low nitrate conditions (Thompson, 1996). In *C. reinhardtii*, a 15-fold increase in lipid bodies occurs under nitrogen deprivation. Notably, 25% of these lipids are of the C18:1 type, containing an 18-carbon chain with one degree of unsaturation, which is optimal for biodiesel production (Z. T. Wang, et al., 2009). This increase in neutral lipid production is associated with energy reserves due to the simultaneous production of starch for energy conservation (Z. T. Wang, et al., 2009). During the post nutrient-deprivation

phase, these neutral lipids, consisting primarily of energy rich triglycerides, can be used by the cell to anabolize polar lipids for functional purposes. The stored lipids can thus be utilized for production of other necessary lipid types upon return to the normal state (Thompson, 1996). These stored triglycerides would also be the optimal choice for biodiesel production. These lipid molecules yield 3 long hydrocarbon chains compared to the 2 produced from all other lipid types, which results in easier extraction from cells and higher yield from transesterification. However, triacylglycerides do not constitute the majority of lipids in algae. Thus, they cannot be the sole resource of lipids for biodiesel.

#### **2.2.3.4 Role in Signal Transduction**

Although it is commonly believed that lipids are utilized in algae only for their energy storage and structural support, they also have important functions in signal transduction cascades. It has been observed that they act as mediators within signal transduction pathways. Phosphatidylinositol groups on lipids are important binding sites for many signaling proteins. More specifically, studies reveal that lipids help in mediating stress-related signal transduction. In one study, the alga *Chlamydomonas moewusii* was subjected to osmotic stress, and it was shown that quantity of phospholipids increased in response (Guschina & Harwood, 2006). Lipids, thus, have diverse but vital roles in a cell. Due to this, they are abundantly present under normal conditions. Changes to these conditions in their culture environment can have dramatic consequences on not only lipids in the algae, but also

the growth rates. The effects of these changes will be discussed in subsequent sections. These lipids share structural similarity with membrane lipids. Their hydrocarbon chains, once separated from the polar functional groups, can be used in biodiesel.

#### **2.2.4 Lipids to Biodiesel: A Practical Solution**

Due to their vital roles in cellular functions, lipids from algae are a reliable and accessible resource for biodiesel production. In general, biodiesel created from neutral lipids of algae are the most favorable. These lipid types enhance engine performance in automobiles due to their higher carbon content relative to other lipid structures (Guckert et al., 1987). However, individually, lipid molecules have a great degree of structural variety. Thus, when assessing a lipid's potential utility for biodiesel production, a couple of properties of lipids must be considered. The most important property is the ratio between number of unsaturated fatty acid chains and saturated fatty acid chains of lipids in the algae. Higher saturation fatty acid chains give the highest theoretical energy yield for a given number of carbon atoms; however, they also have higher viscosity than unsaturated chains. Higher viscosity results in increased drag and pressure in the combustion engine during use, creating excessive mechanical stress. Cetane numbers quantify the amount of time it takes for a fuel to begin combustion after it is injected into an engine; therefore, a lower cetane number shows poorer overall combustion. Saturated fatty acid chains have a higher

ignition delay resulting from a lower cetane number than unsaturated chain lipids. On the other hand, biodiesel from unsaturated lipids performs better at cold temperature than biodiesel produced from highly saturated lipids due to a lower cloud point and pour point. Cloud point indicates the temperature at which solids precipitate out of the liquid phase, while pour point is the temperature below which the liquid becomes too viscous to pour properly. Highly unsaturated chain lipids, however, present the problem of lower oxidative stability than monounsaturated chains (García, Gonzalo, Sánchez, Arauzo, & Peña, 2010). These properties, reflective of saturated and unsaturated fatty acid chains, are succinctly summarized in Table 6.

**Table 6: Chemical properties of saturated and unsaturated fatty acids as source for biodiesel production (a=desirable property for conversion of lipid to biodiesel)**

<b>Properties</b>	<b>Saturated Chains</b>	<b>Unsaturated Chains</b>
<b>Energy Yield</b>	<sup>a</sup> Higher	Lower
<b>Viscosity</b>	Higher	<sup>a</sup> Lower
<b>Cetane Number</b>	Lower	<sup>a</sup> Higher
<b>Cloud Point</b>	Higher	<sup>a</sup> Lower
<b>Pour Point</b>	Higher	<sup>a</sup> Lower
<b>Oxidative Stability</b>	<sup>a</sup> Higher	Lower

Accounting for these properties of fatty acid chains of lipids, the ideal lipid composition is C18:1. The biodiesel produced from this type of lipid provides the best combination of energy yield, combustion quality, and storage life necessary to be competitive with traditional gasoline (García, et al., 2010). While the composition of individual esters produced from the lipids is important, the actual type of the lipids produced by the algae is not as important. Each lipid molecule, regardless of the type

of functional group attached, produces two identical fatty acid esters, assuming equivalent carbon chain length of the esters. The type of lipids produced only becomes important if the lipids differ in terms of transesterification rates or in ease of waste disposal based on attached functional groups. To this end, engineering the algae to favor a certain lipid over another would yield higher efficiency to the biodiesel production. This study does not address selecting production of a particular type of lipid more than other types. We will, however, examine the changes in relative number of the different lipids present in *C. reinhardtii*. This will allow us to better understand the effects of phytohormones on the algae based on changes in the ratio of lipids. As stated earlier, the amounts of total lipids can be altered under changes to environmental factors. The following sections present a discussion of manipulating the algae through environmental variables towards higher yield biofuel production.

### **2.2.5 Manipulation through Environmental Factors**

Environmental conditions have a multitude of affects on algal growth and lipid production. Since these processes are interlinked through ensuring survival of the algal cell, the processes are dynamically related, meaning that the cells constantly adjust to environmental changes. Investigations into improving lipid production for biodiesel from algae have focused on capitalizing on this relationship. Researchers induced stress on the algae and observed the changes to biomass productivity and

lipid yields. Though these changes can provide benefits to biodiesel production, their many compromises that must be made between the two variables are major problem.

#### **2.2.5.1 Light**

The flux of light through algal cells can have dramatic impact on their growth. Given the costs and energy required to maintain artificial light levels, the effects of light on growth rate and lipid yields must be carefully weighed. Exposure of algae to low or high extremes of light intensity may result in an imbalance between light energy absorbed and light energy metabolized (Huner, Öquist, & Sarhan, 1998). As illustrated in Table 6, light intensity between 3 and 12 k-lux (i.e. ranging from indirect sunlight to direct sunlight) can produce a range of growth rates between 0.60 and 4.50 day<sup>-1</sup> in *C. reinhardtii*. Other studies have shown a more narrow range of specific growth rates for a similar range of light intensity: under low light conditions of 40 µmol photons per m<sup>2</sup>/s [2.96 k-lux], the specific growth rate for *C. reinhardtii* was 1.15 day<sup>-1</sup>, while under a higher intensity light with 325 µmol photons per m<sup>2</sup>/s [24 k-lux], it increased to 1.55 day<sup>-1</sup> (Peers et al., 2009).

The differences in the growth rates may be attributable to the sources and quantity of carbon made available to the algae. Algae can also be grown under varying durations of photoperiod cycles. For *C. reinhardtii*, dark and light cycles, using high intensity light alternating every 13-87s, result in a decrease in specific growth rate from 3.84 day<sup>-1</sup> to 2.64 day<sup>-1</sup> (Andersen, 2005). Though growth at high intensity light may first appear as the most appealing option, factoring in costs of

energy for lighting proves otherwise. Accounting for increases in production cost associated with maintaining high intensity of light, the higher growth rates from higher intensity light sources are impractical. Growth outdoors, under near-direct sunlight, can provide approximately 10 k-lux light. Though this light comes without costs, it can negatively impact biomass productivity due to negative consequences of oxidative damage and increase in temperature. Indirect sunlight, however, at approximately 6 k-lux, can provide optimal lighting. Continuous lighting at this level of intensity, thus, is the simplest, least expensive solution. Another factor to consider is the self-shading of algae cells. Algae that are closer to the surface of the growth container have better access to light, while those at the bottom do not absorb as much light. This leads to differential growth among the algae cells, which is dependent on the shape and size of the container.

Growth at high intensity light is made more impractical by the reduction in lipid yields. The fatty acid content of *C. reinhardtii* changes with respect to light intensity, as illustrated in Table 7. At low light conditions, fatty acids total 57.0 mg/g, consistent with cells exposed to very high intensity of light. At more optimal light conditions around 6 klux, cells can contain as much as 77.4 mg/g of fatty acids (Akimoto, et al., 1997). Thus, the optimal intensity of light, under constraints costs for artificial lighting, is approximately 6 klux.

The efficiency of light utilization can also be reduced by clumping of algae and oxidative damage to their photosystems. Clumping is a major problem in scattering of light available to algae (Melis, 2006, 2009). Increasing the intensity of

light does not compensate for losses incurred under clumping. Excessive intensity of light, instead, causes oxidative damage to cells. While higher plants have the ability to manipulate their leaves to avoid absorbing excessive sunlight, algae employ alternative chemical mechanisms for coping with excess light energy (Demmig-Adams & Adams, 2003; Ringlstetter, Kaiser, & Müller-Seitz, 2006). They relieve stress via a non-photochemical quenching process to dissipate excess energy from singlet-excited chlorophylls. This helps to protect the photosynthetic processes in environments with excess light (Anderson et al.).

**Table 7: Effects of light intensity on growth rates, cell yields, and lipid yields in *C. reinhardtii***

<b>Light Intensity (k-lux)</b>	<b><sup>a</sup>Approximate growth rate, <math>\mu</math> (day<sup>-1</sup>)</b>	<b><sup>a</sup>Total cell yield (g/L)</b>	<b><sup>a</sup>Total fatty acid content (mg/g)</b>
<b>3</b>	0.60	0.38	57.0
<b>6</b>	1.50	1.11	77.4
<b>9</b>	3.50	2.23	76.4
<b>12</b>	4.50	2.64	61.9

<sup>a</sup>Akimoto et al 1997

### **2.2.5.2 Temperature**

Another environmental factor important to algal growth is temperature. Generally, under constant light conditions, specific growth rates in *C. reinhardtii* can range from 0.79-1.50 day<sup>-1</sup> relative to a temperature range of 15 to 35°C, as listed in Table 8 (Akimoto, et al., 1997). Though this range yields dramatic improvements to the speed of biodiesel generation, maintaining growth conditions above or below ambient temperature requires additional energy input. To avoid this and to maintain

lower costs of production, it is best to grow the algae at room temperature, 25°C. Of note in *C. reinhardtii*, growth at room temperature would not only reduce overall energy input, but it also provides the optimal specific growth rate, 1.50 day<sup>-1</sup> (Akimoto, et al., 1997). Studies on *Nanochloropsis oculata* and *C. vulgaris* show that temperatures greater than the optimum level stunt the algal growth rate significantly.

Furthermore, the lipid content of these two species also decreases with rising temperature (Luque de Castro & García-Ayuso, 1998). However, the lipid content varies only slightly in relation to the temperature and does not decline sharply at temperatures beyond optimal, unlike the growth rate. The fatty acid levels in *C. reinhardtii* show similar effects of temperature. Temperatures between 15-35°C results in 72.0-84.1 mg/g algae of fatty acids, respectively, as listed in Table 8 (Akimoto, et al., 1997). Across a range of temperatures from 15-35°C, the total lipid content stays consistent, with only small changes in the ratios of individual lipid constituents. A study on the effects of temperature on acidic lipids in *C. reinhardtii* revealed no changes in the total content of these lipids (Sato, et al., 2000). This suggests that little to no effects of temperature variation occur on chloroplast membrane integrity and efficiency of photosynthetic processes (Sato, et al., 2000). Another study shows that *Chlamydomonas geitleri* grows asexually in the temperature range of 4-30°C, but sexual reproduction is limited to a smaller range of 18-25°C (Nečas, 1982). Temperature, thus, is a key variable in the expected observable level of growth.

**Table 8: Effects of temperature on growth rates, cell yields, and lipid yields in *C. reinhardtii*.**

<b>Temperature (constant light, 6 klux)</b>	<b><sup>a</sup>Specific Growth Rate, <math>\mu</math> (day<sup>-1</sup>)</b>	<b><sup>a</sup>Cell yield (g/L)</b>	<b><sup>a</sup>Total free fatty acids (mg/g)</b>
<b>15°C</b>	0.79	0.90	72.0
<b>20°C</b>	1.31	0.96	72.1
<b>25°C</b>	1.50	1.11	77.4
<b>30°C</b>	1.39	1.06	81.1
<b>35°C</b>	1.23	0.97	84.1

<sup>a</sup>Akimoto et al 1997

### **2.2.5.3 Salt**

A third important environmental factor is the salt content of the algal growth media. Halotolerance the ability to survive in media of high salinity, differs from one algal strain to the next and determines how a species will react and adapt to salt stress (Alyabyev et al., 2007). Algae are grown in culture medium which provides necessary nutrients as salts. The level of these salts must be carefully selected. Unusual salt concentrations can trigger biochemical and bioenergetic responses in algal cells. These responses may include increased rates in lipid catabolism, changes in the rates of energy-yielding processes, and changes in membrane permeability (Alyabyev, et al., 2007). Additionally, if algae were to be grown in natural water systems, their ability to cope with salinity level changes would be valuable. Studies have been conducted to determine the effects of environmental salinity manipulation on the bioenergetic responses in algae. For example, the freshwater alga *C. vulgaris* showed increased heat production at low salt concentrations and decreased heat

production at high salt concentrations (Alyabyev, et al., 2007). The rate of heat production is directly proportional to the metabolic rate of the algae. An increase in heat production would raise the temperature of the algae, negatively impacting its growth rates. Additionally, it also increases catabolism of lipid reserves to supply the increase in energy demand under a high metabolic rate. Heat production is, thus, a survival mechanism to cope with stress from salts.

The effects of salts on heat production vary, however, amongst algae. The halotolerant alga *Dunaliella maritime* showed significantly increased heat production in saline environments (Alyabyev, et al., 2007). In another test, cultures of the algae *Dunaliella tertiolecta* were exposed to different NaCl concentrations (Takagi, Karseno, & Yoshida, 2006). The effects of increased environmental salinity on the intracellular accumulation of lipids were recorded. Cells placed in a 1.0 M NaCl solution experienced a higher intracellular lipid content of 67% as compared to those grown in a 0.5 M NaCl solution, which only amassed 60% lipid content (Takagi, et al., 2006). The lipid yields are not drastically different but will provide benefits to biodiesel production. The effects of this factor, however, do not show a clear relationship for every alga. Thus, manipulation of salts may not promise benefits to biodiesel production.

#### **2.2.5.4 Nitrogen**

A fourth environmental factor of importance is nitrogen. Nitrogen is essential for protein synthesis; therefore, a steady supply of nitrates or ammonia is a necessity

for normal growth. Changes in extracellular nitrate concentrations can induce changes in biological processes, including growth and lipid production. A study on *Neochloris oleabundans* in which extracellular concentrations of nitrates were varied between 3 and 20 mM showed trade-offs between the cellular growth rates and lipid yields. At lower concentrations, lipid production was at its highest while growth was at its lowest. At higher concentrations of nitrates, the opposite effect was observed (Y. Li, Horsman, Wang, Wu, & Lan, 2008). Comparable results were produced for *Chlorella protothecoides* and *Nannochloris oculata* and *C. vulgaris* (Xiong, et al., 2008) (Luque de Castro & García-Ayuso, 1998). Both species saw a two to three fold increase in lipid content under nitrogen deprivation. The growth of *N. oculata* was stunted, while the *C. vulgaris* growth rate did not show significant change.

*C. vulgaris* and *C. emersonii* grown in a low nitrate medium, showed increased lipid yields, but biomass productivity was lower than that of the algae grown in control medium (Illman, Scragg, & Shales, 2000). Because the overall lipid production is a product of cell lipid content and total biomass, a two-stage cultivation strategy has been proposed to account for the trade-off between lipid accumulation and cell growth. The first stage of this proposed process is dedicated to normal cell growth in a nutrient-sufficient medium, while the second stage is carried out in a nitrate-poor medium to facilitate lipid accumulation (Courchesne, Parisien, Wang, & Lan, 2009). Nitrogen-starved conditions, thus, offer a compromise between growth and lipid production. Alternatively, a high concentration of nitrates increases lipid content in the short term. Due to detrimental effects on growth rates however, total

long-term lipid production remains low (Courchesne, et al., 2009; Rodolfi, et al., 2008).

#### 2.2.5.5 Phosphorus and Sulfur

Other environmental effects on algae are observed under phosphorus and sulfur manipulation, referred to in Table 9. With limited phosphorus available in the environment, *C. reinhardtii* shows an increase in SQDG and a decrease in GP. This allows the total acidic lipid content to remain balanced, while phosphorus can be diverted to more important biosynthesis products such as nucleic acids. Decreased sulfur levels lead to similar fluctuations in GP and SQDG in the opposite direction (Hu, et al., 2008; Sato, et al., 2000).

**Table 9: Summary of nutrient effects on algae**

<b>Nutrient</b>	<b>Concentration relative to optimal</b>	<b>Effect on growth</b>	<b>Effect on lipid yields</b>
<b>Nitrogen</b>	High	Increase	Overall Decrease
<b>Nitrogen</b>	Low	Decrease	Overall Increase
<b>Phosphorus</b>	Low	-	Decrease GP
<b>Sulfur</b>	Low	-	Decrease SQDG

#### 2.2.5.6 Alternatives to Environmental Manipulation

Changes to environmental factors in algal cultures produce diverse changes. As has been noted, favorable changes in growth or lipid production often come paired with unfavorable effects on other cellular processes. When growth is high, lipid

production decreases and vice versa. Due to these trade-offs under environmental manipulation, the prospects for future practical applications of these methods are poor. This study investigates a possible method to create similar effects as seen in these environmental manipulation studies, without the compromises. The observable changes to growth and lipid production seen in environmental manipulation has its underpinnings in signal events and genetic control. This study attempts to explore these signaling intermediates via application of growth promoting signals, phytohormones.

## **2.3 Phytohormones: Their Roles in Plants and Algae**

### **2.3.1 General Overview**

Phytohormones (plant hormones, plant growth substances) are naturally occurring chemicals that influence and control a majority of plant growth and developmental processes (T. D. Li, Doronina, Ivanova, & Trotsenko, 2007). They not only regulate the plant growth rates but also control how smaller pieces of the plant work together in order for the organism to function as a whole. Phytohormones regulate the reproductive cycles exhibited by plants and allow them to respond to environmental changes as well (Bradely, 1991). The roles of phytohormones in higher-order plants have been substantially explored, however their functions in algae are rarely investigated. Studies in which algal growth has been manipulated by

external agents have hinted at the possible involvement of phytohormone signaling in algae (Bradely, 1991; Evans & Trewavas, 1991).

Although this topic has not yet been extensively examined, particularly in relation to algal biodiesel production, studies illustrating relationship between plant growth and algal growth show evidence for phytohormone-induced growth and metabolic changes in algal species. One study introduced *Azospirillum brasilense* bacteria, which promote plant growth, to the green alga *C. vulgaris*. The presence of these microorganisms induced comparable growth benefits in the algae. The investigators suspected that the phytohormone indole-3-acetic acid (IAA), a member of the auxin family, was responsible for signaling between the bacterium and the algae. They tested this hypothesis by delivering IAA to isolated algae. Independent of the bacteria, the exposure of *C. vulgaris* cultures to IAA led to a significant increase in growth. This study suggests the possibility of this auxin being the substance responsible for inducing the growth changes in the *C. vulgaris* (Gonzalez-Bashan, Lebsky, Hernandez, Bustillos, & Bashan, 2000). Similarly, abscisic acid and the brassinosteroid family of phytohormones have also been linked to biotic and abiotic stress response signaling (Krishna, 2003; Tsavkelova, Klimova, Cherdyntseva, & Netrusov, 2006). Beyond these activities, phytohormones may play numerous additional roles in algal cells. Their presence and function in algae can be deduced by looking for homology between phytohormone and signaling genes delineated in a model plant organism and a model algal organism.

### 2.3.2 Comparative Genomics

Comparative genomics is the study of relating gene sequences between organisms. Comparing the genomes of two organisms can illustrate their evolutionary relationship and functional similarities between their respective genes and proteins (Kim, Tang, & Mardis, 2008). As mentioned previously, though *C. reinhardtii* may not contain the highest lipid content among algal species, it is an ideal candidate for this research. *C. reinhardtii* is a model species for algal research and for more general studies of genetics and comparative genomics. In particular, *C. reinhardtii* is the best model for lipid biosynthesis research (Walker, Collet, & Purton, 2005). Comparative genomics allows comparison of lipid biosynthesis pathways between land plants such as *Arabidopsis thaliana* and algal species such as *C. reinhardtii*.

Previous work in comparative genomics with *C. reinhardtii* has established a close genomic relation with the *Arabidopsis* family. Many orthologs have been identified between *C. reinhardtii* and *A. thaliana* (Merchant, et al., 2007). Putatively complete nonredundant sets of homologous transcription factors were identified in *C. reinhardtii* and *A. thaliana* (Merchant, et al., 2007). In fact, 2489 protein families are shared between the two organisms (Merchant, et al., 2007). A similar comparison of *C. reinhardtii* with *Arabidopsis lyrata* has revealed 1879 shared protein families (Melis, 2006; Merchant, et al., 2007). A large portion of these families code for kinases, guanylyl cyclases, and adenylyl cyclases, which are involved in secondary messaging systems. Many of these families also share roles in photosynthetic processes within chloroplasts. Additionally, it has been suggested that plant-specific

transcription factor families might play important roles in the regulation of light-dependent processes and other pathways such as those involved in sugar production or starch accumulation (Merchant, et al., 2007). These findings suggest that the genes and proteins which play important roles in higher-level plants may play roles in algae as well.

The identified gene sequences for protein products in *A. thaliana* can be used to search the genome of the *C. reinhardtii*. In one study, chloroplast protein translocation complexes were compared between *A. thaliana* and *C. reinhardtii* (Kalanon & McFadden, 2008). The search for the proteins was conducted using the *A. thaliana* amino acid sequence via the Basic Local Alignment Search Tool for Nucleotides (BLASTN), which finds matching sequences of amino acids between two organisms. Another study concerning comparative genomics between *C. reinhardtii* and *A. lyrata* focused on a particular gene which encodes a blue-light photoreceptor (Small, Min, & Lefebvre, 1995). The use of comparative genomics demonstrated that the first 500 amino acids of the protein are 49% homologous to a sequenced section from *A. lyrata* (Small, et al., 1995). Researchers deduced that the gene in *A. lyrata* for the photoreceptor is similar to the identified gene in *C. reinhardtii*, explaining the exhibition of similar functionality. As used in this work, comparative genomics allows relevant comparisons between the model organisms *C. reinhardtii* and *A. lyrata*. This technique provides reliable deductions of gene homologies across the algal and plant families (Lefebvre & Silflow, 1999; Proschold, Harris, & Coleman, 2005).

The use of these techniques allows comparisons between extant plant phytohormone pathways and the less-explored signaling in *C. reinhardtii*. As a model for lipid biosynthesis and algal growth research, *C. reinhardtii* can be used to determine relations between phytohormone signaling and changes in lipid content. Findings of genomic homologies for phytohormone mechanisms in this model alga could also be broadly applied to other species of algae. Throughout this section of the review, the genes coding for specific responses in different plant species are mentioned as a starting point for comparative genomic analyses with *C. reinhardtii*. Phytohormones identified by this analysis may serve as strong candidates to improve lipid yields or growth rates in this organism.

### **2.3.3 Overview of Molecular Mechanisms of Signal Transduction**

Signaling pathways have been established for several phytohormones, including abscisic acid and different types of auxins, brassinosteroids and cytokinins. Studies to fully delineate other phytohormone cascades continue. Many of these findings have been confirmed by the *Arabidopsis* model.

To understand the observed effects of phytohormones, investigations have been focused on molecular mechanisms of signaling. Leading models suggest two primary mechanisms of signaling for most phytohormones. These may occur separately or in conjunction with each other. Phytohormone effects begin with receptors with kinase activity. In the first model, these receptors produce signal cascades which employ phosphorylation events to eventually control transcriptional

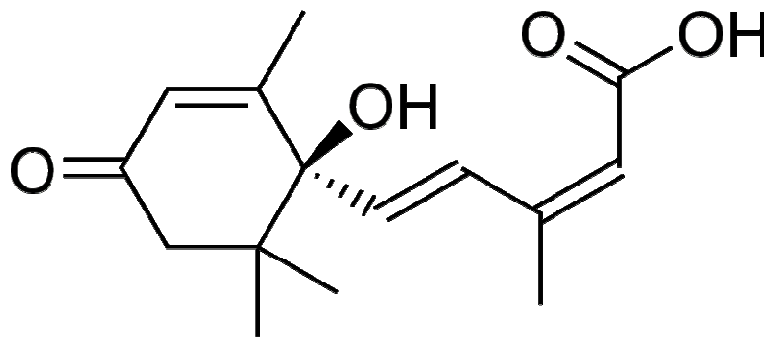
activators and enhancers for growth-regulating genes. Cytokinins, for instance, employ a two-component system with a histidine protein kinase receptor plant (Hwang & Sheen, 2001). Along this signal cascade, regulator proteins can alter the signal of the cytokinin; however, the end process produces a transcriptional event. Similarly, brassinosteroid function is attributed to an intracellular receptor with a leucine-rich repeat receptor-like kinase and a membrane receptor with serine-threonine kinase activity (Friedrichsen, Joazeiro, Li, Hunter, & Chory, 2000; Nam & Li, 2002). The induced signal cascade increases transcription of genes involved in growth regulation.

In the second mechanism of phytohormone signaling, active signal cascades can act on intracellular proteins rather than acting at the transcriptional level. Gibberellins operate by this model, inactivating a protein called GAI/RGA found in the plant. This activity promotes an increase in growth of the cell (Fu et al., 2001). Auxins affect the activity of the cell by a similar means. At the transcription level, auxin response factors (ARFs) dimerize and bind to DNA to allow transcriptional control. However, auxins also bind to receptors that generate signal cascades to induce ancillary proteins to regulate the dimerization of ARFs. This controls the transcription of growth-regulating genes. In the end, both models produce similar effects of altering the transcription of growth-regulating genes. The specific mechanisms are different, but the end results are comparable. Six major classes of phytohormones responsible for this regulation, including their signal transduction pathways, are described in greater detail in the following sections.

## 2.3.4 Common Phytohormones of Plants and Algae

### 2.3.4.1 Absciscic Acid

Absciscic acid (ABA, Figure 8) is synthesized from isopentenyl pyrophosphate (IPP) C5, derived from glyceraldehyde phosphate and pyruvate (Taylor, 1997). The primary role of ABA is in the physiological response of plants to biotic and abiotic environmental stresses. Common abiotic stressors including salt stress, hypoxia, and low temperatures, induce ABA synthesis in organisms. ABA then increases the gene expression of regulatory proteins and regulates stomatal closing due to dehydration (Finkelstein, Gampala, & Rock, 2002). Additionally, ABA initiates many growth and developmental processes in plants, including embryo maturation, synthesis of seed storage, bud dormancy, and the inhibition of seed germination and reproductive growth by signaling for transcription of growth regulating genes. Therefore, it makes sense that ABA levels are found to increase significantly during seed maturation as well as during periods of environmental stress (Xiong, et al., 2008)). ABA also plays a role in the storage of some cellular products (Salisbury & Ross, 1992).



**Figure 8: Absciscic acid, which aids in the response of plants to biotic and abiotic stresses in the environment**

In *A. thaliana*, ABA biosynthesis is controlled by several genes, which are upregulated by salinity, dehydration, and other osmotic factor (Eckardt, 2002; Nambara & Marion-Poll, 2005). Specifically, the expression of the four *A. thaliana* *CYP707A2* genes is promoted by osmotic stress (Xiong, et al., 2008). The four genes in the *CYP707A2* gene family encode ABA 8'-hydroxylases, which catalyze the committed step in the predominant ABA anabolic pathway (Seo et al., 2006; Xiong, et al., 2008). In addition to exogenous signals, gibberellins and brassinosteroids may also upregulate *CYP707A2* expression. Additionally, the exogenous application of ABA has been shown to increase the expression of both biosynthetic and catabolic genes of ABA. This finding indicates that ABA may serve a self-regulating function in *A. thaliana* (Seo, et al., 2006).

Past work in delineating the signal transduction network of ABA has been fraught with findings that were later proven to be invalid. As examples, two receptors were identified, only to be disproven in future studies. The G-protein coupled receptor, GCR2, was found to bind to ABA with high affinity and yield strong ABA responses (Liu et al., 2007). This protein was also identified in *A. thaliana*, providing a promising step forward in ABA research. However, a later study in which these findings were tested by a second team disproved the results of GCR2 in ABA perception (J. Guo, Zeng, Emami, Ellis, & Chen, 2008). The sensitivity of GCR2 to ABA varied greatly with changing environmental conditions. This led to the conclusion that it did not play a role in ABA signaling. Similarly, an RNA metabolism protein, FCA, was found to demonstrate receptor-like behavior upon

binding with ABA (Razem, El-Kereamy, Abrams, & Hill, 2006). Again the conclusions of this study were invalidated by subsequent work (Marris, 2008). Other receptors, including ABA insensitive receptor protein ABI1 and ABI3 (Leung et al., 1994; Merchant, et al., 2007), GPCR-type G proteins GTG1 and GTG2 (Pandey, Nelson, & Assmann, 2009), and a receptor-like protein kinase RPK1 (Hong, Jon, Kwak, & Nam, 1997), have been associated with ABA signaling.

Although findings of ABA receptors have come under fire, other biochemical events have been established for ABA signaling. ABA has been confirmed to activate calcium ion channels in regulation of the calcium-modulated phosphatases part of the ABA signal pathway (Leung, et al., 1994; Merchant, et al., 2007). The role of these phosphatases, linked with ABI1, ABI2, and ABI3, is integral to the signaling events beyond the receptors (Himmelbach, Yang, & Grill, 2003; Leung, Merlot, & Giraudat, 1997). Plant mutants lacking these phosphatases have been shown to lose all growth characteristics associated with ABA responses (Leung, et al., 1997). These findings for ABA signaling have not been disputed by recent evidence, suggesting that phosphatases do hold integral roles in these signaling pathways.

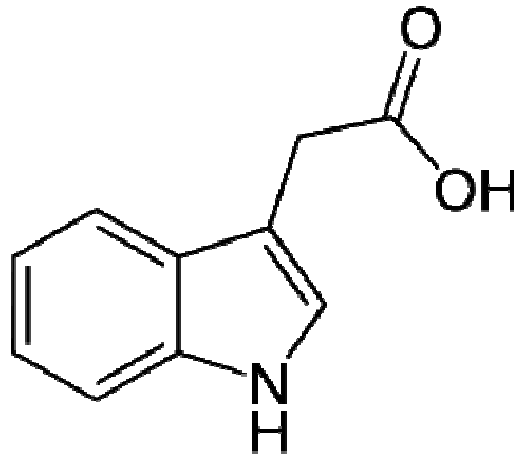
While the biosynthetic pathway of ABA has been well documented in plants, its expression in algae is still relatively unclear. The application of ABA to *C. reinhardtii* has been found to enhance algal resistance to oxidative stress (Nambara & Marion-Poll, 2005; Yoshida, Igarashi, Mukai, Hirata, & Miyamoto, 2003). The expression of the antioxidant enzymes catalase and ascorbic peroxidase increases significantly upon exogenous addition of ABA. This allows increased growth of the

algae during periods of oxidative stress under normal light conditions. Although the evidence is not conclusive, it does suggest the possible role of ABA as a stress signaling hormone. At this point, ABA appears promising as a potential chemical for the acceleration of growth or lipid accumulation. Further comparative genomic analyses may either confirm or refute the utility of introducing this substance to algal cultures.

#### **2.3.4.2 Auxins**

Auxins are a class of phytohormones that primarily increase growth in plants (Bartel, 1997; Galston & Purves, 1960; Woodward & Bartel, 2005). Plants can synthesize their own auxins from tryptophan or indole-3-butyric acid, or they can obtain them from their surroundings (Hagen, Uhrhammer, & Guilfoyle, 1988; Schneider & Wightman, 1974). Indole-3-acetic acid (IAA, Figure 9) is the most common naturally occurring auxin. Originally, the term auxin was used to classify phytohormones that induce elongation in shoot cells. After extensive study of auxin responses, however, they have been found to promote root initiation and inhibit root elongation (Galston & Purves, 1960; Woodward & Bartel, 2005) by regulating the activity of D type cyclin dependent kinase A during the Gap 1( $G_1$ ) and Synthesis (S) phases of the cell cycle (Himanen et al., 2002). Additionally, auxins delay leaf abscission (shedding), inhibit lateral bud formation, induce callus formation, and promote an epinastic (downward-bending) response. On a cellular level, auxins accomplish these tasks by increasing cell wall plasticity, increasing water intake,

altering respiratory patterns, and altering nucleic acid metabolism (Galston & Purves, 1960). Auxins cause these profound changes due to their activity at the transcriptional level (Brummell & Hall, 1987; Himanen, et al., 2002). Their effects can be observed as early as 3 minutes after binding to cellular phytohormone receptors (Brummell & Hall, 1987; Hagen, et al., 1988). Clearly, extensive auxin activity has been documented in plant species. Unlike many other phytohormones, it is known to exist in certain algal species as well, but uncertainty concerning its function remains.



**Figure 9: Indole-3-acetic acid, a member of the auxin class of phytohormones, which are responsible for growth processes in plants**

Auxins are one of the few families of phytohormones that are naturally secreted in algae (W. Jacobs, 1985; W. P. Jacobs, 1951). The most common auxin found in brown algae, red algae, green algae, and diatoms is IAA. However, the concentration of this auxin is much lower than concentrations common in higher order plants (W. Jacobs, 1985; W. P. Jacobs, 1951; Overbeek, 1940). In certain algae of the *chlorophyceae* class, low concentrations of IAA actually have an inhibitory effect on growth, while high concentrations have proven toxic (W. P. Jacobs, 1951).

However, IAA has a positive effect on growth rate and cell size in *C. vulgaris*, *C. pyrenoidosa*, and *Oocystis* while having no effect on *Alaria esculenta* (W. P. Jacobs, 1951). The different responses of algae show that these algal species may possess different auxin signaling pathways. This observation of variant responses to auxins in different algae has spurred discussion on the evolutionary development of auxins as an important molecule in plant metabolism (W. P. Jacobs, 1951). Although auxins are responsible for promoting morphological changes in plants, there is currently no evidence suggesting parallel effects in algae. Nor is there a convincing and definitive answer (no predictive values or mechanisms) as to what effects auxin will elicit in a particular algal species (W. P. Jacobs, 1951).

Auxin has been traced through plant lineages, and its evolutionary pattern suggests a link between *Charyophytes* (water-plants) and *Bryophytes* (land-plants) (W. P. Jacobs, 1951). Evidence suggests a simpler signaling pathway underlies the adaptations seen in current angiosperms (flowering land-plants). Determination of the auxin signaling mechanism has mostly involved identification of transcription level regulation factors. Finding a receptor for auxin perception has proven difficult. The Auxin binding protein, ABP1, was first suspected as a receptor on the endoplasmic reticulum, an unconvincing location for an auxin receptor (S. D. Clouse & J. M. Sasse, 1998; Kepinski & Leyser, 2005). Evidence for ABP1 expression suggests it is maintained at constant levels throughout vegetative plant development, although it was first connected to embryonic development periods in plants. Using conditional ABP1 *A. thaliana* lines, Tromas et al. (2009) demonstrated that ABP1 is

required for post-embryonic shoot development, acting on various cellular responses. However, the necessity of auxin involvement in ABP1-driven downstream responses and its role in plant root growth are not clear.

More convincing evidence for auxin perception has been uncovered in the characterization of the TIR1 protein receptor as a component of the SCFTIR1 complex (Dharmasiri, Dharmasiri, & Estelle, 2005; Kepinski & Leyser, 2005; Tan et al., 2007). TIR1-mutants with a lone SCF complex showed a lack of auxin binding and response. The SCF complex has ubiquitin-ligase activity to inactivate auxin transcription repressors. Enzyme assays also illustrate the capacity of TIR1 to bind promiscuously to various IAA analogues (Tan, et al., 2007). Binding of auxin to TIR1 activates the SCF complex and induces inactivation of ARFs. ARFs bind to downstream elements that control the expression of auxin-related growth genes. Growth is induced by mediating the activity of cyclin D/cyclin dependent kinase (CDK) A for the G<sub>1</sub> to S phase transition and by regulating transcription of CDK inhibitory protein KRP2 and ARFs (Himanen, et al., 2002; Liscum & Reed, 2002). More than 20 ARFs have been identified in the *A. thaliana* genome, serving various functions for auxin responses (Liscum & Reed, 2002).

Auxins and brassinosteroids (see the section on brassinosteroids) also show interdependency in their signal pathways. They share common pathways for promotion of cell growth and elongation in plants. The auxin-response element ARFAT is one factor that is common between the brassinosteroid and auxin pathways (Liscum & Reed, 2002). Many of the ARFs bind to ARFAT motifs and promote

downstream auxin dependent responses involved in cell growth (Ulmasov, Murfett, Hagen, & Guilfoyle, 1997). Therefore, ARFs not only play a significant role in auxin signaling but also participate in brassinosteroid signaling events.

The natural production of auxins within algae cells and their role in plant growth makes these hormones ideal for experimentation. Specifically, IAA is a perfect candidate for the growth and lipid production experiments as it is the most common phytohormone found in most algal species.

#### **2.3.4.3 Brassinosteroids**

Brassinosteroids are naturally occurring phytohormones that promote growth in pollen, seeds, and young plant tissues (Steven D. Clouse & Jenneth M. Sasse, 1998; Hu, et al., 2008; Mandava, 1988). There is evidence that they contribute to cell division, as well as cell expansion, vascular differentiation, etiolation (leaf elongation), and reproductive development of plant cells (Steven D. Clouse & Jenneth M. Sasse, 1998; Hu, et al., 2008). In Chinese cabbage protoplasts, brassinosteroids promoted cell division that varied with the amount of hormone applied, and also enhanced the formation of clusters and colonies. Specifically, the brassinosteroids accelerated the necessary regeneration of the cell wall prior to cell division (Steven D. Clouse & Jenneth M. Sasse, 1998). Applied at nM to  $\mu$ M concentrations, the brassinosteroid brassinolide (pictured in Figure 10) caused significant elongation of cells including hypocotyls, epicotyls, and peduncles of dicot plants, as well as coleoptiles and mesocotyls of monocot plants. Young tissue is



necessary signaling elements when grown in the presence of light (Tsavkelova, et al., 2006). Homologs of these genes may be found in *C. reinhardtii* as well. There is also evidence of brassinosteroid-induced changes in the metabolism of *C. vulgaris* (Bajguz & Czerpak, 1996). The introduction of brassinazole, an inhibitor of brassinosteroid biosynthesis, results in decreased growth in *C. vulgaris* under normal light conditions (Tsavkelova, et al., 2006). Additionally, at concentrations between  $10^{-8}$  M and  $10^{-12}$  M, significant increases in DNA, RNA, and protein content were observed alongside increases in growth after 24 hours. Further work is required to fully develop brassinosteroid models in algae.

The current established signal transduction pathways for Brassinosteroids paint a basic picture of the process. Brassinosteroids bind with receptors at the plasma membrane, specifically the Brassinosteroid receptor protein 1 (BRI1) paired with a serine-threonine kinase (Nam & Li, 2002). When brassinosteroids are received on the membrane, BIN2 is inactivated by an unknown mechanism which allows the hypophosphorylated nuclear proteins bri1-EMS-suppressor 1 (BES1) and brassinazole resistant (BZR1) to gather in the nucleus and suppress transcription. In the absence of brassinosteroids, proteins in the BES1/BZR1 family are hyperphosphorylated by BIN2 and eventually degraded. As steroidal hormones, Brassinosteroids also have intracellular receptor complexes. These receptor complexes have been identified to contain leucine-rich repeat receptor-like kinases, LRR-RLK domains, and brassinosteroid insensitive 1 receptor proteins, BRI1. Overexpression of BAK1 genes, which code for the LRR-RLK and BRI1 proteins,

produces an increase in responses associated with brassinosteroid growth effects in *A. thaliana* (Li, et al., 2002). Brassinosteroids signaling pathways also incorporate elements of the auxin signaling pathway (See Auxins). The ARAFT protein is a target of both auxins and brassinosteroids and involves activation of cell growth genes (Ulmasov, et al., 1997).

Work on identifying more proteins involved in the signaling cascade is ongoing. Results have been more promising in identification of biosynthetic enzymes for brassinosteroids. The Det2 and Dwf4 genes have been identified to code for a highly conserved steroid reductase and hydroxylases, respectively (Steven D. Clouse & Jenneth M. Sasse, 1998). The enzymes from these genes are important in the production of brassinosteroid precursor, cathasterone. Evidence of brassinosteroid production in the green alga *C. vulgaris* may indicate that *C. reinhardtii* possesses brassinosteroid signaling components as well (Tsavkelova, et al., 2006).

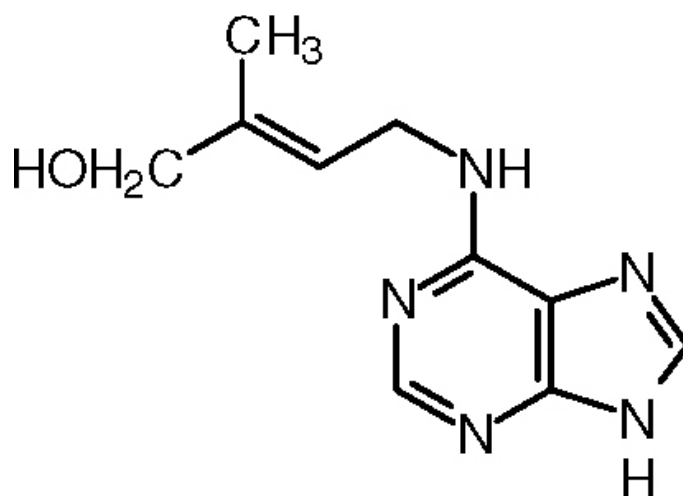
Brassinosteroids hold promise due to their production and role in other species of green algae. The possible existence of the appropriate phytohormone receptor genes in *C. reinhardtii* makes brassinosteroids potential chemicals for experimentation. A more complete genomic analysis may reveal whether brassinolide or other brassinosteroids are strong candidates for enhancing lipid accumulation or growth rate.

#### **2.3.4.4 Cytokinins**

Cytokinins are plant growth substances which play a role in senescence and chloroplast development, primarily by promoting cell division (Riou-Khamlichi, Huntley, Jacqmard, & Murray, 1999; Tarakhovskaya, Maslov, & Shishova, 2007). An example of a cytokinin is trans-zeatin, which appears in Figure 11 below. It has been shown that plants with lower levels of cytokinins develop stunted shoots, with leaf cell production at only 3-4% of that for plants with regular levels of cytokinins (Werner, Motyka, Strnad, & Schmülling, 2001). These phytohormones also impose upper limits on the speed of growth in order to prevent overgrowth in plants (Werner, et al., 2001). There is a clear relationship between auxin and cytokinins, with the combination playing an essential role in the formation of roots and their growth (Skoog & Armstrong, 1970). By comparing the genomes of plants to *C. reinhardtii*, it is possible to determine the presence of cytokinins in algae. *C. reinhardtii* has 15143 genes, with no CHASE sequences (code for protein domains involved in cytokinin receptors), 1 HPT sequence (code for phosphotransmitter proteins), and 4 RR sequences (code for response regulator proteins). The frequency of these sequences coding for cytokinin receptors is much lower in *C. reinhardtii* than in terrestrial plants (Pils & Heyl, 2009). This distinction between land plants and *C. reinhardtii* may have arisen due to evolutionary development leading to adaptations for land plants. However, it has been shown that cytokinin signaling remains possible despite of the lack of CHASE gene sequences.

Furthermore, endogenous cytokinin-like activity has been documented in various algae (Stirk, Ördög, Van Staden, & Jäger, 2002; Ördög, Stirk, Van Staden,

Novák, & Strnad, 2004). While the signaling features are present, they are not as common as in normal plants. This would likely lead to a less pronounced effect of cytokinins in algae due to fewer receptors. Thus, studying the documented endogenous effects of cytokinins poses a challenge. No cytokinin biosynthesis mutants have been isolated in plants (Werner, et al., 2001). Effects of cytokinins have been determined by overexpression of cytokinin genes or exogenous addition of cytokinins. Similar methods in algae, if resulting in marked growth, would further cement the genomic relationship to terrestrial plants. The signaling processes of cytokinins in plants are discussed in greater detail below.



**Figure 11: Trans-zeatin, a member of the cytokinin class**

Cytokinin signal transduction pathway begins with binding to a two-component receptor system, involving the cytokinin receptor, CRE1 (Inoue et al., 2001). *Arabidopsis* mutants with mutant CRE1 proteins are unresponsive to the presence of external cytokinin. The CRE1 receptor is suspected to be paired with a histidine kinase, AHK4, which induces phosphorylation of subsequent proteins as

part of the signal cascade (Hwang & Sheen, 2001; Yamada et al., 2001). Homologs of this receptor kinase have been observed to participate in cytokinin signaling across the plasma membrane, as seen in *A. thaliana*, suggesting its integral role in this signal cascade.

Along these pathways, regulatory proteins play a critical role in increasing and decreasing the cytokinin signal. *Arabidopsis* response regulators, ARR, can both downregulate cytokinin signal, ARR-A, and upregulate the signal, ARR-B (Rashotte et al., 2006). In total, 4 response regulator proteins (RR) sequences have been identified in *C. reinhardtii* (Pils & Heyl, 2009). These serve as transcription factors and control regulation of the Arabidopsis AP2 family. The functions of the AP2 family genes have not been determined yet (Rashotte, et al., 2006). Homologs of RR have been associated with AP2 genes in *C. reinhardtii* (Merchant, et al., 2007).

The cytokinin signal also activates transcription of D-type cyclin, involved in cell cycle activation. The effect of increased growth from cytokinins is a product of the activation of these regulators of the cell division cycle and differentiation (Riou-Khamlichi, et al., 1999; Sheen, 2001). Cytokinins increase transcription activation of a cyclin D3, involved with the G<sub>1</sub>-to-S phase transition. Transgenic overexpression of the cyclin D3 resulted in cytokinin-like growth increases in *A. thaliana* (Riou-Khamlichi, et al., 1999).

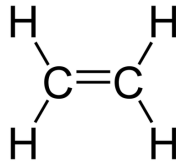
Thus, cytokinins, as cell-division promoting substances, may induce a faster growth rate in algae cells as they do in higher-level plant species. This fact, along with the detection of cytokinin-like activity in algae cells, is encouraging and

highlights the potential for these substances to promote enhanced biodiesel production from algae.

#### **2.3.4.5 Ethylene**

The gas ethylene (Figure 12) plays a central role in the growth and development of terrestrial plants. It is produced to some degree by all higher plants. Ethylene affects the growth and shape of developing cells (Dugardeyn & Van Der Straeten, 2008). As a phytohormone, it is responsible for flower and fruit growth, as well as senescence of cells and abscission of leaves (Wray, 1992). Ethylene is produced in plants as one product of the degradation of the amino acid methionine. The hormone can be detrimental to plant health if found in abnormally large concentrations. In plants such as tomatoes and potatoes, concentrations as low as 50 micrograms per cubic meter can yield reduction in cellular growth. Growth inhibition experiments suggest that algae can withstand only low concentrations of ethylene, as higher levels have proven toxic. After 72 hours of culturing the alga *Selenastrum capricornutum* in growth medium with dissolved ethylene concentrations ranging from 8.2 to 131 mg/L, the concentration of ethylene for growth inhibition was found to be 40 mg/L. This result suggests that algae have no natural mechanism to utilize ethylene. On the other hand, ethylene biosynthesis has been observed in certain species of algae, which indicates that it must serve some purpose. In *Haematococcus pluvialis*, the ethylene biosynthesis pathway is nearly identical to that of *S.*

*capricornutum*, with the breakdown of methionine into intermediates which yield ethylene (Maillard, Thepenier, & Gudin, 1993). While the evidence for ethylene activity in algae is scarce, the specific processes by which it operates in other plants are well documented.



**Figure 12: Ethylene, a gaseous phytohormone which aids in the growth and development of many land plants**

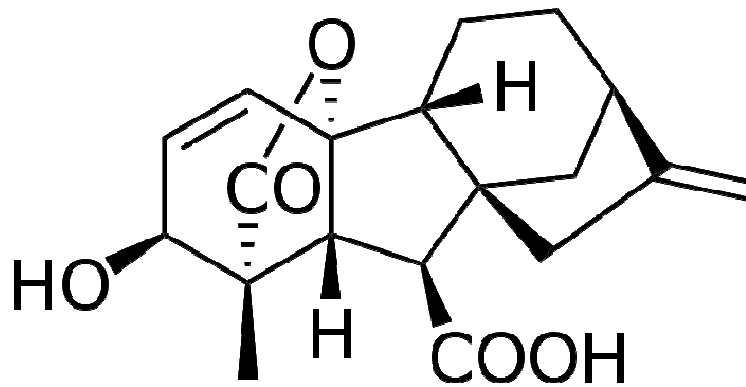
Ethylene affects its target cells through a family of membrane receptors, including the Ethylene Response Sensor 1 and 2 proteins. Ethylene binds to its receptors through a copper ion, which is carried by a protein transporter. In the presence of ethylene, the membrane receptors are inhibited (H. Guo & Ecker, 2004; Hua & Meyerowitz, 1998). Inhibition of the receptors prevents the activation of a Raf-like serine/threonine kinase, which is a negative regulator of the ethylene response in *A. thaliana*. Ethylene response in *A. thaliana* consists of differential gene expression through the activation of transcription factors known as EIN1, 2, and 3 (Ethylene Insensitive) (H. Guo & Ecker, 2004). These transcription factors aid in the growth response to ethylene. The ethylene receptor ETR1 is located on the endoplasmic reticulum (Chen, Randlett, Findell, & Schaller, 2002). This receptor follows the same pattern of signal transduction as Ethylene Response Sensor 1 and 2 proteins. Ethylene uses repressors for regulation of transcription of growth regulation genes. The downstream protein CTR1 serves as an important repressor in the ethylene

signal transduction pathway (K. L. C. Wang, Li, & Ecker, 2002). Evidence suggests that CTR1 acts similarly to MAPK kinases. Therefore, a MAPKK cascade may be involved with ethylene signaling as well (K. L. C. Wang, et al., 2002).

The evidence for the use of ethylene in algal species described above is limited, and the findings may not apply to other green algae. The lack of convincing evidence for positive growth or lipid accumulation in algae suggests that when introduced to *C. reinhardtii*, ethylene is not likely to enhance biodiesel production.

#### **2.3.4.6 Gibberellins**

Gibberellins are diterpenoid acids that affect many areas of plant growth. They promote stem elongation and fruit generation, and they allow seed germination (M. Nakajima et al., 2006). Some gibberellin biosynthetic enzymes, such as GA 3 $\beta$ -hydroxylase, are coded by sGA3ox1. GA 3 $\beta$ -hydroxylase converts GA20 to bioactive GA1 (Figure 13) using the CaMV-35S promoter. Application of Gibberellin caused lettuce cells to increase in size (Gonai et al., 2004). Little evidence for endogenous gibberellin activity has been observed in green algae. Jennings observed increased growth of algae in response to gibberellin effects, but in *C. reinhardtii*, gibberellin activity has not been demonstrated (Jennings, 1968) (Kato, Purves, & Phinney, 1962).



**Figure 13: Gibberellin 1 (GA1) is a member of the gibberellin class of phytohormones**

Current knowledge of the gibberellin signaling pathway revolves around the gibberellin insensitive dwarf receptor protein, GID1, which interacts with F-box proteins similar to the Auxin pathway for transcriptional regulation (Masatoshi Nakajima et al., 2006). Downstream repressor proteins, DELLA, are thereby inactivated to yield transcription of cell growth genes. Identified *A. thaliana* orthologs of these genes include AtGID1a, AtGID1b, and AtGID1c, along with the DELLA ortholog, AtDELLA (Nakajima et al. 2006). Gibberellin binding also results in suppression of GAI/RGA found in the plants (Richards et al. 2001). The function of GAI/RGA has not yet been completely determined and work on characterizing the gibberellin signaling processes continues.

Although increased growth in response to gibberellins has been documented in algae, there is scarce evidence for its actions beyond those in higher-order plants. Specifically, gibberellin activity has not been demonstrated in *C. reinhardtii*, so it is unlikely to play a role in lipid production or growth enhancement in this species.

## 2.4 Concluding Remarks about the Literature

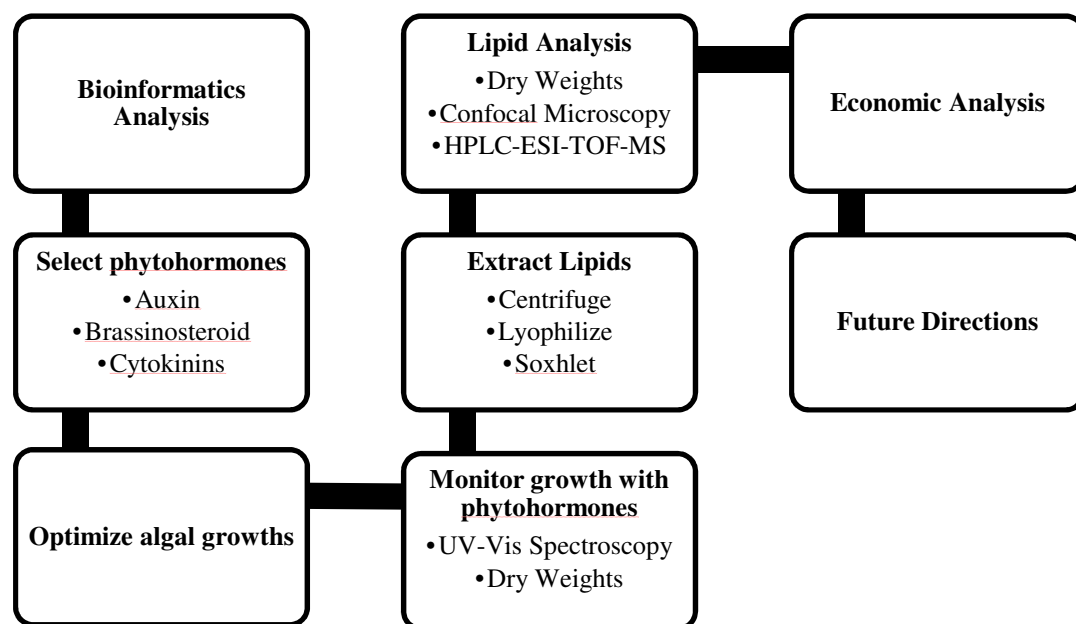
The demand for abundant, clean, and renewable energy sources has led to the development of several potential substitutes for traditional fossil fuels. Hydroelectric power, wind power, solar power, nuclear power, and biofuels are major candidates to fill this niche as the dominant producer of the world's energy. Even a partial replacement of fossil fuels may dramatically improve the environment, human health, or the global economic situation. However, only biofuels are compatible with the currently preferred modes of transportation, which require liquid fuels to fill the tanks of modern automobiles and airplanes. Beyond transportation, liquid fuels are important in heating, and even in electricity production. Within the scope of biofuels, biodiesel is the optimal candidate to replace fossil fuels. Biodiesel has a higher energy density than ethanol, and is perfectly compatible with today's internal combustion engines. Additionally, unlike ethanol, it can be transported through the network of pipelines which currently distributes petroleum across this nation. The most logical source of biodiesel production is microorganisms, such as algae. Production from algae does not compete with the food supply, and it does not require the same large land investment as biodiesel from corn or other crops.

The ideal alga for biodiesel research is *C. reinhardtii*, which has a fully-sequenced genome and has served as a model research species for decades. Although *C. reinhardtii* has lower lipid content than many other algae, changes found in this organism will be easily translatable to other algal species. Past research has focused on genetic modifications and manipulating environmental variables for stimulating

growth rates and lipid production. However, a novel approach to enhancing lipid accumulation is the introduction of phytohormones. These substances promote growth and other responses in higher-order land plants, such as *A. thaliana*. They are naturally produced in many algal species as well, and their introduction to cultures of algae may have similar growth-enhancing effects as in plants. A detailed comparative genomic analysis, examining homologies in phytohormone receptors between plants and algae, will help determine the ideal candidates for experimentation. Specifically, *A. thaliana* phytohormone receptors which are also detected in the *C. reinhardtii* genome will allow for the identification of phytohormones which will serve as starting points for this study.

### 3. Methodology

To determine which phytohormones could potentially induce a response in *C. reinhardtii*, we performed a comparative genomics analysis to identify analogous phytohormone receptor genes in *A. thaliana* and *C. reinhardtii*. After identifying phytohormones that *C. reinhardtii* could potentially respond to, we conducted three sets of experiments to determine the effects of various phytohormones on the growth rate and lipid yield on the algae. First, *C. reinhardtii* was grown in minimal media containing various concentrations of phytohormones. Next the lipids were extracted from each of the cultures in order to purify and isolate the cellular fraction that could potentially be transformed into biofuels. Afterwards, we performed a confocal microscopy analysis and a HPLC-ESI-TOF-MS analysis to determine the types and quantities of lipids generated by the algae. Finally, we determined the economic efficiency of adding phytohormones to algae to increase biofuel production. The flowchart below (Figure 14) describes the basic approach of our method.



**Figure 14: Flow chart depicting the basic outline of the project starting from bioinformatics analysis.**

### 3.1 Bioinformatics

We first conducted a literature review of the molecular mechanisms of the phytohormone signal transduction pathway in *A. thaliana*. Receptor and signal proteins integral to eliciting a cellular response were identified. The gene sequences of the most common proteins and receptors, listed in Table 10, involved in each pathway were obtained from Arabidopsis Information Resource (www.arabidopsis.org). The sequences were aligned with the *C. reinhardtii* genome using the Basic Local Alignment Search Tool (BLAST, blast.nlm.nih.gov). The bit scores, which indicate the quality of an alignment, and Expect values, which

represent the number of hits expected to be obtained by chance, were obtained for the best matches in *C. reinhardtii*. The alignments were evaluated for the number of matches, the lowest Expect values, and the highest bit scores. Alignments producing matches with an Expect value less than 0.01 were considered significant. A phytohormone signal transduction pathway was considered similar if at least two genes associated with the pathway provided matches with the criteria described above. Using these parameters, the likelihood of similarities in phytohormone signal transduction pathways between *A. thaliana* and *C. reinhardtii* was determined.

**Table 10: The phytohormone receptor genes we decided to compare between *A. thaliana* and *C. reinhardtii* were found from the Arabidopsis Information Resource.**

Phytohormone	Absciscic Acid	Brassinosteroids	Cytokinins	Auxin	Ethylene	Gibberellins
Receptor Genes	GCR2	BZR1	CRE1	ABP1	ERS1	GID1
	ABI3	BRI1	AHK4	TIR1	EIN1	GAI
	FCA	BIN2	AP2	KIP2	CTR1	RGA
	ABI2	BES1	ARR	ARF1	ERS2	RGL2
	RPK1	DET2	CYCD3	ARF19	EIN3	RGL3
	GTG1	DWF4	AHK5	ARF8	ETR1	RGL1
	ABI1		ARR5	ARF6	EIN2	
	GTG2		ARR2	CYCD3		
	RPK1		ARR19	ATCUL1		
				SKP1		
				RBX1		
				UBC3		
<b>Total</b>	<b>9</b>	<b>6</b>	<b>9</b>	<b>12</b>	<b>7</b>	<b>6</b>

## 3.2 Algal Growth Methods

### 3.2.1 Growth Conditions

*C. reinhardtii* (UTex strain 89) was obtained from the Culture Collection of Algae at the University of Texas at Austin. The algae were grown in minimal salt *C.*

*reinhardtii* medium, as specified in the CRC Handbook of Microalgal Mass Culture (Table 11), in 250 mL Erlenmeyer flasks covered with cheesecloth wrapped in cotton balls to prevent contamination. Minimal media was chosen in order to eliminate possible interactions the phytohormones would have with rich media. Minimal media would also serve as a better model for inexpensive large-scale growth.

The ultimate growth conditions for algae were determined after initial optimization trials. The algae were inoculated into the medium (pH 7.0) at a ratio of 1:9 (stock algae : medium). The algae were grown under constant light source of 1900 lumens at a temperature of 22 °C. The flasks were rotated at a constant 100 rpm on the New Brunswick Gyrotory Shaker Model G2. The carbon source for the algae was atmospheric CO<sub>2</sub>.

### **3.2.2 Determining Cell Density from Absorbance**

Algal cultures were examined by microscopy and hemocytometer to establish a relationship between *C. reinhardtii* cell densities and OD readings. This relationship helped us to determine the growth rates of the algae. Such a procedure allows for standardization of the spectrophotometer data, eliminating the need for physical cell counting in subsequent readings. To obtain the conversion rate between OD and cell density, a sequence of samples were created by serially diluting the stock solution by tenfold 4 times. The OD reading for each sample was taken. Afterwards, 1 mL of each sample was added to the hemocytometer. The number of algae on the 5x5 grid on the hemocytometer was counted at 100x magnification. A graph of number of

algae versus the OD was then created to determine the conversion rate (75000 algae/mL) (Chlamydomonas Sourcebook).

**Table 11: The minimal salt *C. reinhardtii* media as found in the CRC Handbook of Microalgal Mass Culture is shown below. To prevent precipitates in the Hutner's Trace Elements, we first added everything to the solution except FeSO<sub>4</sub> and EDTA. The solution was brought to a boil. EDTA and FeSO<sub>4</sub> were slowly added to the solution. The final Hutner's Trace Elements solution was a dark purple color. The minimal media was autoclaved before inoculation with *C. reinhardtii*.**

<i>C. reinhardtii</i> Minimal Media	Concentration (L <sup>-1</sup> )	Hutner's Trace Elements	Concentration (mg/mL)
NH <sub>4</sub> Cl	0.05 g	EDTA Disodium Salt, Dihydrate@	66.6
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.02 g	ZnSO <sub>4</sub> ·7H <sub>2</sub> O	29.33
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.01 g	H <sub>3</sub> BO <sub>3</sub>	15.2
K <sub>2</sub> HPO <sub>4</sub>	0.072 g	MnCl <sub>2</sub> ·4H <sub>2</sub> O	6.8
KH <sub>2</sub> PO <sub>4</sub>	0.036 g	FeSO <sub>4</sub> ·7H <sub>2</sub> O	6.66
Hutner's Trace Elements	1 mL	CoCl <sub>2</sub> ·6H <sub>2</sub> O	2.13

### 3.2.3 Addition of Phytohormones

The phytohormones tested in this study were Indole-3-acetic acid (IAA), Epibrassinolide, Trans-zeatin, and Trans-zeatin-riboside (Table 12, Figure 15). These were provided free of charge by Sigma-Aldrich. Each experiment tested a particular phytohormone at concentrations 10<sup>-12</sup> M, 10<sup>-11</sup> M, 10<sup>-10</sup> M, 10<sup>-9</sup> M, and 10<sup>-8</sup> M. This range was chosen after initial tests showed that concentrations of phytohormones over 10<sup>-6</sup> generally killed the algae and literature review showed that *C. reinhardtii* actually produces auxin and brassinosteroids at concentrations around 10<sup>-13</sup> M. These cultures were otherwise grown in the conditions as above. The initial four phytohormone treatments were IAA, Epibrassinolide, Trans-zeatin, and Trans-zeatin-riboside. An additional two phytohormone treatments were conducted after analyzing

the preliminary data. These treatments were a combination of IAA and Trans-zeatin and a combination of IAA and Trans-zeatin-riboside (Table 13). These combination treatments had the potential to further increase growth because the literature review revealed that auxins and cytokinins work synergistically to increase growth rate in plants.

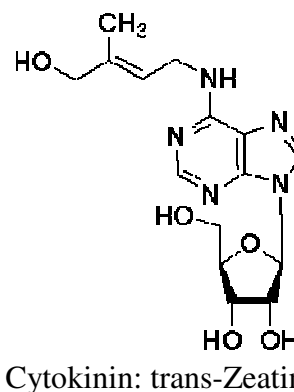
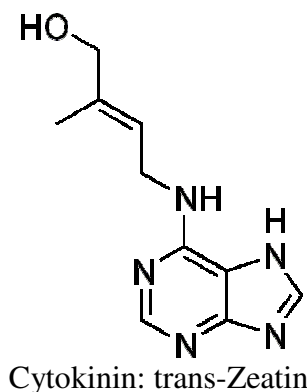
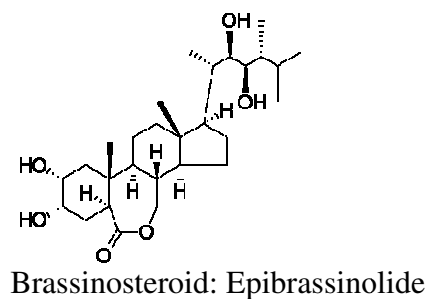
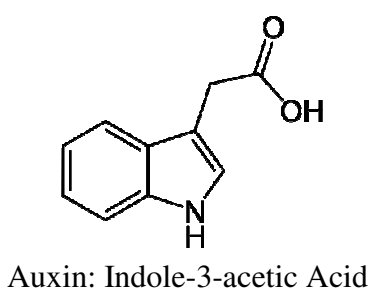
Each trial consisted of a set of 5 cultures with concentrations of phytohormones for each order of magnitude ranging from  $10^{-8}$  to  $10^{-12}$  M. These solutions were created from 1 mM stock solutions of phytohormones dissolved in ethanol. The ethanol was used to help dissolve the phytohormones. In order to maintain a consistent level of ethanol in the cultures, additional ethanol was added to each solution until a final concentration of 0.001% ethanol by volume. Two additional cultures were grown without the addition of phytohormones. One had 0.001% ethanol by volume while the other was simply grown in the growth conditions mentioned in the previous section. The additional cultures serve as controls to compare the effects of the applied phytohormones and to ensure that ethanol has no impact on the algae. For each culture, OD was measured consistently twice a day for a total of 10 days. Times for measurement were consistent day to day. At the end of 10 days, cells were isolated by centrifugation and dried by lyophilization. The dry algal mass of these samples was then measured. A thorough description of procedures for this process follows.

**Table 12: List of the phytohormones and their properties**

Phytohormone	Category	Formula	Molecular Weight	Catalog Number
Indole-3-acetic Acid (IAA)	Auxin	C <sub>10</sub> H <sub>9</sub> NO <sub>2</sub>	175.18	I2886
Epibrassinolide	Brassinosteroid	C <sub>28</sub> H <sub>48</sub> O <sub>6</sub>	480.68	E1641
Trans-Zeatin	Cytokinin	C <sub>10</sub> H <sub>13</sub> N <sub>5</sub> O	219.24	Z0876
Trans-Zeatin-Riboside	Cytokinin	C <sub>15</sub> H <sub>21</sub> N <sub>5</sub> O <sub>5</sub>	351.36	Z0375

**Table 13: The treatments of phytohormones and concentrations of these treatments along with the number of trials are shown below. The data used to analyze the effects of phytohormones only come from this data set. Although other trials were conducted, these 40 trials were conducted in the exact same conditions as possible in a very small time frame (103 days).**

Treatment	Concentrations of Phytohormones	Number of Trials
IAA	10 <sup>-12</sup> - 10 <sup>-8</sup>	8
Epibrassinolide	10 <sup>-12</sup> - 10 <sup>-8</sup>	8
Trans-Zeatin	10 <sup>-12</sup> - 10 <sup>-8</sup>	8
Trans-Zeatin-Riboside	10 <sup>-12</sup> - 10 <sup>-8</sup>	8
IAA + Trans-Zeatin	10 <sup>-11</sup> + 10 <sup>-12</sup> - 10 <sup>-8</sup>	4
IAA + Trans-Zeatin-Riboside	10 <sup>-11</sup> + 10 <sup>-12</sup> - 10 <sup>-8</sup>	4



**Figure 15: The molecular structures of the phytohormones are shown above (Chemdraw).**

### **3.3 Extraction Methods**

#### **3.3.1 Isolating Algae**

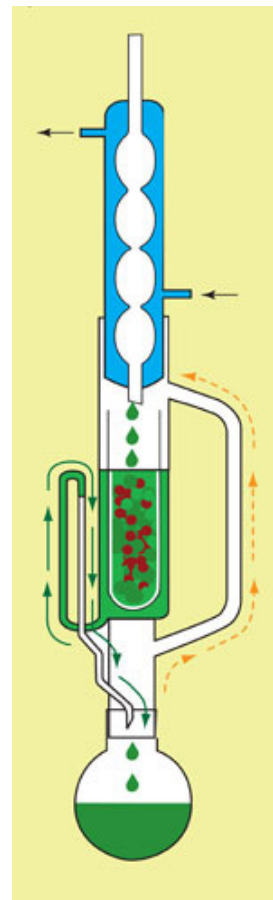
In order to prepare the algae for extraction and analysis, 125 mL of each culture ( $V_C$ ) were centrifuged for 5 minutes in 50 mL Falcon tubes at 5000 g using a Beckman Avanti J-25I Centrifuge 5 (Goldschmidt-Clermont, 1989). Multiple rounds of centrifugation were conducted for each culture to ensure all 125 mL worth of algae could be condensed into a pellet inside the 50 mL Falcon tubes. After each round of centrifugation, all but 5 mL of the supernatant was discarded. When the cultures were finished being centrifuged, the pellet was then resuspended in the remaining supernatant and frozen with liquid nitrogen. The frozen mass was then lyophilized by the Labconco Freezone 2.5 for 18 hours at 0.012 Torr to remove all remaining water. The resuspension of the cell pellet allowed for more even drying and easier handling of the dry algae during the Soxhlet protocol. A rotary evaporator could be used in place of the lyophilizer for the same results (Jiang, 1975). The mass of the dry pellet ( $M_T$ ) was determined by taking the difference between the mass of the Falcon tube and the pellet after lyophilization and the Falcon tube before the isolation procedure. The dry pellet was then extracted for lipids by the Soxhlet protocol.

#### **3.3.2 Soxhlet Protocol**

The Soxhlet procedure is efficient at extracting about 90% of the total lipid yield (Cooksey, et al., 1987). The dry algal content was transferred into a 10 mm

Whatman cellulosic thimble. Not all of the algae could be transferred so the mass of the extracted algae ( $M_E$ ) was determined by difference between the mass of the cellulosic thimble with the algae and the mass of the cellulosic thimble. The cellulosic thimble was then placed into the Soxhlet thimble. The Soxhlet thimble was then connected to a roundbottom flask and a condenser. The roundbottom flask contained a mixture of 7 mL hexane and 7 mL isopropanol, which was chosen in order to be able to extract the greatest amount and types of lipids (Manirakiza, Covaci, & Schepens, 2001). The Soxhlet apparatus (Figure 16) was set onto a heating block and heated to 82.5 °C. The solvent was refluxed in the Soxhlet apparatus for a total of 4 hours. After 4 hours, the lipids in the algae will have sequestered into the solvent in the roundbottom flask. The remaining content inside the thimble includes all of the non-soluble content of the algal cell including various organelles and proteins. A total of 6 extractions were carried out at the same time in order to process 1 entire trial of phytohormone treatment at the same time. The solutions in the roundbottom flasks were further processed to isolate the dry lipids. We are grateful to Dr. Thomas Castonguay, Nutrition Department at the University of Maryland, for guidance and use of the Soxhlet extraction equipment.

**Figure 16: The Soxhlet thimble is the piece of glassware between the roundbottom flask and condenser. The cellulosic thimble was placed inside the main body of the thimble. When heated, the solvent in the roundbottom flask evaporates and travels along the outer arm (orange arrow). The solvent then condenses inside the condenser and drips into the main thimble chamber. The cellulosic thimble allows lipid soluble components to pass through the cellulosic thimble while retaining other unwanted particulates. Eventually when enough solution has accumulated inside the main thimble chamber, the solution will drain through the Soxhlet arm (green arrow), thus causing the lipids dissolved in the main thimble body to drain and concentrate into the roundbottom flask. This process repeats for 4 hours. (Image Source: <http://www.rsc.org/chemistryworld/Issues/2007/September/ClassicKitSoxhletExtractor.asp>)**



### 3.3.3 Isolating Lipids

The 14 mL of solvent containing the lipids after Soxhlet extraction was transferred to scintillation vials and then evaporated using a N<sub>2</sub> evaporator until dry. The tips of the evaporator were constantly adjusted to be 1 cm away from the top of the solution. The flow rate of the evaporator was adjusted to ensure that no splashing of the solution occurred. The evaporation of the solvent typically took about 20 minutes, leaving only dry lipids inside the scintillation vial. The mass of the lipids (M<sub>L</sub>) was determined by taking the difference in mass between the scintillation vial with lipid after evaporation and the empty scintillation vial. This mass was used to determine the total grams of lipid created per liter of culture, according to the calculation below.

$$\text{lipid concentration } \left(\frac{g}{L}\right) = \left(\frac{M_T}{M_E}\right)(M_L)/(V_C)$$

$M_T$  = total mass of algae growth (g)

$M_E$  = mass of algae used for extraction (g)

$M_L$  = mass of extracted lipids (g)

$V_C$  = volume of culture (g)

### 3.4 Lipid Analysis Methods

#### 3.4.1 Liquid Chromatography/Mass Spectrometry

We identified the lipids found in our extract by liquid chromatography/mass spectrometry (LC/MS). Liquid chromatography was used to first separate the lipids based on polarity. Mass spectrometry was then used to identify the individual separated lipids by matching the resulting mass to charge ratios of the lipids with known literature values. Our LC/MS apparatus interfaced the Agilent 1100 HPLC with the JEOL AccuTOF-CS ESI-TOF Mass Spectrometer to form a high pressure liquid chromatography – electrospray ionization – time of flight (HPLC-ESI-TOF) setup, in the Chemistry Department of University of Maryland. We are grateful to Dr. Yue Li for guidance in adapting LC-MS protocols to our needs and in use of the equipment.

The dry lipid extracts were resuspended in isopropanol before LC/MS analysis. A gradient for the HPLC mobile phase was used. Mobile Phase A was 90% methanol, 9.5% acetonitrile and 0.5% acetic acid. Mobile Phase B contained 9.5%

methanol, 90% acetonitrile, and 0.5% acetic acid. The gradient started with 50% Mobile Phase A and B and ramped up to 100% Mobile Phase B over the course of 30 minutes. This final condition was maintained for 40 minutes, after which a ten minute flushing period occurred with Mobile Phase B. The flow rate was 0.25 mL/min. The UV/Vis spectrometer on the Agilent 1100 HPLC was set to monitor absorbance at 220 nm. The sample size of each trial was 10  $\mu$ L (Vieler, Wilhelm, Goss, Süß, & Schiller, 2007b). Originally the gradient used was 0% Mobile Phase B to 100% Mobile Phase B over a period of 30 minutes, but most of the lipids did not elute until the very end, so the initial concentration and time span of Mobile Phase B were increased. This yielded a better resolution for the lipid separation. The disadvantage of using an acetonitrile mobile phase is that the acetonitrile has some absorbance at 200 nm, the wavelengths absorbed of the lipids. Although this interference makes it more difficult the ability to accurately quantify the lipids, the ability to accurately identify the lipids is not affected.

### **3.4.2 Confocal Microscopy**

To determine whether a particular phytohormone treatment had caused a culture to produce more lipids and to assess subcellular distribution of lipids, a small sample of each culture was stained with Nile Red and then observed under the Zeiss LSM 510 Confocal Microscope. Nile Red binds to lipids so the quantity bound to the algal sample can be used to determine the total lipid content. Excitation and emission spectrum were obtained in order to determine the optimal wavelengths for measuring

the lipid concentration of the cells. It was determined that the optimal excitation and emission wavelengths were 488 nm and 540 nm, respectively. Chlorophyll is also excited at 488 nm, but the emission of chlorophyll at 540 nm was determined to be insignificant in comparison to the emission of the Nile Red dye. The resulting images were used to qualitatively determine whether lipid production increased or decreased. Morphological changes including size and shape were also assessed from the images.

## 4. Results

Our investigation of the effects of phytohormones on *Chlamydomonas reinhardtii* began by assessing the possible genetic basis for phytohormone signaling, followed by testing of the phytohormones by addition to algal cultures. We first wanted to explore the underlying evolutionary links between signaling components of phytohormones in *C. reinhardtii* based on those determined in *Arabidopsis thaliana*. Using the Basic Local Alignment Search Tool (BLAST) we compared the nucleotide sequences of genes across the two organisms.

After this, we selected phytohormones, Indole-3-acetic acid (IAA), Epibrassinolide, Trans-Zeatin, and Trans-Zeatin-Riboside, which showed signs of homology between the two organisms and also had supporting evidence for action in algae in literature. Having established this, we proceeded to test these phytohormones at concentrations ranging from  $10^{-12}$  M to  $10^{-8}$  M. We measured the growth rate of cultures using spectrophotometry and also determined their dry algal mass. After isolating and drying the algae we extracted lipids from the cells via Soxhlet extraction. We measured the mass of the extracted lipids, which we refer to as the specific lipid mass. This specific lipid mass was used in conjunction with the dry algal mass to calculate the efficiency of the cultures, referred to as the lipid concentration. This value accommodates the productive capacity of a culture for lipid quantity and rate of growth.

Beyond these quantitative measures, we sought to look closer at the intracellular effects of the algae. Using confocal imaging with Nile Red dye, we were able to visualize changes in lipid bodies of cells. Additionally, we performed a High Pressure Liquid Chromatography-Electrospray Ionization-Time of Flight- Mass Spectrometry (HPLC-ESI-TOF-MS, referred to as LC-MS) analysis of the extracted lipids. This allowed us to determine the changes in the metabolism of lipids. By comparing findings from these various experimental techniques we were able to understand the effects of phytohormones on *C. reinhardtii*.

#### **4.1 Genomic Analysis**

We used the BLAST tool to search for *C. reinhardtii* genes that were homologous to known genes of *A. thaliana* phytohormone receptors and signaling intermediates. We searched for specific genes whose receptors were most commonly found in *A. thaliana*. We examined the Expect values (E-values) and bit scores for each receptor. A low E-value (0.01 or lower) indicates that the results are more significant, meaning less likely to have happened by chance. A high bit score signifies a better degree of sequence alignment between the gene and the receptor. We analyzed the search results to find which phytohormones could possibly induce changes in growth rate or lipid content.

#### 4.1.1 Absciscic Acid Signaling Components

Gene ABI1 from *A. thaliana* generated a match with a hypothetical protein in *C. reinhardtii* that had a low E-value of 0.009 and a relatively high bit score of 42.8. Homology could not be determined because the functions of hypothetical proteins are unknown and have yet to be annotated. The genes involved in the absciscic acid response for *A. thaliana* generated matches predominantly with hypothetical proteins in *C. reinhardtii*.

Previous research on the signal transduction pathway for absciscic acid response has been ambiguous. Candidates for the ABA receptor such as the GCR2 and FCA proteins in *A. thaliana* have been invalidated while the ABA insensitive proteins have been shown to receive phytohormone molecules on the surface of the *A. thaliana* cell membrane, although there is no general consensus on these (Anderson, et al.; Leung, et al., 1994). This ambiguity was reflected in the bioinformatics analysis as no protein involved in the *A. thaliana* phytohormone cascade that was examined demonstrated strong matches with the *C. reinhardtii* genome. Due to this, absciscic acid was not selected for further investigation.

**Table 14: Genomic analysis results for *A. thaliana* query genes involved in absciscic acid recognition matches in *C. reinhardtii*.**

Query Gene	# of Matches	Lowest E-Value	Highest Bit Score	Matches w/E-value <.1	Best Match
GCR2	7	3.5	33.7	0	Hypothetical Protein
ABI3	5	9.8	31.9	0	Hypothetical Protein
FCA	10+	1.4	37.4	0	Hypothetical Protein

Query Gene	# of Matches	Lowest E-Value	Highest Bit Score	Matches w/E-value <.1	Best Match
ABI2	0	N/A	N/A	0	No Match
RPK1	0	N/A	N/A	0	No Match
GTG1	2	4.0	33.7	0	Hypothetical Protein
<b>ABI1</b>	<b>4</b>	<b>.009</b>	<b>42.8</b>	<b>1</b>	<b>Hypothetical Protein</b>
GTG2	2	.89	37.4	0	Hypothetical Protein
RPK1	2	4.0	33.7	0	Hypothetical Protein

#### 4.1.2 Auxin Signaling Components

As for abscisic acid, the matches for the genes involved in auxin response in *A. thaliana* were mostly hypothetical proteins in *C. reinhardtii*. However, the auxin response factors (ARFs), which dimerize and bind to DNA to allow transcriptional control in *A. thaliana*, provided matches for ARF8 and ASK2 in the *C. reinhardtii* genome, with low E-values of less than 0.01 as well as relatively high bit scores. For instance, one protein in *C. reinhardtii*, the low CO<sub>2</sub>-inducible protein (LCI1), with an E-value of .038, a bit score of 41.0, and a maximal identity of 100% was found to resemble the ARF8 protein.

ARF8 mediates the phytohormone response in *A. thaliana* by expressing auxin regulated genes and, along with ARF6, controls stamen elongation and flower maturation, while ASK2 aids in controlling the mitotic cell cycle (Mattsson, Ckurshumova, & Berleth, 2003). The LCI1 gene, which was considered to be a strong match with ARF8 in *A. thaliana*, has been observed to be involved in the encoding

and regulation of a transporter that increases the uptake of CO<sub>2</sub>, but it has not been characterized biochemically (Ohnishi et al., 2010). Thus, it is difficult to determine whether the LCI1 protein is relevant to a possible auxin response mechanism in *C. reinhardtii* or if it contains a conserved transporter domain.

Additionally, the SKP1 protein in *C. reinhardtii*, which is an ubiquitin ligase, had an E-value on the order of 10<sup>-48</sup> and a maximal identity of 84%, indicating possible homology. SKP1 was a strong match with ASK2 and has been shown to be involved in cell division. However, it is not known whether it is regulated by auxin. Although LCI1 and SKP1 have not been shown to be directly involved in phytohormone response, previous research regarding the application of auxin to *C. reinhardtii* has demonstrated that *C. reinhardtii* may respond to the phytohormone and does secrete small concentrations of auxin (Cooke, Poli, Szein, & Cohen, 2002; W. Jacobs, 1985; W. P. Jacobs, 1951). Thus, other genes that have not been annotated may be involved in phytohormone response. It is possible that these genes may not resemble or share any homology with the genes involved in phytohormone response in *A. thaliana*. Due to the fact that literature indicates a possible role for auxin and bioinformatics reveal possible homologous proteins found in *C. reinhardtii*, auxins were selected for further analysis.

**Table 15: Genomic analysis results for *A. thaliana* query genes involved in auxin recognition matches in *C. reinhardtii*.**

<b>Gene Name</b>	<b># of Matches</b>	<b>Lowest E-Value</b>	<b>Highest Bit Score</b>	<b>Matches w/E-value &lt;.1</b>	<b>Best Match</b>
ABP1	1	3.6	33.7	0	ZYSB1
TIR1	1	6.5	33.7	0	Hypothetical Protein
KIP2	5	2.1	33.7	0	Hypothetical Protein
ARF1	0	N/A	N/A	0	No Match
ARF19	4	2.9	35.6	0	Hypothetical Protein
<b>ARF8</b>	<b>10+</b>	<b>.003</b>	<b>44.6</b>	<b>6</b>	<b>Hypothetical Protein</b>
ARF6	9	.70	37.4	0	Hypothetical Protein
CYCD3	1	2.7	33.7	0	Hypothetical Protein
<b>ATCUL1</b>	<b>6</b>	<b>5e-16</b>	<b>87.8</b>	<b>1</b>	<b>CUL3</b>
<b>SKP1</b>	<b>4</b>	<b>2.3</b>	<b>33.7</b>	<b>0</b>	<b>Hypothetical Protein</b>
RBX1	1	3.3	33.7	0	Hypothetical Protein
<b>UBC3</b>	<b>1</b>	<b>1e-17</b>	<b>91.5</b>	<b>1</b>	<b>UBC10</b>
UBC4	0	N/A	N/A	0	No Match
<b>UBC5</b>	<b>1</b>	<b>2e-8</b>	<b>60.8</b>	<b>1</b>	<b>UBC2</b>
<b>ASK2</b>	<b>10+</b>	<b>5e-48</b>	<b>192</b>	<b>8</b>	<b>SKP1</b>
ASK9	4	.25	35.6	0	Hypothetical Protein
<b>ASK11</b>	<b>3</b>	<b>4e-24</b>	<b>111</b>	<b>1</b>	<b>SKP1</b>

#### 4.1.3 Brassinosteroid Signaling Components

Proteins involved in the brassinosteroid response in *A. thaliana* provided matches only for the BRZ1 and BES1 proteins. These matches returned low E-values of less than 0.01, high bit scores, and maximal identities near 100%. The BZR1 protein, which has a DNA binding domain, had numerous matches in the genome of *C. reinhardtii* with E-values less than .003, including the zinc-metalloproteinase-like

protein (ZMP1), flagellar associated protein (FAP92), and VPS34-like PI-3 kinase (VPS34).

The signal transduction pathway for brassinosteroids is better understood than those of other phytohormones. Brassinosteroids bind with receptors at the plasma membrane composed of the Brassinosteroid receptor protein 1 (BRI1) paired with a serine-threonine kinase, BIN2 (Nam & Li, 2002). When brassinosteroids bind BRI1, BIN2 is inactivated by an unknown mechanism, which allows hypophosphorylated nuclear proteins such as Brassinazole resistant (BZR1) to suppress transcription (He, Gendron, Yang, Li, & Wang, 2002).

The BES1 protein, which encodes a signaling protein that accumulates in the nucleus, and the BZR1 protein, which has a DNA binding domain, had numerous matches in the genome of *C. reinhardtii* with E-values less than .003. However each of these proteins, including ZMP1 and VPS34, has known functions related to locomotion and are not associated with the phytohormone response, indicating that the proteins only share conserved domains. In addition, since BES1 and BZR1 are not specific solely to the brassinosteroid response, and thus their strong matches may not be meaningful for our purposes. Proteins more closely associated with the brassinosteroid response pathway, such as BIN2 and BRI1, did not give any strong matches. The relationship between brassinosteroid signaling components in *A. thaliana* and *C. reinhardtii* is not completely elucidated yet. Further work will contribute to understanding this phytohormone's response.

**Table 16: Genomic analysis results for *A. thaliana* query genes involved in brassinosteroid recognition matches in *C. reinhardtii*.**

<b>Gene Name</b>	<b># of Matches</b>	<b>Lowest E-Value</b>	<b>Highest Bit Score</b>	<b>Matches w/E-value &lt;.1</b>	<b>Best Match</b>
<b>BZR1</b>	<b>10+</b>	<b>7e-05</b>	<b>89.1</b>	<b>10+</b>	<b>ZMP1</b>
BRI1	6	.69	37.4	0	Hypothetical Protein
BIN2	1	5.6	33.7	0	Hypothetical Protein
<b>BES1</b>	<b>10+</b>	<b>.002</b>	<b>44.6</b>	<b>9</b>	<b>Hypothetical Protein</b>
DET2	0	N/A	N/A	0	No Match
DWF4	4	9.7	33.7	0	Hypothetical Protein

#### 4.1.4 Cytokinin Signaling Components

Cytokinin-like activity has been observed in *C. reinhardtii*; thus it is likely that the algal genome contains at least a rudimentary signal transduction pathway (Stirk, et al., 2002; Ördög, et al., 2004). The cytokinin binding receptor CRE1, a histidine kinase in *A. thaliana*, generated some matches with E-values of .024 and 0.069, respectively, with the nitrate assimilation regulatory protein (NIT2) and RWP-RK transcription factor. Given the dissimilar suspected functions and bit scores, it is likely that they all contain conserved domains common among histidine kinases and associated proteins due to their diverse functions in *C. reinhardtii*.

AHK4, which is also a histidine kinase in *A. thaliana*, gave a similar list of matches that included NIT2, confirming that the proteins only had conserved domains in common. Other proteins associated with the cytokinin response in *A. thaliana* generated hypothetical proteins or few or no matches. However, due to the findings of

previous research and the possibility that NIT2 and RWP-RK may have some role in phytohormone response, cytokinins were selected for further investigation.

Particularly, Trans-Zeatin and Trans-Zeatin-riboside were selected on the basis of their prevalence in the regulation of cell division for *A. thaliana*.

**Table 17: Genomic analysis results for *A. thaliana* query genes involved in cytokinin recognition matches in *C. reinhardtii*.**

<b>Gene Name</b>	<b># of Matches</b>	<b>Lowest E-Value</b>	<b>Highest Bit Score</b>	<b>Matches w/E-value &lt;.1</b>	<b>Best Match</b>
<b>CRE1</b>	<b>10+</b>	<b>.069</b>	<b>41.0</b>	<b>1</b>	<b>Hypothetical Protein</b>
<b>AHK4</b>	<b>10+</b>	<b>.069</b>	<b>41.0</b>	<b>1</b>	<b>Hypothetical Protein</b>
AP2	3	5.0	33.7	0	Hypothetical Protein
ARR	0	N/A	N/A	0	No Match
CYCD3	1	2.7	33.7	0	Hypothetical Protein
AHK5	6	9.6	33.7	0	Hypothetical Protein
ARR5	0	N/A	N/A	0	No Match
<b>ARR2</b>	<b>5</b>	<b>.047</b>	<b>41.0</b>	<b>1</b>	<b>Hypothetical Protein</b>
ARR19	1	4.5	33.7	0	F1F0

#### 4.1.5 Ethylene Signaling Components

None of the genes searched produced low E-values or high bit scores. Most matches consisted of hypothetical proteins, which could not be further investigated.

Previous research has demonstrated that ethylene has no role in *C. reinhardtii*, confirming that ethylene was not a good candidate for further research.

**Table 18: Genomic analysis results for *A. thaliana* query genes involved in ethylene recognition matches in *C. reinhardtii*.**

Gene Name	# of Matches	Lowest E-Value	Highest Bit Score	Matches w/E-value <.1	Best Match
ERS1	1	1.7	35.6	0	Hypothetical Protein
EIN1	2	7.2	33.7	0	Hypothetical Protein
CTR1	14	.88	37.4	0	MRPL30
ERS2	2	.49	37.4	0	Hypothetical Protein
EIN3	4	5.9	33.7	0	Hypothetical Protein
ETR1	2	7.2	33.7	0	Hypothetical Protein
EIN2	1	.98	37.4	0	Hypothetical Protein

#### 4.1.6 Gibberellin Signaling Components

None of the genes searched produced low E-values, high bit scores. Most matches consisted of hypothetical proteins. Most research has been centered on the gibberellin insensitive dwarf receptor protein (GID1), which interacts with F-box, which mediates protein-protein interactions to regulate transcription (Vandenbussche et al. 2007; Ueguchi-Tanaka et al. 2005). It is an F-box protein that is critical to the response to gibberellins in *A. thaliana* to gibberellins. Alignment search for this gene did not yield any matches. Additionally, signaling intermediates of the gibberellins pathway have not been completely determined. Proteins that have been proposed to be involved in the pathway, in Table 19, did not give any noteworthy matches in *C. reinhardtii*. Thus, we conclude that gibberellins were not likely to initiate a response in *C. reinhardtii*.

**Table 19: Genomic analysis results for *A. thaliana* query genes involved in ethylene recognition matches in *C. reinhardtii*.**

<b>Gene Name</b>	<b># of Matches</b>	<b>Lowest E-Value</b>	<b>Highest Bit Score</b>	<b>Matches w/E-value &lt;.1</b>	<b>Best Match</b>
GID1	1	4.6	33.7	0	REM1
GAI	3	4.3	33.7	0	Hypothetical Protein
<b>RGA</b>	<b>8</b>	<b>1.3</b>	<b>35.6</b>	<b>2</b>	<b>Hypothetical Protein</b>
RGL2	8	.34	37.4	0	Hypothetical Protein
RGL3	6	.33	37.4	0	Hypothetical Protein
RGL1	1	3.7	33.7	0	NS

#### **4.1.7 Overall Interpretation of Bioinformatics**

Most of the *C. reinhardtii* proteins that matched proteins involved in phytohormone responses in *A. thaliana* were hypothetical proteins. Thus, it was difficult to determine whether the pathways found in *A. thaliana* exist in *C. reinhardtii*. However, since auxins are secreted by some species of algae, and cytokinin-like substances are produced by *C. reinhardtii* under certain conditions, auxins and cytokinins were chosen for further investigation (Bajguz & Czerpak, 1996; Cooke, et al., 2002; Stirk, et al., 2002). In addition, brassinosteroids were chosen in part because their possible roles in algae have not been fully examined.

## **4.2 Growth Analysis**

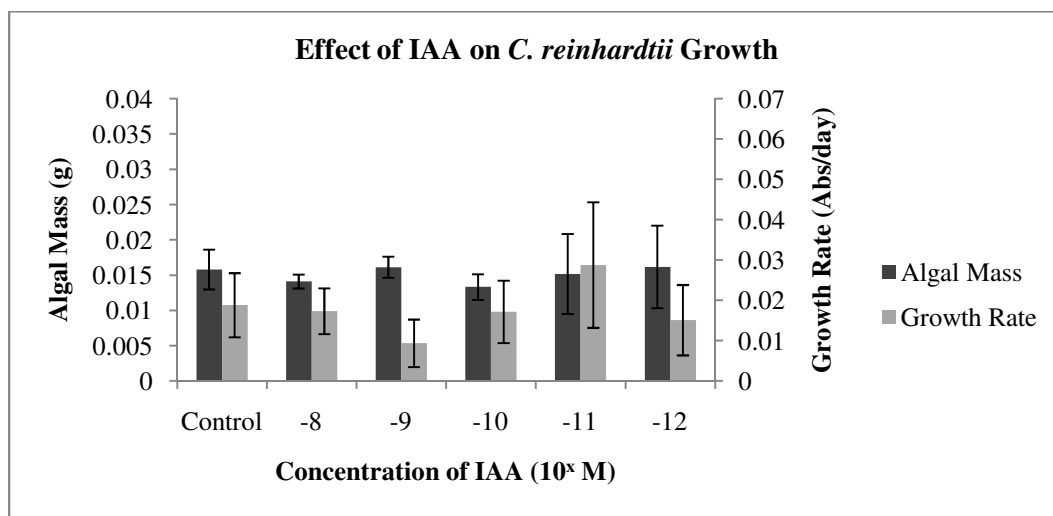
### **4.2.1 Spectroscopy and Algal Mass**

Calculations of growth rates were performed by linear regression analysis of daily measurements of algae samples' absorbance at 670 nm. The error bars shown below in Section 4.2.1.1 and following sections represent the standard deviation of the growth rates calculated. Larger changes in growth rate often occur at lower concentrations of phytohormones, such as in the auxin trials (See Fig. 17). The potential reasons for this occurrence will be explored in the Discussion. In addition to spectrophotometric measurements of growth, dry algal mass of cultures was also measured and averaged across trials. Eight trials were repeated for each phytohormone at each concentration. The growth rates and dry algal masses were plotted together to compare the effect of the phytohormone for each measure of growth. The spectrophotometric growth rate can only account for changes in number of cells, whereas the algal mass includes changes to cell sizes (Representative raw data is provided in appendix A.1 and A.2). The algal mass can only be determined at the end of the growth period.

#### **4.2.1.1 IAA**

The application of auxin, also known as indole-3-acetic acid, was found to cause an increase in growth rates of algae with increases of  $52.9 \pm 36.3\%$  at the  $10^{-11}$  M concentration over a set of 8 trials. The other concentrations of auxin did not show

this large increase in growth rate. The application of IAA slightly increased the final mass of the algae at  $10^{-12}$  M concentration by  $2.4 \pm 1.0$  % relative to controls; however, treatment at all other concentrations caused a decrease in algal mass. The final algal masses remained more or less the same when taking into account experimental uncertainty. Figure 17 provides the measurements at all concentrations and for the controls.

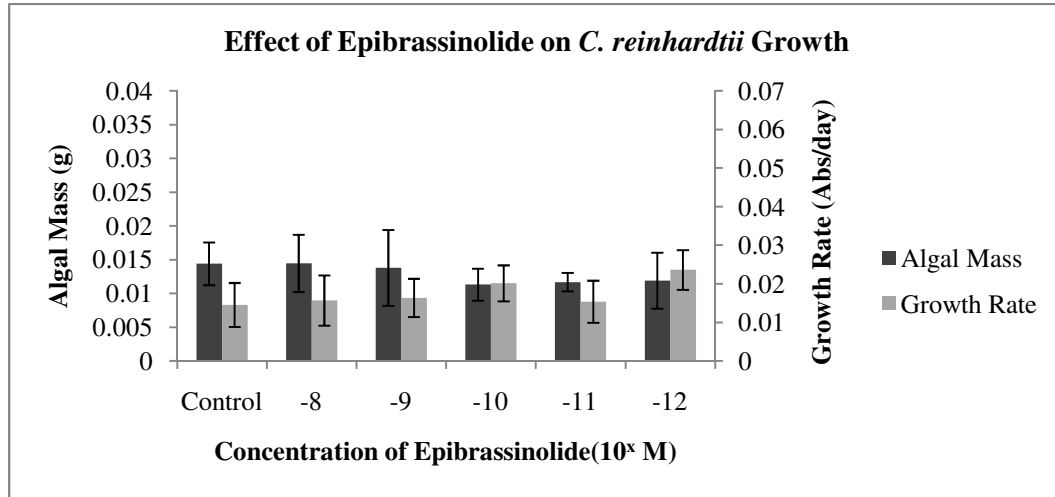


**Figure 17: A concentration of  $10^{-11}$  M of IAA increases the growth rate by 52.9%. The algal mass was not affected significantly by IAA.**

#### 4.2.1.2 Epibrassinolide

The application of various concentrations of Epibrassinolide had similar results: an increase at certain concentrations of the phytohormones, but large variation. There was a smaller increase in growth rate in comparison to IAA, but at the  $10^{-12}$  M concentration of Epibrassinolide, the growth rate increased by approximately  $62.7 \pm 28.1$ %. Application of Epibrassinolide caused a slight decrease

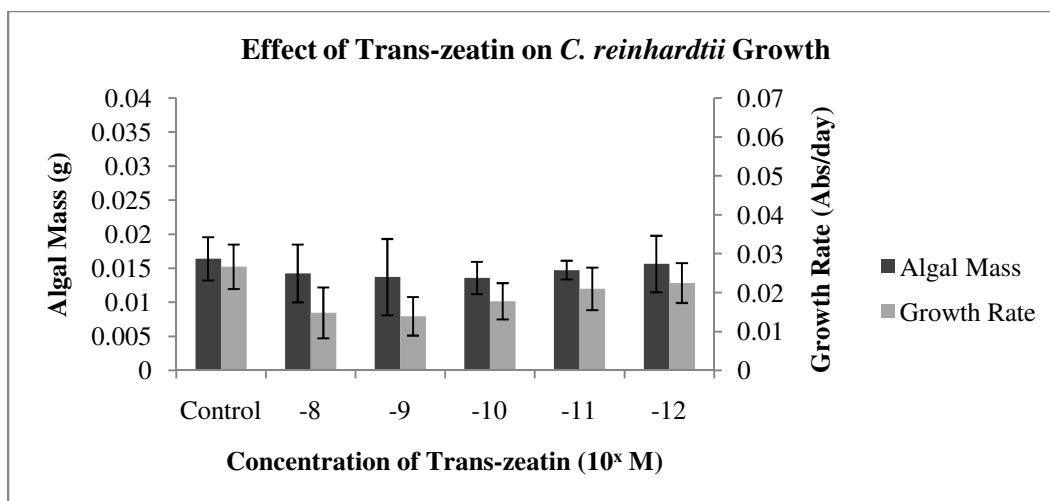
in algal mass at all concentrations, with larger decreases occurring with the more dilute concentrations of phytohormone. Figure 18 provides the measurements at all concentrations and for the controls.



**Figure 18:** A treatment of brassinolide at 10<sup>-12</sup> increased the growth rate of *C. reinhardtii* by 62.7%. However, the algal mass was not really affected by the brassinolide treatment.

#### 4.2.1.3 Trans-zeatin

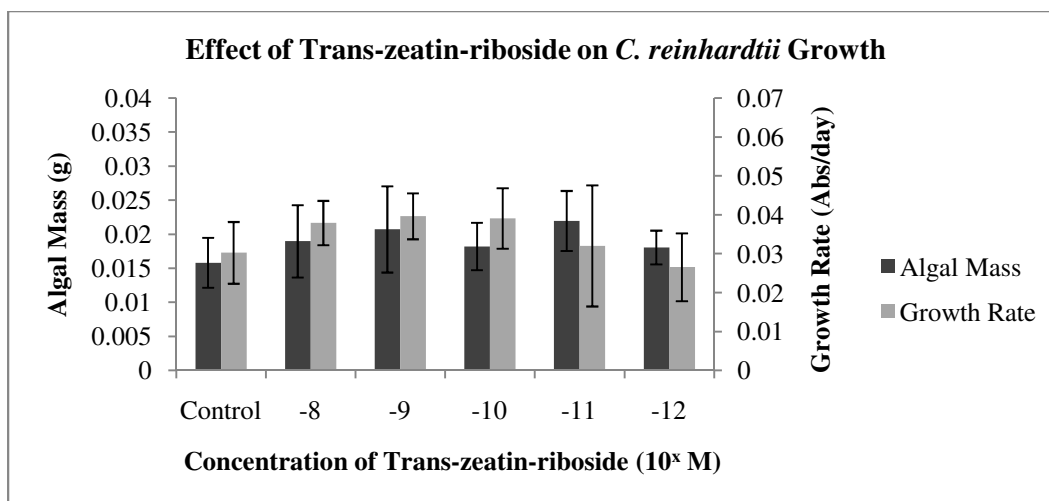
The application of Trans-zeatin had an adverse effect on algal growth at high concentrations. Trans-zeatin caused decreases in growth rates across all concentrations. The greatest decrease was  $47.6 \pm 38.6\%$  at a concentration of 10<sup>-9</sup> M. A slight decrease in algal mass was also noted at all concentrations, with the greatest decrease of  $17.3 \pm 4.8\%$  at concentrations of 10<sup>-10</sup> M. These changes were generally mirrored between growth rate and dry algal masses, seen in Fig. 19.



**Figure 19: Trans-zeatin negatively effects algae mass and growth rate at all concentrations of treatment.**

#### 4.2.1.4 Trans-zeatin-riboside

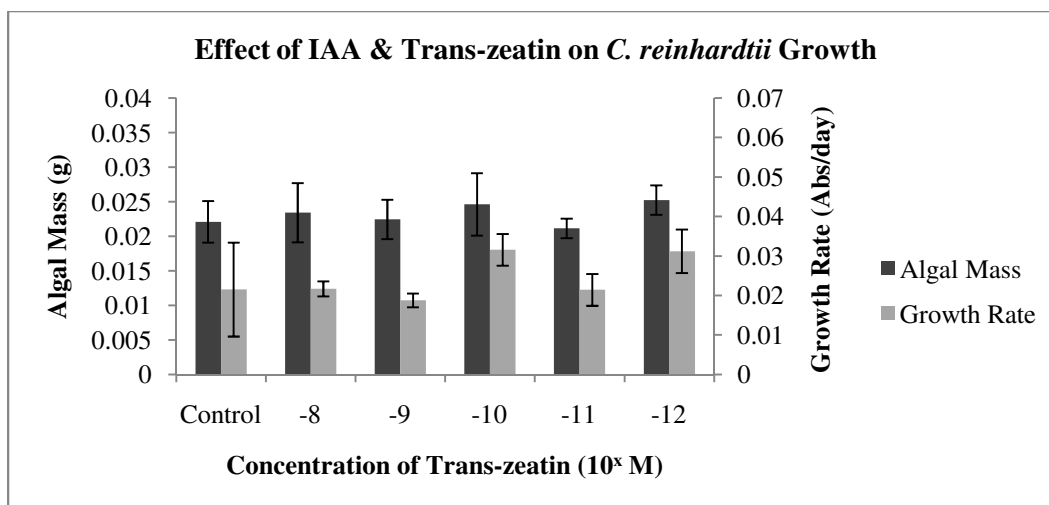
The application of trans-zeatin-riboside has a positive effect on the growth rate of the algae at higher concentrations. The highest growth rate was a  $31.1 \pm 9.4\%$  increase in growth rate found in the  $10^{-9}$  M concentration of trans-zeatin-riboside. Treatment of algae with trans-zeatin-riboside caused an increase in algal mass at all concentrations, with the greatest increase of  $38.9 \pm 11.9\%$  occurring at concentrations of  $10^{-11}$  M. A near exact increase in dry algal mass also occurs at a concentration of  $10^{-9}$  M. Figure 20 provides the measurements at all concentrations and for the controls.



**Figure 20: Trans-zeatin-riboside increased the algae mass and growth rate of *C. reinhardtii*. Specifically, the growth rate increased the most at the concentration of  $10^{-9}$  M while the mass increased the greatest at a concentration of  $10^{-11}$ .**

#### 4.2.1.5 IAA & Trans-zeatin Combination

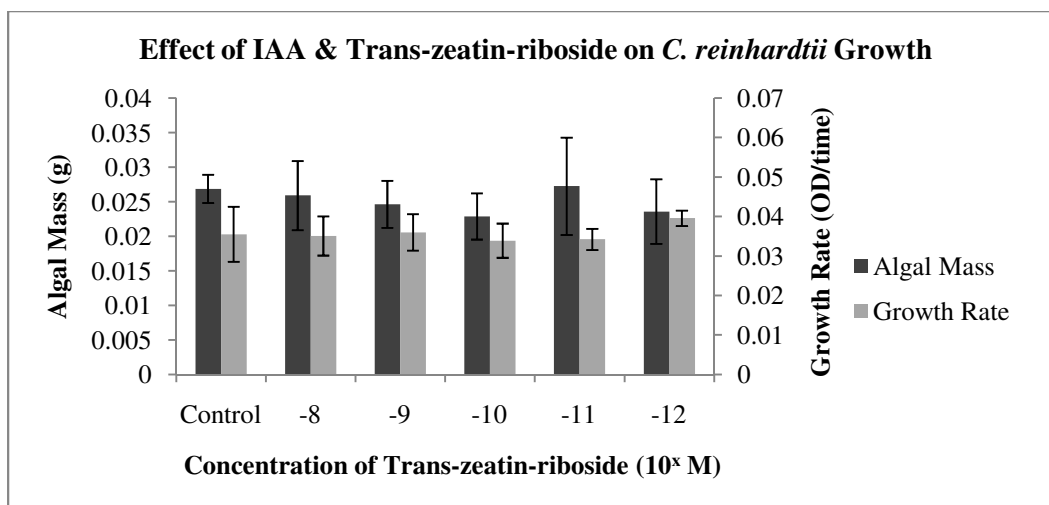
The application of both IAA and Trans-zeatin did not appear to affect the growth rate significantly except for at Trans-zeatin concentrations of  $10^{-10}$  and  $10^{-12}$  M. Specifically at a concentration of  $10^{-10}$  M, a  $46.9 \pm 26.6\%$  increase was observed. Treatment of *C. reinhardtii* with IAA & Trans-zeatin combination also produced a slight increase in algal mass at all concentrations except for  $10^{-11}$  M. Most noticeable increases in dry algal mass occurred at concentrations of  $10^{-10}$  M and  $10^{-12}$  M, with the greatest increase of  $14.2 \pm 2.3\%$  relative to control at  $10^{-12}$  M. Figure 21 provides the measurements at all concentrations and for the controls.



**Figure 21:** The combination of IAA ( $10^{-11}$  M) and Trans-zeatin showed greatest increase in growth rate at Trans-zeatin concentration of  $10^{-10}$  M. The dry algal mass increased most at  $10^{-12}$  M Trans-zeatin.

#### 4.2.1.6 IAA & Trans-zeatin-riboside Combination

The application of both IAA and Trans-zeatin-riboside seemed to have a varying effect on the growth rates of the algae. The highest growth rate was observed at  $10^{-12}$  M concentration Trans-zeatin-riboside (concentration of IAA was held at  $10^{-11}$  M); however, at  $11.5 \pm 2.3\%$ , it was only a slight increase in growth rate relative to the controls. Application of IAA and Trans-zeatin-riboside decreased algal mass at all concentrations except for  $10^{-11}$  M. At this concentration the mass was increased slightly by  $1.4 \pm 0.4\%$  relative to controls. Figure 22 provides the measurements at all concentrations and for the controls.

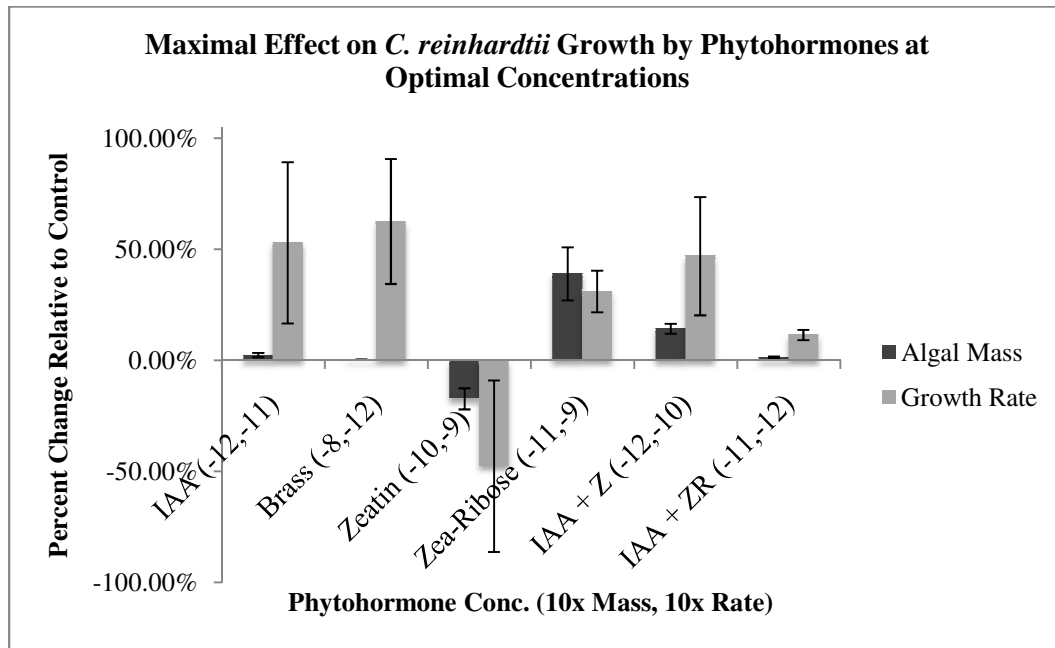


**Figure 22: The combination of IAA ( $10^{-11}$  M) and Trans-zeatin-riboside showed greatest increase in growth rate at Trans-zeatin concentration of  $10^{-12}$  M. The dry algal mass increased most at  $10^{-11}$  M Trans-zeatin. The concentration of IAA is  $10^{-11}$  M.**

#### 4.2.1.7 Overall Effects on Growth

The overall changes in growth rate varied widely, but select concentrations of certain phytohormones showed some positive effect on the growth of *C. reinhardtii*. The most positive increases in growth rate were chosen for each phytohormone and graphed in Figure 23. The data shows that it is likely, but not certain, that epibrassinolide by itself has the most positive effect on growth rate of the algae of  $62.5 \pm 28.1\%$ . IAA by itself poses a close contender. IAA and Trans-zeatin together also seem to have a positive effect on the growth of the algae. However, the most Trans-zeatin-riboside has the most consistent and significant effect on growth of *C. reinhardtii*. The spectrophotometrically determined growth rate increases by  $31.1 \pm 9.4\%$  relative to controls, and the dry algal mass increases by  $38.9 \pm 11.9\%$ . Across the various treatments, the optimal concentration effects of the phytohormones show

discrepancies between growth rates and dry algal masses. The results of Trans-zeatin-riboside, as stated above, are of particular importance because they show consistency in these two measures. The basis for differences between these two measures is explored in the Discussion section.



**Figure 23:** A graphical representation of the six most extreme changes due to phytohormone treatments on the change in growth rate of the algae. The addition of Epibrassinolide and IAA produced the greatest increases in growth rate. The addition of Trans-zeatin-riboside produced the greatest increase in algal mass. The concentrations of phytohormones were  $10^{-12}$  and  $10^{-11}$  M for IAA,  $10^{-8}$  and  $10^{-12}$  M for Epibrassinolide,  $10^{-10}$  and  $10^{-9}$  M for Trans-Zeatin,  $10^{-11}$  and  $10^{-9}$  M for Trans-zeatin-riboside,  $10^{-12}$  and  $10^{-10}$  M for IAA and Trans-Zeatin, and  $10^{-11}$  and  $10^{-12}$  M for IAA and trans-zeatin-riboside. Taken together, Trans-zeatin-riboside shows the most consistency between these two measures. Due to this, it is most effective at increase *C. reinhardtii*'s growth.

#### 4.2.2 Statistical Analysis of Growth Measurements

An unpaired, two-sample t-test was performed between each phytohormone treatment and all of the controls. For this test, the MATLAB function `ttest2(A,B,.95,'right')` was used, with the first argument being a column vector with

all runs of a particular treatment, and the second argument being a column vector representing the control group of the tested metric (see Appendix A.7). The '.95' means that the test looks at the 95% confidence level, and 'right' signifies the one-sided test, looking only for cases where the mean of the treatment group was higher than that of the control group. No treatment of IAA, Epibrassinolide, or Trans-zeatin affected any aspect of the growth. All trials of combined IAA and Trans-zeatin increased mass of both the total growth and extracted lipids. Trans-zeatin-riboside increased the total mass in the  $10^{-9}$  and  $10^{-11}$  treatments, and increased the growth rate in all but the  $10^{-12}$  concentrations. When combined with IAA, zeatin-riboside increases growth rate and total mass in all concentrations, and also increases lipid mass at the  $10^{-11}$  concentration.

These results are encouraging for combination treatments, but by themselves they are not proof of any effects. Because all the controls were averaged together, they do not represent the bias present in any particular set of trials. Since the combined trials were performed last, we were not able to do 8-10 iterations, as we were with the earlier trials, instead performing only 3 or 4. This means that the bias in these particular trials was not well-represented in the average control, and so these would be more likely to stand out against the control. Table 20 summarizes the groups with significant p-values. All p-values can be found in the Appendix A.8.

Because of these issues with the t-test, a one-factor analysis of variance (ANOVA) was also performed, to look at the variation among treatment groups of the same phytohormone. Based on these variances, we can tell to a specified confidence

factor whether the groups are all from the same data pool (i.e. accept the null hypothesis that there is no statistical difference between groups), or whether one or more of the groups are statistically different from one or more of the rest of the groups (reject the null hypothesis). For our experiments, the groups of interest were the different concentrations of phytohormones compared to each other and a control. In other words, each ANOVA calculation looked at a single metric (growth rate (absorbance/day), final mass of algae (g), final dry mass of algae (g)) of a single hormone, with 6 groups (1 group of controls and 5 groups, each being a single phytohormone or combination treatment at a single concentration). The confidence interval used was 95%.

A Matlab m-file was written to test the various concentrations of phytohormones against their respective controls (see Appendix A.9), and to see if a trend presented itself. For this test, the Matlab command *anova1* was used, which operates under the following three assumptions:

- All sample populations are normally distributed.
- All sample populations have equal variance.
- All observations are mutually independent.

Though we did not see a perfect normal distribution within our groups, nor equal variance, the ANOVA test is known to be robust with respect to violations of the first two criteria. We expect the third assumption to be upheld, since the equipment, techniques, and methodologies were held constant throughout the growth trials, lessening the probability of confounding variables.

In order to reject the null hypothesis (which is that the phytohormone treatments had no effect on the metric being tested), a p-value of 0.05 or less was needed; that is, there was less than a 5% chance that the samples were all taken from the same group. If a p-value of 0.05 or less was found, the m-file provided a graphical readout showing the pair-wise comparisons and any groups that were shown to be statistically different from each other. For all p-values, a box plot was displayed which showed the relative variances within and among groups.

Only one phytohormone treatment showed a statistically significant improvement over the control. The growth rate of the algae treated with  $10^{-12}$  M Epibrassinolide showed an improvement, with a 1% chance that the difference was a result of random chance (Table 20). Additionally, effects Trans-zeatin concentrations of  $10^{-8}$  M and  $10^{-9}$  M on growth were shown to be statistically lower than controls. This supports the inhibition of growth shown by evidence for these treatments effects. These results from ANOVA support our findings despite the sources of large error in the results. The fact that none of the other groups show statistically significant evidence of improved growth does not imply a lack of phytohormone effects for other treatment groups. The variance in measurements for all groups, including controls, is large. Sources of this error are discussed in the Discussion and support for practical effects of other phytohormone treatments are supported by growth metrics. P-values from ANOVA of all other treatments are provided in Appendix A.10.

**Table 20: T-test and ANOVA significant results for growth rate and dry algal mass measurements. All values are below  $p = 0.05$  significance level. Statistically significant effects at respective concentrations which correspond to the greatest changes in the metric are denoted by \*.**

<b>Phytohormone</b>	<b>Metric</b>	<b>Concentration (10<sup>x</sup> M)</b>	<b>T-test p-value</b>	<b>ANOVA p-value</b>	<b>Change Relative to Control</b>
<b>Epibrassinolide</b>	Growth rate	-12*	-	0.0122	Increase
<b>Trans-zeatin</b>	Growth rate	-8	-	0.0168	Decrease
		-9*	-	0.0168	Decrease
<b>Trans-zeatin- riboside</b>	Growth rate	-8	0.002	-	Increase
		-9*	0.0001	-	Increase
		-10	0.0001	-	Increase
		-11	0.0096	-	Increase
	Dry Algal Mass	-9	0.017	-	Increase
		-11*	0.0016	-	Increase
<b>IAA &amp; Trans- Zeatin</b>	Dry Algal Mass	-8	0.0015	-	Increase
		-9	0.0038	-	Increase
		-10	0.0004	-	Increase
		-11	0.0145	-	Increase
		-12*	0.0001	-	Increase
<b>IAA &amp; Trans- zeatin-riboside</b>	Growth Rate	-8	0.0275	-	Increase
		-9	0.019	-	Increase
		-10	0.0432	-	Increase
		-11	0.037	-	Increase
		-12*	0.0037	-	Increase
	Dry Algal Mass	-8	0.0001	-	Increase
		-9	0.0003	-	Increase
		-10	0.0024	-	Increase
		-11*	0.0001	-	Increase
		-12	0.0014	-	Increase

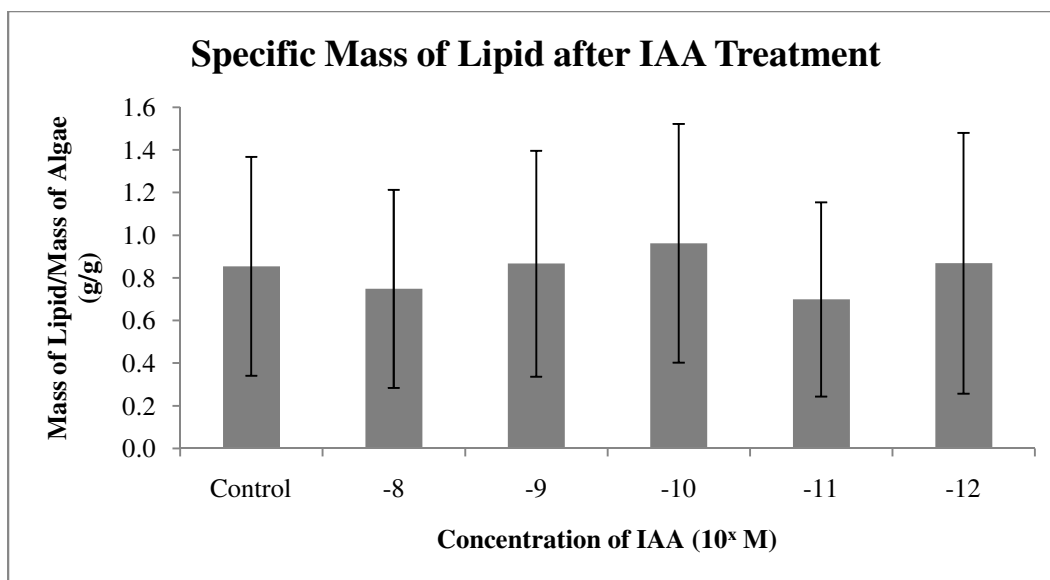
## **4.3 Lipid Analysis**

### **4.3.1 Specific Lipid Mass**

The dry mass of the lipids after extraction was divided by the dry mass of the algae from which the lipids were extracted to give the specific mass of lipid after a phytohormone treatment. This information reveals the percentage of algal mass that is actually extractable lipid, giving us a preliminary measure of efficiency of lipid production by the algae. Coupled with the growth rate of algae, the specific lipid mass is a good measure of a phytohormone's potential in increasing lipid yields.

#### **4.3.1.1 IAA**

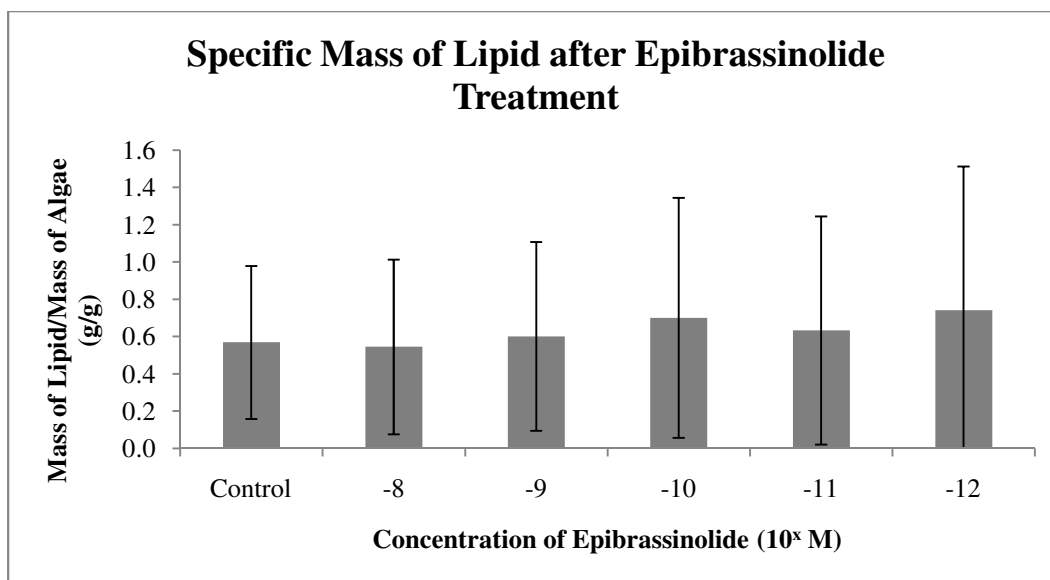
The application of IAA resulted in higher specific lipid mass at  $10^{-10}$  M concentration, while causing decreases in specific lipid mass for certain phytohormone concentrations. Taking into account the experimental uncertainty, however, makes it difficult to draw any conclusions—the yields are approximately the same. Of note, however, the control group also displays a great degree of variance, implying that the variance in treatment groups may not discount observed effects. An examination of these errors and the metric's validity is provided in the Discussion.



**Figure 24:** The error on the specific lipid mass of IAA makes it difficult to determine which concentration of IAA has the greatest impact on lipid yields. Discounting this variance, the  $10^{-10}$  M treatment shows the greatest increase.

#### 4.3.1.2 Epibrassinolide

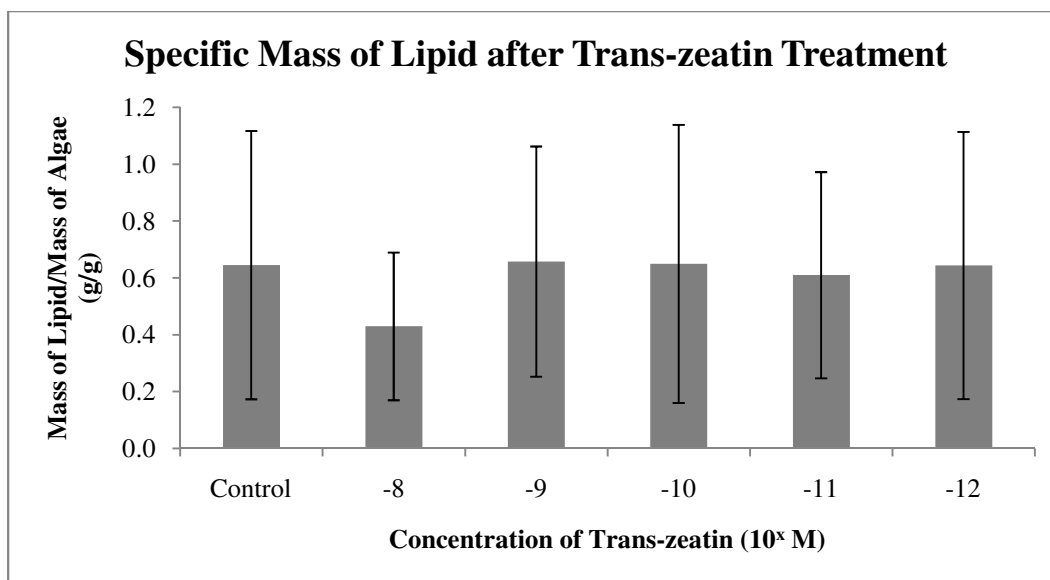
Application of Epibrassinolide to algae resulted in generally negligible changes in specific lipid mass at all concentrations. However, as with IAA, the error is very large so it is difficult to determine whether the brassinosteroid is having an effect on the lipid yields. An examination of these errors and the metric's validity is provided in the Discussion.



**Figure 25:** Epibrassinolide treatments also show an negligible difference in specific lipid mass. Discounting for the variance, Epibrassinolide at concentration of  $10^{-12}$  M shows the greatest increase.

#### 4.3.1.3 Trans-zeatin

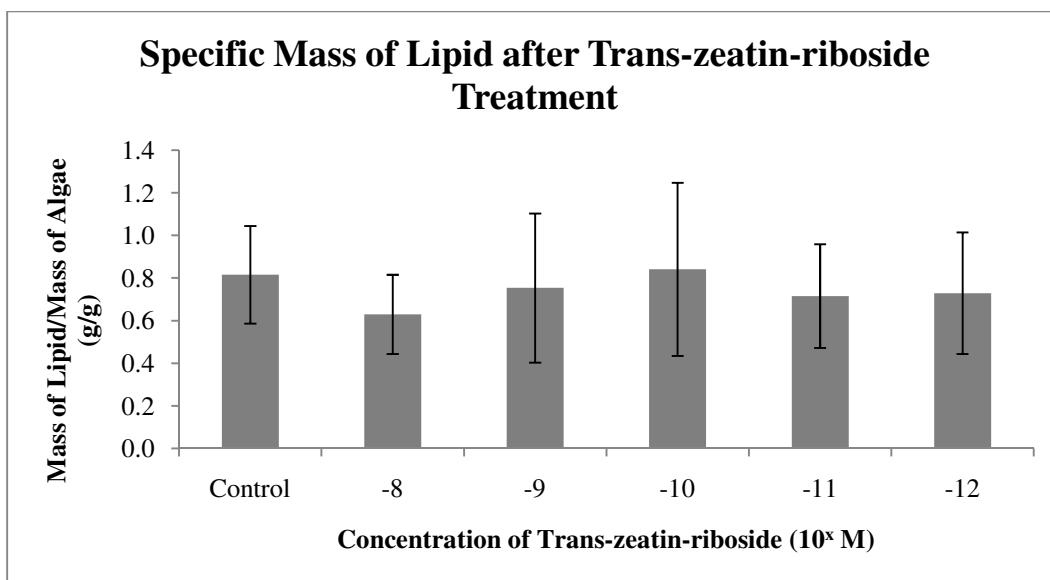
Lipid yield resulting from application of Trans-zeatin was changed very little in all but  $10^{-8}$  M concentration. At this treatment concentration, algae produced fewer lipids per gram of cell weight.



**Figure 26:** Trans-zeatin appears to cause a decrease in lipid concentration at a phytohormone concentration of  $10^{-8}$  M. Other concentration treatments show a negligible change.

#### 4.3.1.4 Trans-zeatin-riboside

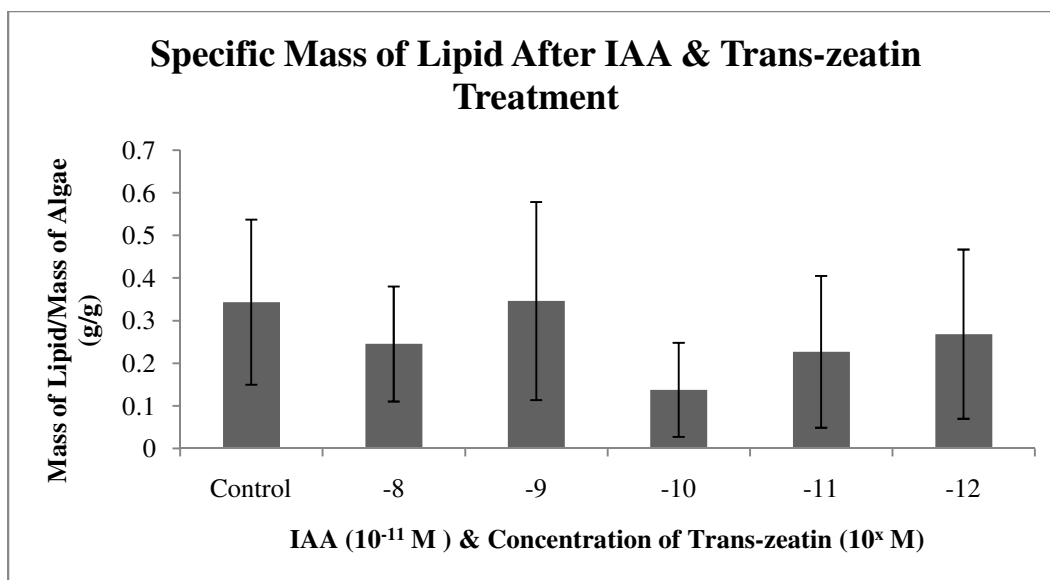
Application of Trans-zeatin-riboside decreased specific lipid mass slightly at most concentrations. Noticeable decreases were observed at  $10^{-8}$  M concentration. The  $10^{-10}$  M concentration of Trans-zeatin-riboside allowed it to have the least detrimental effect on the algae's specific mass of lipid.



**Figure 27:** Trans-zeatin-riboside treatment at  $10^{-10}$  M had the least detrimental effect on lipid production. Treatment of  $10^{-8}$  M concentration decreased lipid production the greatest relative to controls.

#### 4.3.1.5 IAA & Trans-zeatin Combination

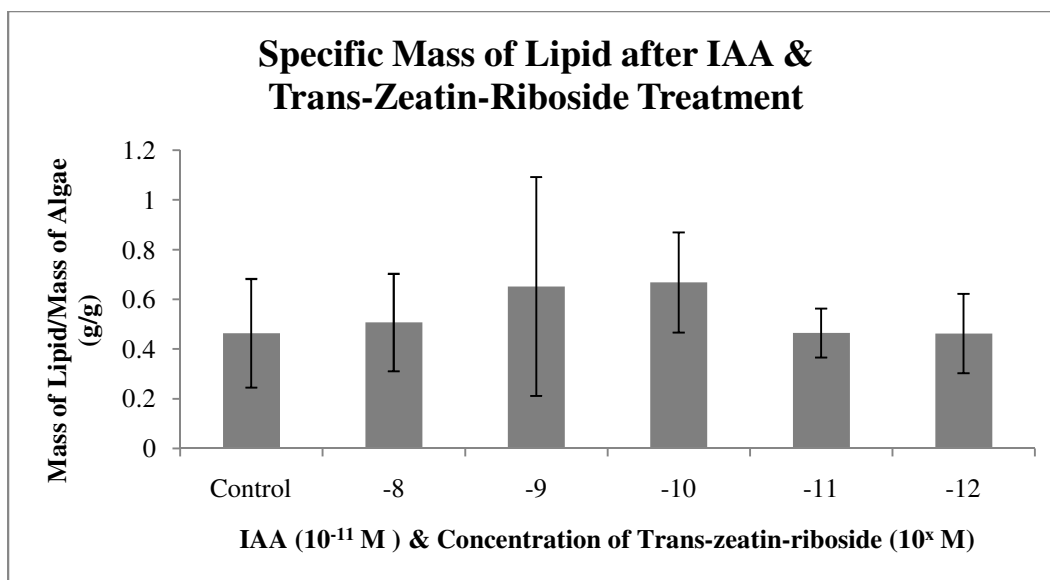
Application of IAA & Trans-zeatin substantially decreased lipid yield at all concentrations except for  $10^{-9}$  M, at which lipid yield remained the same relative to controls. A treatment of IAA and Trans-zeatin at a concentration of  $10^{-10}$  yielded the greatest decrease in specific lipid mass. Ignoring variation in these measures, a consistent dose response curve is likely possible for this treatment if the effects at  $10^{-9}$  M are not accurate. However, the same could also be argued for the effects at the other concentrations. An examination of these errors and the metric's validity is provided in the Discussion.



**Figure 28: IAA & Trans-zeatin treatment had generally detrimental effects on lipid production. Discounting for variance of  $10^{-9}$  M, which shows a negligible change, a consistent dose response curve to this treatment is possible.**

#### **4.3.1.6 IAA & Trans-zeatin-riboside Combination**

Application of IAA and Trans-zeatin-riboside combination increased specific lipid mass noticeably at  $10^{-9}$  M and  $10^{-10}$  M concentrations, but it produced little change at other concentrations. It should be taken into consideration that the experimental error makes it difficult to ascertain whether the specific mass of lipid indeed had an increase the treatment of IAA & Trans-zeatin-riboside at a concentration of  $10^{-9}$  M. An examination of these errors and the metric's validity is provided in the Discussion.



**Figure 29:** Treatments of IAA & Trans-zeatin-riboside at a concentration of  $10^{10}$  M clearly yields the highest increase in specific lipid mass.

#### 4.3.1.7 Overall Effects on Specific Lipid Mass

The phytohormone treatments had varying effects on algal specific lipid mass. IAA and Epibrassinolide treatments increased specific lipid mass, and IAA & Trans-zeatin combination treatments decreased specific lipid mass. Epibrassinolide, Trans-zeatin, Trans-zeatin-riboside and the combination treatment of IAA & Trans-zeatin-riboside yielded little substantial effects. The IAA and Epibrassinolide treatments yielded very high error in their results; therefore, it is very difficult to determine whether the phytohormones effects are valid. It is interesting to note that the Trans-zeatin-riboside treatments, which we will see in the next section have the highest lipid concentration, shows no real increase in specific lipid weight. This caveat is explored in our discussion section about lipid concentrations.

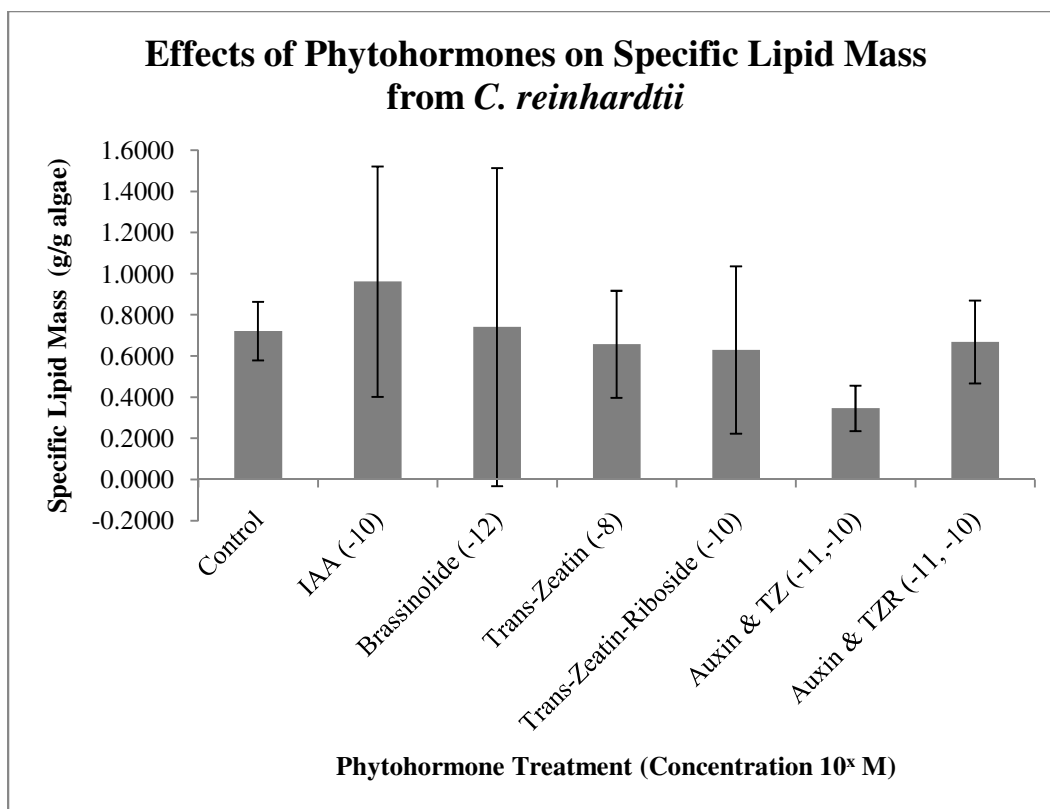


Figure 30: of various phytohormones on algal specific lipid mass.

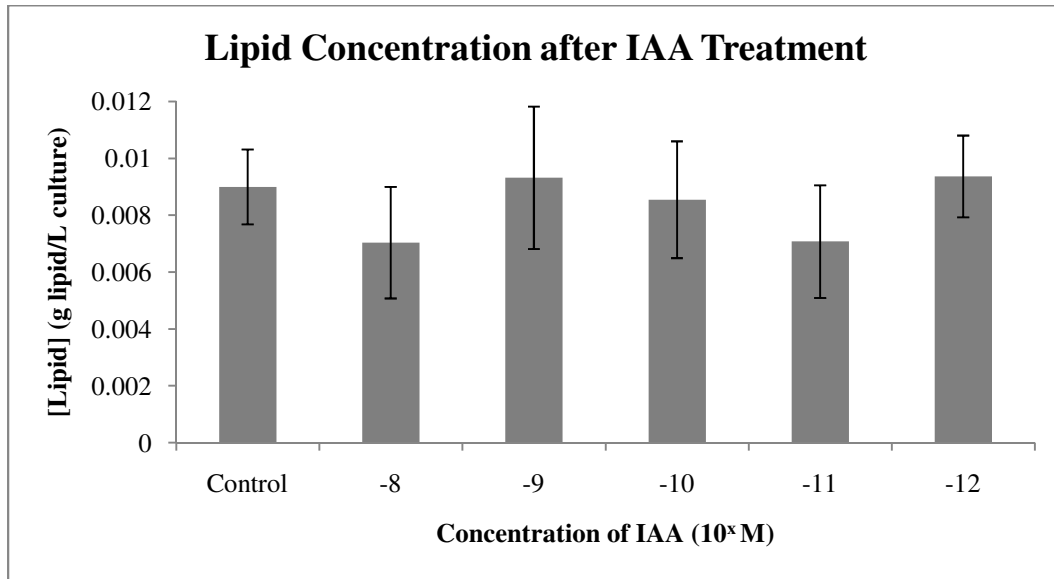
### 4.3.2 Lipid Concentration

The lipid concentration was calculated as in Appendix A.6. The lipid concentration takes the specific lipid masses one step further and incorporates the growth capacity of algae in the culture. This measure provides the efficiency of a culture by involving the quantity of product and the rate at which it can be produced.

#### 4.3.2.1 IAA

The application of IAA resulted in higher lipid yield at  $10^{-9}$  M concentration, an increase of  $29.2 \pm 35.8\%$  relative to controls, even though it causes decreases in

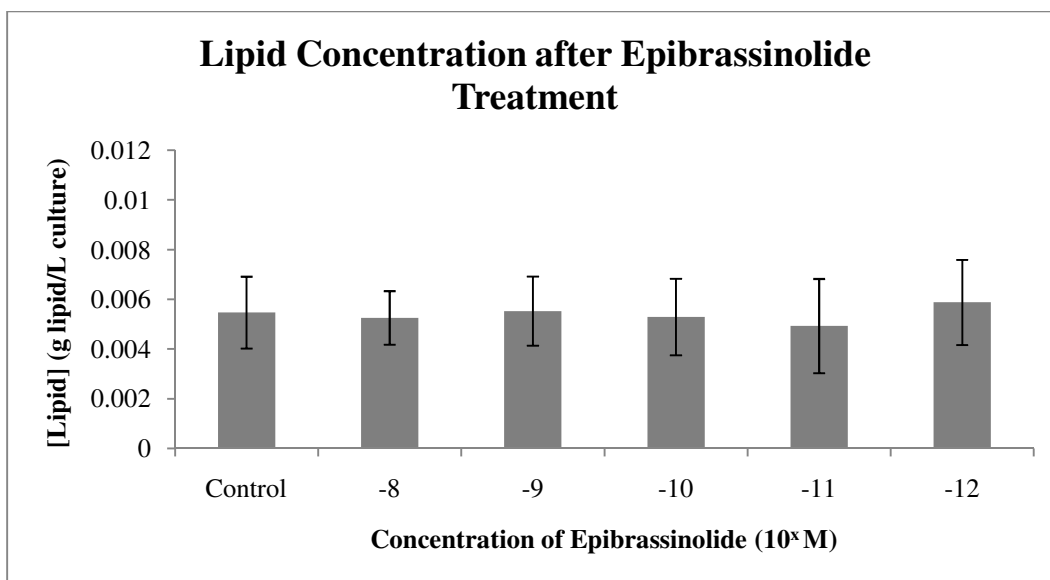
specific lipid mass. Taking into account the experimental uncertainty, however, makes it difficult to draw any conclusions—the yields are approximately identical.



**Figure 31:** The application of IAA shows a large amount of experimental uncertainty; therefore, it is difficult to determine the actual effect of IAA on *C. reinhardtii*.

#### 4.3.2.2 Epibrassinolide

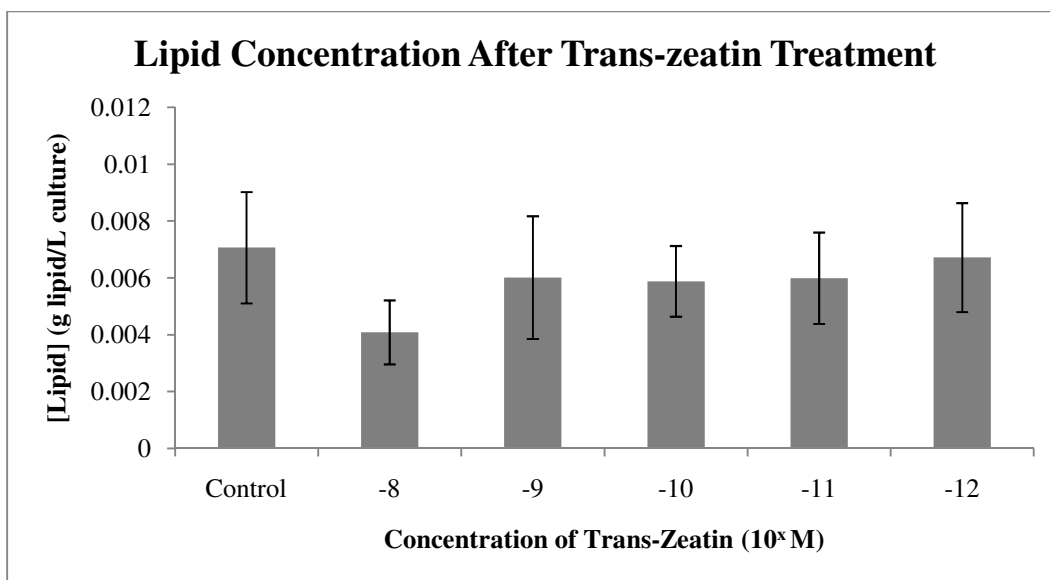
Application of Epibrassinolide to algae resulted in a negligible increases in lipid concentration at all treatment concentrations except for  $10^{-12}$  M. At this concentration, the lipid concentration decrease by  $18.9 \pm 30.2\%$  relative to controls.



**Figure 32:** Treatment of Epibrassinolide causes an increase in lipid concentration at  $10^{-12}$  M. At all other concentrations, Epibrassinolide appears to have no impact on lipid treatment.

#### 4.3.2.3 Trans-Zeatin

Lipid concentration resulting from application of Trans-zeatin was unaffected in all but the  $10^{-8}$  M concentration treatment. At the  $10^{-8}$  M concentrations, lipid concentration decreases by  $7.4\% \pm 36.7\%$  relative to control. The error in this measurement is examined in the Discussion.

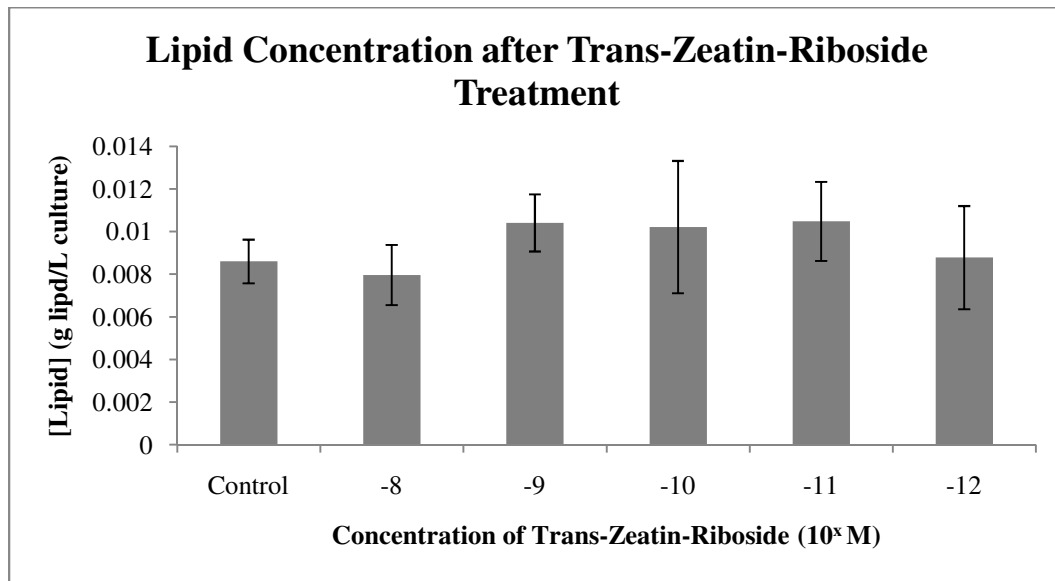


**Figure 33:** treated with trans-zeatin clearly shows a decrease in lipid concentration at a treatment of  $10^{-8}$  M. Treatments with lower levels of trans-zeatin do not significantly affect lipid concentration.

#### 4.3.2.4 Trans-zeatin-riboside

Application of trans-zeatin-riboside increased lipid concentrations at all treatment concentrations except  $10^{-8}$  M. The greatest increase occurred at a concentration of  $10^{-11}$  M; however, a treatment at  $10^{-9}$  M yielded a rather similar increase. It is interesting to note that the greatest increase in growth rate occurred at a treatment of trans-zeatin-riboside at  $10^{-9}$  M and the greatest increase in algal mass occurred at a concentration of  $10^{-11}$  M. The effect at treatment of  $10^{-11}$  M corresponds to a lipid concentration increase of  $44.6 \pm 42.1\%$  relative to controls. Though at first this variance may appear drastically greater than the measure of the effect on lipid concentration, in reviewing the individual variance in control and treatment groups, the variance in this percent change is less valuable. The actual effect of approximately

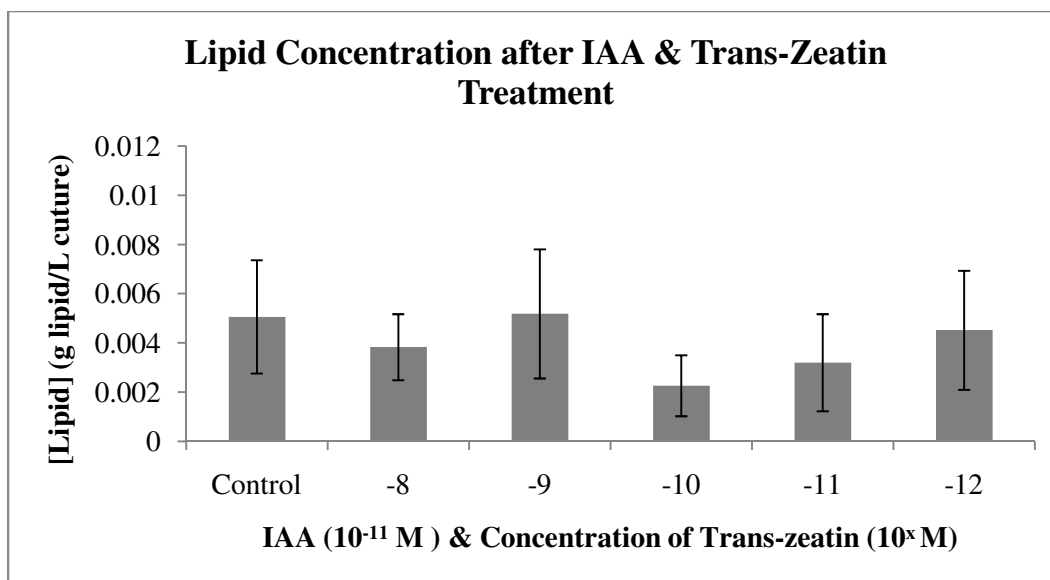
44% is valid. The sources of this variation and validity of the actual effect are examined in the Discussion.



**Figure 34:** Significant increases in lipid concentrations of approximately 21% occur with treatments of Trans-zeatin-riboside  $10^{-9}$  M and  $10^{-11}$  M relative to controls.

#### 4.3.2.5 IAA & Trans-Zeatin Combination

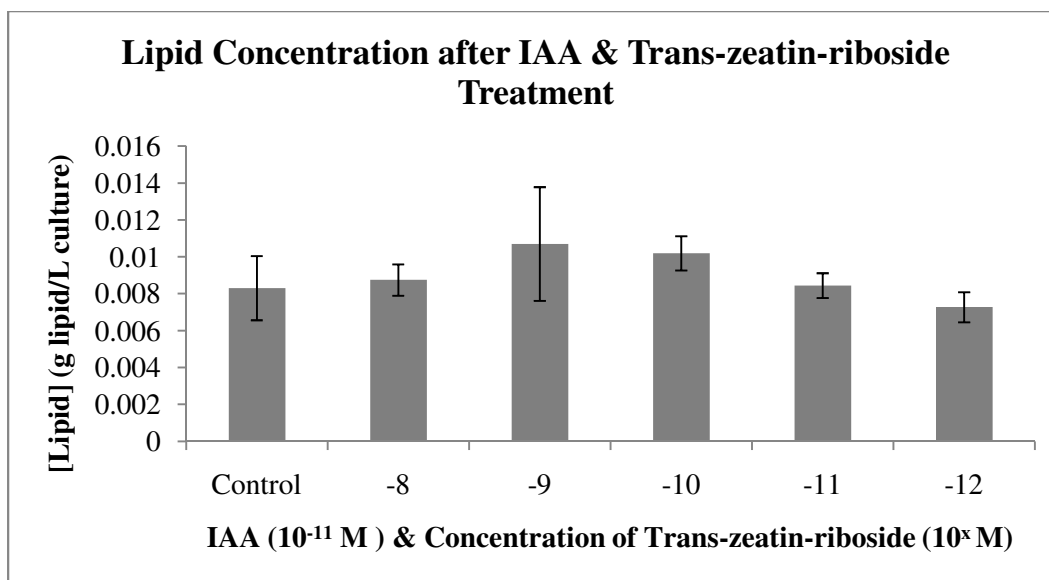
Application of IAA & Trans-zeatin substantially decreased lipid concentration at all concentrations except for the treatment at  $10^{-9}$  M Trans-zeatin. At this concentration lipid concentration remained the similar to control. The greatest decrease in lipid concentration,  $28.5 \pm 39.9\%$  relative to controls, was at a treatment of  $10^{-10}$  M



**Figure 35: Treatments of IAA & Trans-zeatin decreases lipid concentration at most concentrations of treatment, except  $10^{-9}$  M.**

#### **4.3.2.6 IAA & Trans-zeatin-riboside Combination**

Application of IAA and Trans-zeatin-riboside combination increased lipid concentration noticeably at  $10^{-9}$  and  $10^{-10}$  M concentrations, but it produced little change at other concentrations. It should be taken into consideration that the experimental error makes it difficult to determine with certainty that the specific weight of lipid had an increase at those concentrations. The increase of lipid concentration at  $10^{-9}$  M is 28.8%, however, the error is 33.5%. The error is larger than the actual measurement because this value comes from a calculation uses a variety of data gathered. An examination of these sources of error is presented in the Discussion.



**Figure 36:** A treatment of IAA & Trans-zeatin-riboside appears to yield an increase at a treatment of  $10^{-9}$  M; however, the error in the measurement is large.

#### 4.3.2.7 Overall Effects on Lipid Concentration

The phytohormone treatments had varying effects on algal lipid concentration. Figure 62 and Table 32 in Appendix A.11 summarize the changes in lipid concentration of treatment groups, relative to controls. IAA, Trans-zeatin-riboside, and the IAA & Trans-zeatin-riboside combination increased lipid concentration. Epibrassinolide, Trans-zeatin, and the IAA & Trans-zeatin combination decreased lipid yield. Generally, most concentrations for all treatments showed negligible changes relative to control. The greatest increase in lipid concentration was a treatment of Trans-zeatin-riboside at  $10^{-11}$  M, which yielded an increase of  $47.6 \pm 54.5\%$ . Though the error in this measurement is large, the actual effect as indicated by the increase is representative of the phytohormone's effect. This increase implies that the efficiency of a production system incorporating Trans-zeatin-riboside would

expect to see increases of 48% in its biodiesel production capacity. This increase would be through a combination of increased lipid content and rate of growth.

#### **4.3.3 Statistical Significance Analysis of Lipid Measurements**

As with the growth measurements, the lipid data was analyzed using the two-argument, one-sided t-test, with a 95% level of significance (for m-file see Appendix A.7). Of all the phytohormone and their respective concentrations tested, only three treatments showed an improved lipid concentration. Treatments of Trans-zeatin-ribose at  $10^{-10}$ ,  $10^{-11}$ , and  $10^{-12}$  M all showed statistical increases over the control. Notably, at  $10^{-10}$  M specific lipid mass shows the greatest increase. Table 21 below summarizes these finding, listing the treatments with significant p-values. P-values for all treatments can be found in the Appendix A.8.

The data was also run through ANOVA to test for statistical significance (for sample code see Appendix A.9). Again, large error sources marred the efficacy of the test, and no treatment could be shown to increase lipid mass or specific weight over the control to a confidence level of 95%. Trans-zeatin-ribose treatments were statistically different from each other ( $10^{-9}$  M had a higher lipid specific weight than both  $10^{-8}$  and  $10^{-12}$  M), but were inconclusive when compared against the control. Table 21 shows the results below. The large error associated with our measurements is likely a result is examined in the Discussion. P-values for all treatments can be found in Appendix A.10.

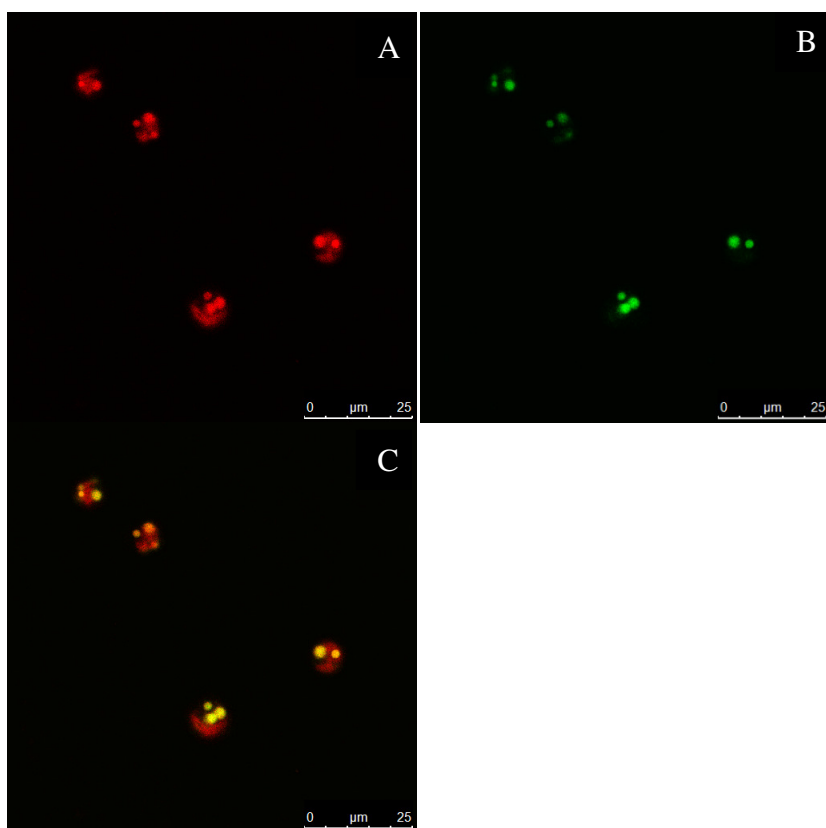
**Table 21: T-test and ANOVA significant results for specific lipid mass and lipid concentration measurements. Only values below  $p = 0.05$  significance level are shown. Statistically significant effects at respective concentrations which correspond to the greatest changes in the metric are denoted by \*.**

Phytohormone	Metric	Concentration ( $10^x$ M)	T-test p-value	ANOVA p-value	Change Relative to Control
<b>Trans-zeatin- riboside</b>	Lipid Conc.	-10	0.003		Increase
		-11*	0.0221		Increase
		-12	0.0077		Increase

## 4.4 Confocal Microscopy

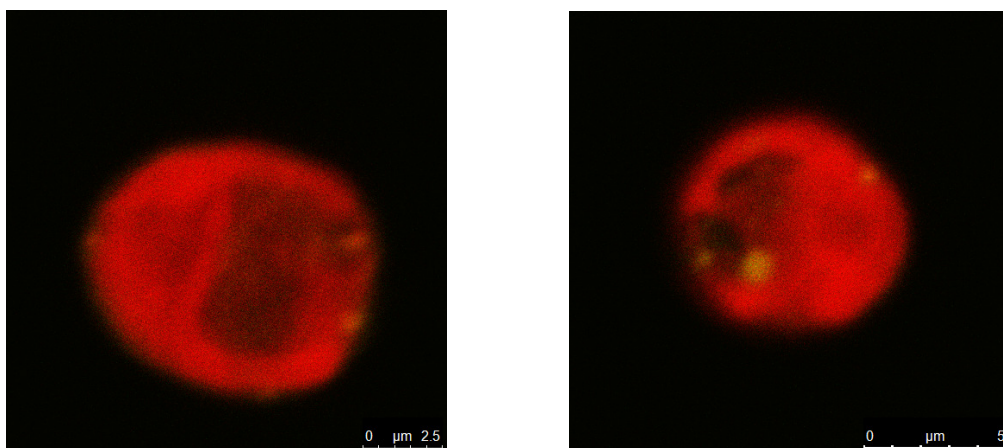
### 4.4.1 Visualizing Lipid Bodies

After cells fixed and dyed with Nile Red, there were two photomultiplier tubes (PMTs) that were activated to detect two ranges of wavelengths. The excitation and emission wavelength were 488 nm and 540 nm, respectively. The first PMT detected any natural fluorescence from the chlorophyll in the algae. The second PMT detected any fluorescence from the Nile Red dye binding to lipid droplets inside the algae. The resulting two images could then be overlaid to view the complete image as shown in Fig. 37. In those images, it appears that the lipid droplets show autofluorescence. This occurs because the algae were dyed with Nile Red prior to examination with the confocal microscope. Nile Red's emission spectrum includes the wavelengths that the first PMT was detecting, and thus red dots appear in Fig. 37A.



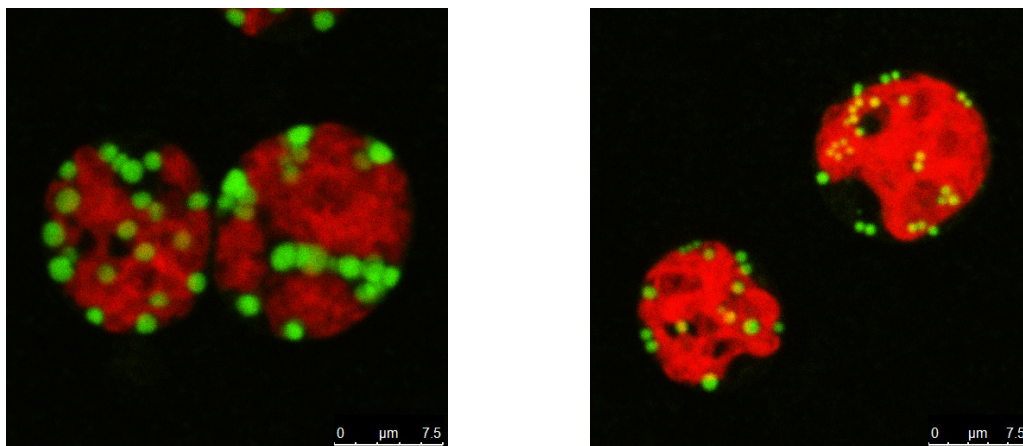
**Figure 37: Algae fluorescing naturally (A). Lipid droplets dyed by Nile Red fluorescing (B). Overlay of both images (C).**

All figures shown below will have two example images to illustrate the effects of phytohormone treatments. Due to time and monetary constraints, analysis of the combination treatments was not possible. Similar confocal imaging of these treatments is necessary.



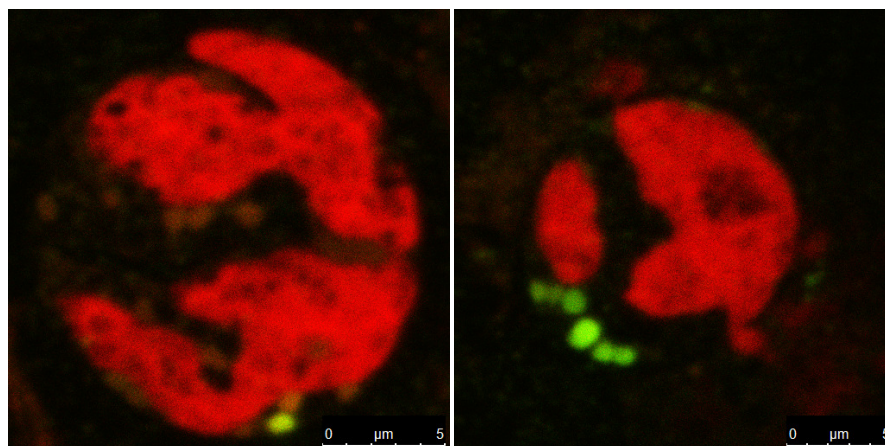
**Figure 38: Control cells dyed with Nile Red. Notice the small number of lipid bodies.**

It was found that the control cells showed some fluorescence from the Nile Red dye, but very little. The algae treated with IAA, however, had a very different appearance, as shown in Fig. 39. They had many lipid droplets that were stained by the Nile Red that seemed to be of small size relative to the cell.



**Figure 39: Cells with IAA applied to them. There are a greater number of lipid bodies but in smaller sizes. Relative to control, the lipid bodies here are in greater quantity.**

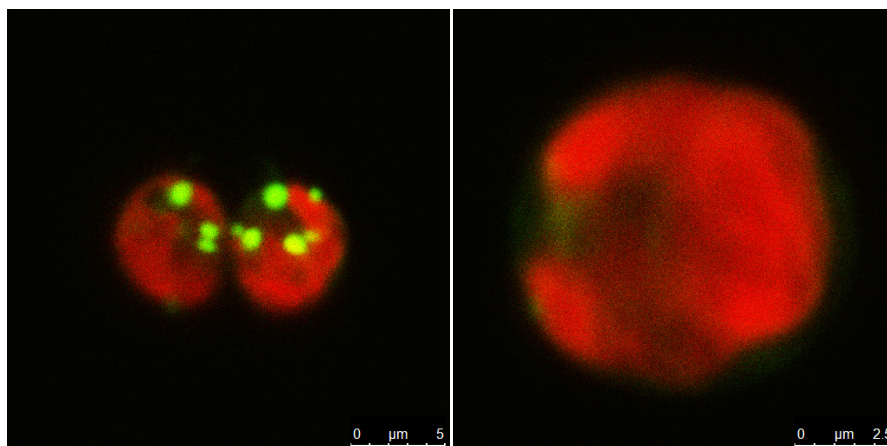
Cell sizes are difficult to judge; there is great degree of variability noted for imaged cells. The application of Epibrassinolide illustrates this phenomenon.



**Figure 40: Cells with Epibrassinolide applied to them. Application of this phytohormone also produces spaces which do not autofluoresce. This indicates inactivated chloroplasts since the fluorescence is from chlorophyll. These regions could also arise from increases in other cellular organelles.**

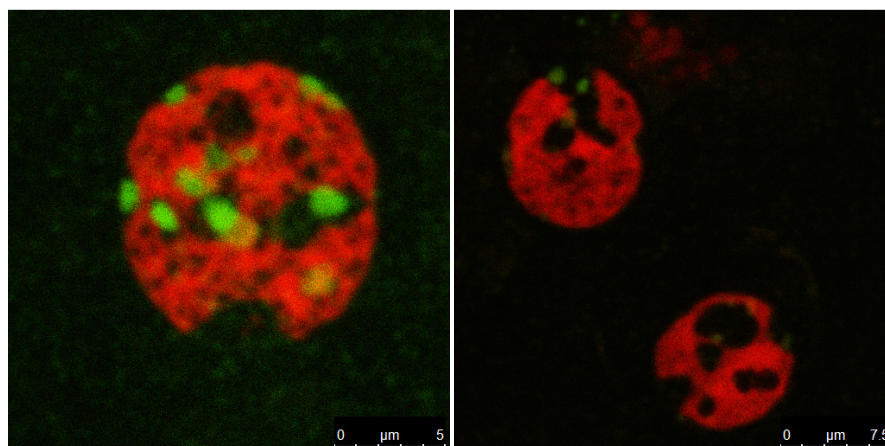
The addition of Epibrassinolide resulted in cell morphology, illustrated in Fig. 40, where there appeared to be large gaps in the auto fluorescence of the cell. Despite this, the quantity of lipid bodies still increase as stained by Nile Red. It is possible that the regions which do not autofluoresce have inactivated chloroplasts. Since chlorophyll is the source of the autofluorescence of the cells, this effect is possible. Alternatively, there could be changes in the size of other organelles such as the reticulum. The reason for this effect is unclear.

Upon application of Trans-zeatin there are more lipid bodies, as seen in Fig. 41 than control groups. The lipid bodies sizes relative to cell size are larger compared to other groups.



**Figure 41: Cells treated with Trans-zeatin. Though the number of lipid bodies is lower than those seen with other phytohormone treatments, the size of these lipid bodies is larger. Quantification of these lipid bodies was not possible under our time constraints.**

Trans-zeatin-riboside caused an increase in the number and size of lipid bodies relative to controls. Compared to other phytohormone treatments, the quantity of lipid bodies was lower for Trans-zeatin-riboside. The size of these lipid bodies, however, was greater than most other treatments. Similar effects as seen with Epibrassinolide were observed. As seen in Fig. 42, large regions do not exhibit autofluorescence. This could be due to inactivation of chloroplasts or increases in size of cellular organelles.

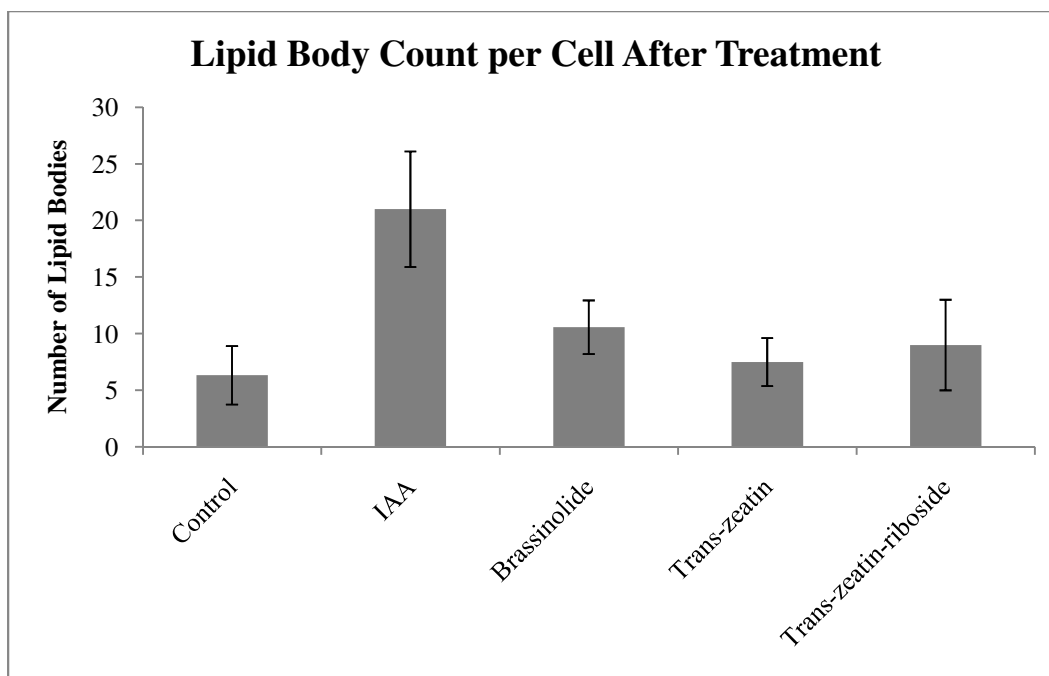


**Figure 42:** Cells treated with Trans-zeatin-riboside. As with Trans-zeatin, there are fewer lipid bodies; however, the size of these lipid bodies is larger than with those seen for other phytohormone treatments. Additionally, regions lacking autofluorescence are observed similar to those seen with Epibrassinolide treatment.

#### **4.4.2 Analysis of Confocal Images in Context of Previous Metrics**

The lipid body per cell count indicates changes in lipid metabolism visually.

An increase in this count correlates directly with increased lipid content and effects of phytohormones on metabolism. The counts the various treatments are provided in Fig. 43. While, this count is important, it provides only a relative estimate under the assumption of constant lipid body sizes. True quantification by fluorescence for these lipid bodies would actually indicate relative changes in lipid bodies due to phytohormone treatments. The lipid body count, in the scope of our study, only provides guide to visualize the underlying effects for previous metrics of lipid content.



**Figure 43: Treatment with IAA and Epibrassinolide resulted in greatest increases in lipid bodies per cell as compared to those of controls. Other phytohormone treatments also show increases, however, the counts are lower relative to IAA and Epibrassinolide. This method of quantification is not true representative of actual increases in lipid content. It only provides a guide for visualizing underlying effects observed through the lipid content measurements. The errors illustrated are derived from the differences in lipid body counts for 4 cells within each group.**

Based on this, the phytohormone treatments can be compared to each other and controls. The lipid body per cell count increased most dramatically after treatment with IAA, but also had a significant increase with Epibrassinolide. Due to time constraints, we were unable to examine the cells that had grown in the presence of IAA and Trans-zeatin or Trans-zeatin-riboside. The difference in lipid body counts, however, is an interesting facet that may shed light on the metabolism of the algae. It is interesting that there are large sections of algal cells under certain treatments that seem to lack both Nile Red and chlorophyll autofluorescence. Epibrassinolide, Trans-zeatin, and Trans-zeatin-riboside treatments produce these

regions the most in *C. reinhardtii*. This may be due to inactivation of chloroplasts in that region of the cell as the algae begin to stop growth and create more lipids.

It is interesting that IAA causes the largest increase in lipid body counts—around a  $231 \pm 57\%$  increase in lipid body counts. IAA acts in plants as a growth stimulating agent, and at high concentrations can stimulate the production of ethylene that may cause the plant to die. The algal growth rate under IAA was increased, as seen earlier, but this does not explain the increase in lipid body production. While the production of lipids may be a response to stress from the presence of IAA, further exploration will be necessary to draw conclusions about the response pathway for auxin.

For Epibrassinolide, there was a potential increase in lipid body production in *C. reinhardtii*. Interestingly, in plants, Brassinosteroids may increase metabolism though it has an unclear role in cell growth, as it was shown to accelerate senescence in some cultured cells. This acceleration, if it is also present in algal pathways, may be the cause of the chloroplast inactivation throughout the cell. There were large regions of non-fluorescence, or dark regions, in the Epibrassinolide-treated cells. Again, this is speculation based on the physiology of plants—it is unknown whether these same pathways can be applied to *C. reinhardtii*.

The treatments with cytokinins did not seem to have a major effect on lipid body production in the algae. In plants, cytokinins are known to stimulate cell division, but only in the presence of other phytohormones. If we make the assumption

that phytohormones operate in algae in the same manner, then it follows that there is little change in metabolism in algae after addition of the phytohormones.

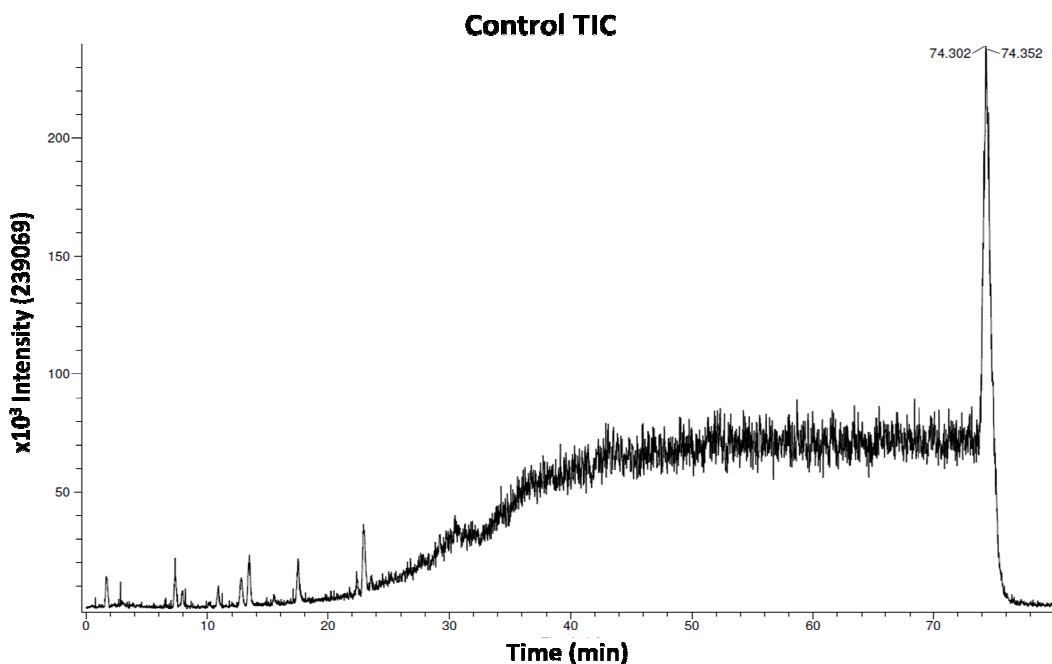
## **4.5 Liquid Chromatography – Mass Spectrometry**

### **4.5.1 LC–MS**

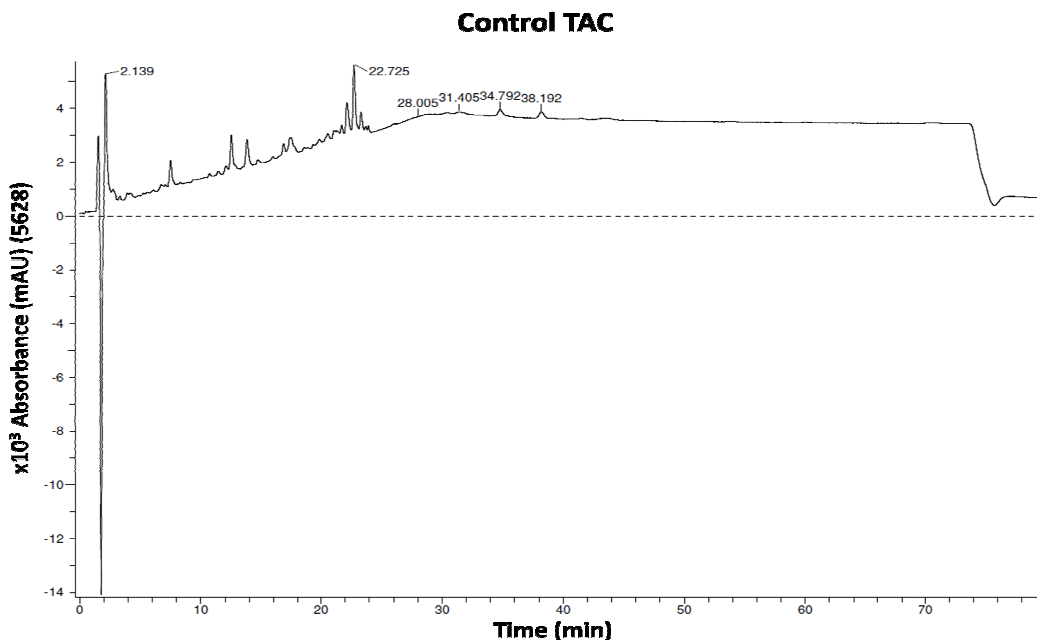
A liquid chromatography-mass spectrometry (LC-MS) analysis was conducted on the lipids we extracted by Soxhlet extraction. The LC-MS machine gave two outputs: total ion current (TIC) chromatograms and total absorption chromatograms (TAC). The TIC chromatograms show the sum of the intensity of all the products being detected by the MS at any given time. The TAC measures the absorbance of the sample passing the detector in the HPLC at any given moment. Both of these chromatograms contain peaks which indicate that a substance has been detected by either the HPLC or MS machines. Each peak in the TIC chromatograms has a corresponding mass chromatogram. These mass chromatograms contain  $m/z$  ratios which can be used to identify the extracted lipids. Ideally we could integrate the peaks in each chromatogram to determine the quantity of the lipids. Unfortunately, the type of mobile phase we used made it difficult to determine a reliable baseline for the peak integrations because the acetonitrile in the mobile phase was strongly detected by both the TIC and TAC. However, the analysis allowed us to qualitatively determine if there were any salient changes in the quantity of lipids produced.

#### 4.5.2 Control Results

Figures 44 and 45 show typical TIC and TAC chromatograms for the control group, respectively. Because of the gradient, the acetonitrile did not effect the TIC baseline beyond 20 minutes. The solvent blocking effect maxed out at 40 minutes. The TAC showed a linear increase in the solvent blocking effect from the beginning. The final peak at the end of the TIC at minute 74 was the rest of the solvent at higher volume being passed through during the flushing phase.



**Figure 44: Total intensity chromatogram for a typical control sample. The noticeable increase in the baseline is due to the concentration of acetonitrile increasing.**



**Figure 45: Total absorbance chromatogram for a typical control sample. Peaks can be seen past the 20 minute mark that cannot be seen in the TIC. It is possible that other peaks are being hidden by the solvent. However those peaks are relatively small, so their impact on the total lipid analysis is not too large.**

From these two figures, we determined which mass chromatograms best represented the lipids to be found. In order to do that, we identified the time points of the salient peaks in the TIC and TAC simultaneously. Generally, peaks in both TIC and TAC occur at similar time points. The peaks in the TIC matched the peaks in the TAC for the first 30 minutes. After that point, the mobile phase absorbance effects started to block possible peaks found in the TIC, so the remaining peaks to be sampled were taken from the TAC only. Even the TAC the peaks become weaker as the mobile phase B is ramped up, which indicates that the mobile phase absorbance is masking some of the weaker peaks. However, since any possible hidden peaks were at such a low intensity they were determined to contain insignificant quantities of

lipids. Also, it is important to note that the same peaks in the TIC and TAC occur approximately 10 seconds apart. This is because the time it took for the sample to pass between the two detectors was about 10 seconds. Figures 46 and 47 below are magnifications of Figures 44 and 45, respectively. The peaks are marked by time stamps as shown in the figures.

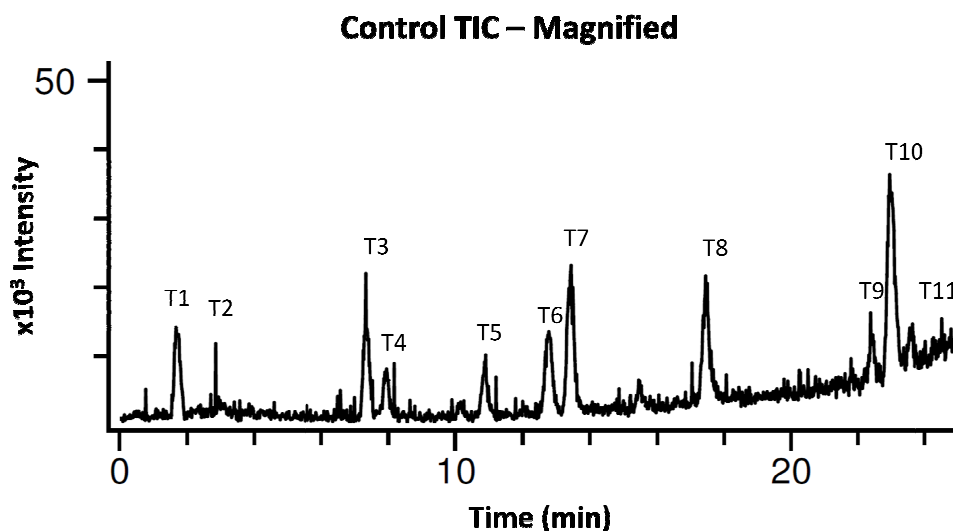


Figure 46: Magnified view of the Control TIC in Figure 44.

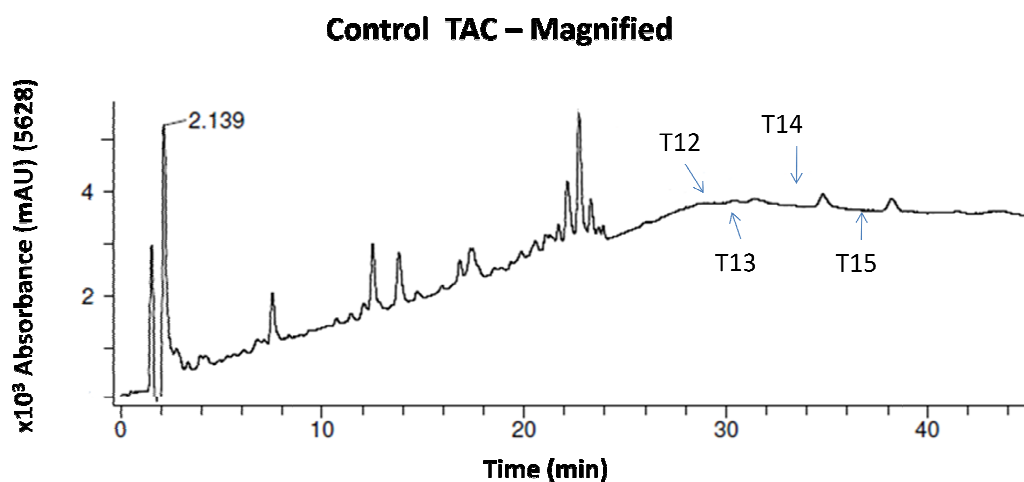
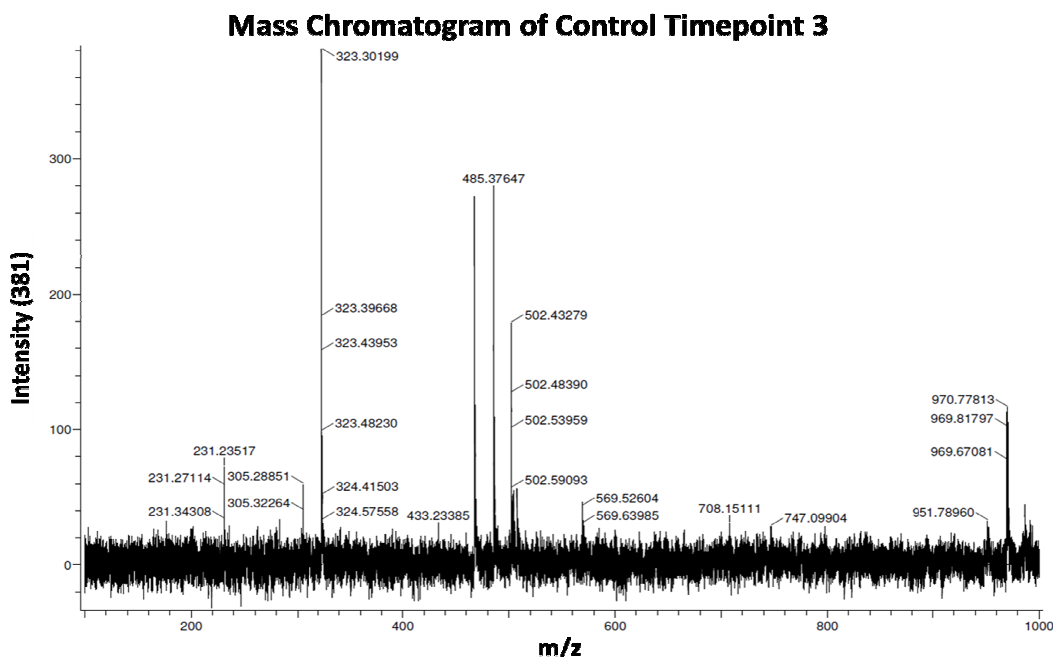


Figure 47: Magnified view of the Control TAC in Figure 45.

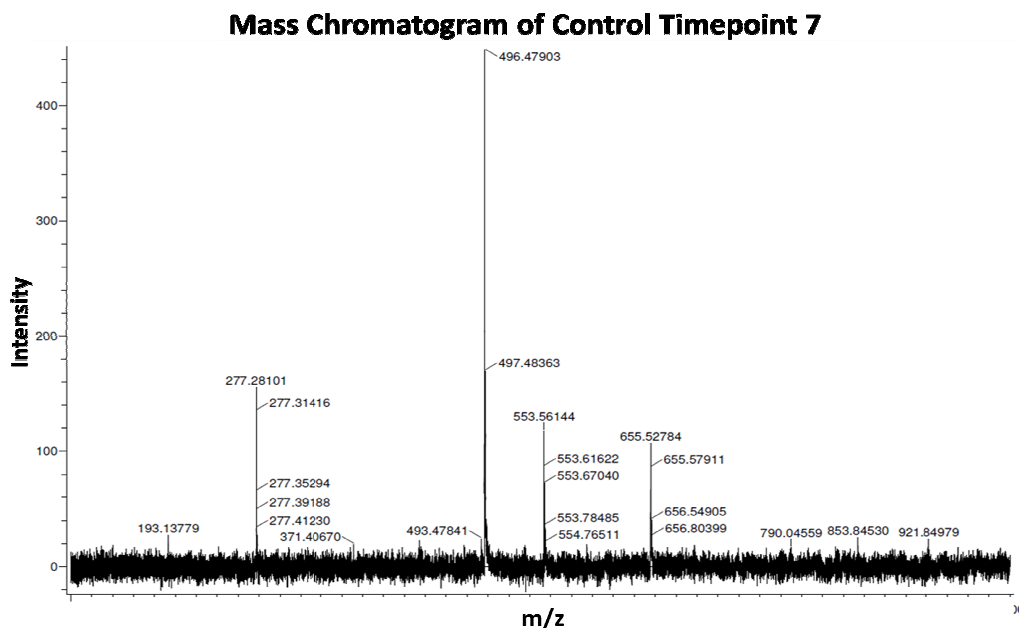
After marking the peaks in the chromatograms, each peak was analyzed to determine which lipids were present in the peak. Shown below in Figures 48 and 49 are two sample mass chromatograms, from time point T3 and T7, respectively. The intensities of the  $m/z$  peaks are graphed. Each  $m/z$  peak corresponds to a particular lipid. The identity of the lipids was determined by corresponding  $m/z$  values found in literature or an online lipid database (Vieler et al. 2007; Nature Lipidomics Database). In order to identify these lipids from databases, we reduced the significant figures of the identified  $m/z$  values till only the decimal. This choice was due to the noisy baselines and solvent interference we observed. Though the system is capable of high-resolution analysis, we chose to reduce the resolution. Most matched lipids, however, were only different in structural isomers and matched those previously documented for *C. reinhardtii*. The identity of the lipids at T3 can be found in Table 22. The identity of the lipids at T7 can be found in Table 23. All of the identified lipids found at each time point across all control samples were aggregated and displayed in Table 24. Note that the thick black lines in the mass chromatograms of Figure 48 and 49 at zero intensity indicate the noise from the MS detector. Any peaks identified by the Agilent software that were too small relative to the size of the noise were disregarded.



**Figure 48:** Mass chromatogram of timepoint 3 which corresponds to the peak at 7 minutes and 30 seconds in the TIC and TAC. The identities of the peaks are shown in Table 22.

**Table 22:** Compilation of m/z peaks and corresponding lipids at T3. The classes of the lipids found are glycopospholipids, diacylglycerols, and free fatty acids.

m/z	Name
231.3	Myristic Acid
305.3	Clavepictine B
323.3	Anandamide
485.4	Phosphoglycerol (PG 16:0)
502.4	Docosanediol-1,14-disulfate
569.5	Diacylglycerol (16:0)
708.2	Diacylglycerophosphate
969.8	Triacylglycerol



**Figure 49:** Mass chromatogram of timepoint 7 which corresponds to the peak at the TIC and TAC at 13 minutes and 30 seconds. The identities of the lipid were are shown in Table 23.

**Table 23:** Compilation of m/z peaks and corresponding lipids at T7. The classes of the lipids are glycerophospholipids, diacylglycerols, and triacylglycerides.

m/z	Name
277.3	Crucigasterin
496.5	Monoacylglycerophosphocholine
553.6	Sulfoquinovosyl-monoacylglycerol
655.5	Diacylglycerol (17:1/22:5)
790	Glycerophosphoserine
853.8	Triacylglycerol (16:0/16:1/24:3)
921.8	Diacylglycerol (16:0)

**Table 24:** Compilation of m/z peaks are corresponding lipids for the entire control sample. The composition of the lipids in the cells are varied but typical according to literature sources. The main classes of lipids found are free fatty acids, fatty amides, glycerophospholipids, diacylglycerides, and triglycerides. Fatty acids, fatty amides, and glycerophospholipids are lipids typically associated with cell reproduction. Diacylglycerides and triglycerides are lipids typically associated with energy storage.

m/z	Molecule Assignment	Class of Lipid
105	2-Amino-3-hydroxypropanoic acid	Amino Fatty Acids
146	3-hydroxy-3-methyl-2-oxo-pentanoic acid	Free Fatty Acids

<b>m/z</b>	<b>Molecule Assignment</b>	<b>Class of Lipid</b>
206	1,2-dithiolane-3R-pentanoic acid	Free Fatty Acids
222	Dodecatetraenedioic acid	Free Fatty Acids
222	Dodecatetraenedioic acid	Free Fatty Acids
225	(E)-4-Nitrostilbene	Miscellaneous
231	Myristic Acid	Fatty Acid
241	N-(3-oxo-octanoyl)-homoserine lactone	Fatty Amides
241	N-(3-oxo-octanoyl)-homoserine lactone	Fatty Amides
241	Tetradecasphinga-4E,6E-dienine	Sphingolipids
241	Trans-2-(4-nitrophenyl)-3-phenyloxirane	Miscellaneous
241	Trans-2-(4-nitrophenyl)-3-phenyloxirane	Miscellaneous
249	2E,6Z,8Z,12E-hexadecatetraenoic acid	Free Fatty Acids
268	7-heptadecenoic acid	Free Fatty Acids
277	Crucigasterin	Sphingolipids
282	Octadecenoic acid	Free Fatty Acids
305	Clavepictine B	Sphingolipids
323	Anandamide	Fatty Amides
323	Anandamide	Fatty Amides
339	N-oleoyl glycine	Fatty Amides
339	Elmiric Acid	Fatty Amides
353	Anandamide	Fatty Amides
377	N-arachidonoyl dihydroxypropylamine	Fatty Amides
399	1-nonyl-2-methyl-sn-glycero-3-phosphocholine	Glycerophospholipids
474	Vitexin 2-acetate	Flavonoids
483	Taurolithocholic acid (W)	Sterol Lipids
485	Phosphoglycerol (PG 16:0)	Glycerophospholipids
485	Phosphoglycerol (PG 16:0)	Glycerophospholipids
494	Monoacylglycerophosphocholine	Glycerophospholipids
496	Monoacylglycerophosphocholine	Glycerophospholipids
496	Monoacylglycerophosphocholine	Glycerophospholipids
502	Docosanediol-1,14-disulfate	Free Fatty Acids
502	Docosanediol-1,14-disulfate	Free Fatty Acids
553	Sulfoquinovosyl-monoacylglycerol	Glycerolipids
569	Diacylglycerol (16:0)	Diacylglycerides
593	Diacylglycerol (16:0/18:2)	Diacylglycerides
609	Diacylglycerol (16:1)	Diacylglycerides
611	Diacylglycerol (16:0/19:0; 17:0/18:0)	Diacylglycerides
625	Diacylglycerol (18:0/18:0)	Diacylglycerides
639	Diacylglycerol (16:0/21:0)	Diacylglycerides

<b>m/z</b>	<b>Molecule Assignment</b>	<b>Class of Lipid</b>
639	Diacylglycerol (17:0/20:0)	Diacylglycerides
639	Diacylglycerol (18:0/19:0)	Diacylglycerides
639	End of run flush	
639	Triauroylglycerol(12:0)	Triacylglycerides
655	Diacylglycerol (17:1/22:5)	Diacylglycerides
675	Diacylglycerol (18:2/22:1)	Diacylglycerides
705	Diacylglycerophosphate	Diacylglycerides
732	Glycerophosphocholine (16:0)	Glycerophospholipids
743	Monogalactosyl-monoacylglycerol (MGMG 32:6)	Glycerolipids
743	Monogalactosyl-monoacylglycerol (MGMG 32:6)	Glycerolipids
767	Phosphatidylglycerol	Glycerophospholipids
770	Monogalactosyl-diacylglycerol (MGDG 20:5/16:3))	Glycerolipids
790	Glycerophosphoethanolamine (18:0)	Glycerophosphoethanolamine
790	Glycerophosphoserine (18:0)	Glycerophosphoserine
852	Triacylglycerol (17:0/17:1/17:0)	Triacylglycerides
853	Triacylglycerol (16:0/16:1/24:3)	Triacylglycerides
916	Glycerophosphocholine (19:0/26:0)	Glycerophospholipids
916	Glycerophosphocholine (20:0/25:0)	Glycerophospholipids
921	Diacylglycerol (16:0)	Diacylglycerides
969	Triacylglycerol (16:0/22:0/22:3))	Triacylglycerides
969	Triacylglycerol (18:1/20:2/22:0))	Triacylglycerides

#### 4.5.2.1 Phytohormone Application

The following figures represent samples that illustrate important changes and deviations we found with different phytohormone treatments. The tables following each set of figures show additional lipids we found that differ from the control samples. Only salient changes in lipids or lipids not found in controls are listed in the tables; lipids present in the controls usually are still present in all phytohormone-treated samples at the various concentrations. An up arrow indicates an increase in

lipid from the control, a down arrow indicates a decrease in lipid from the control, and a star indicates a completely new lipid from the control.

#### 4.5.2.2 IAA at $10^{-8}$ M

Figure 50 shows the TIC for IAA at  $10^{-8}$  M. This plot looks very similar to the control as the same peaks are observed at the identical times. Figure 51 shows the m/z values of peaks found at timepoint T10. There are a number of important lipids that were observed for this treatment that are in different amounts than the controls. Table 25 summarizes these changes. Specifically, increased levels of the free fatty acid dodecatetraenedioic acid and fatty amide N-(3-oxo-octanoyl)-homoserine lactone were found. The diacylglyceride glycerophosphoinositol (20:4) was found to be present whereas it was absent in the control. There was also a decrease in diacylglycerols (16:0/18:2).

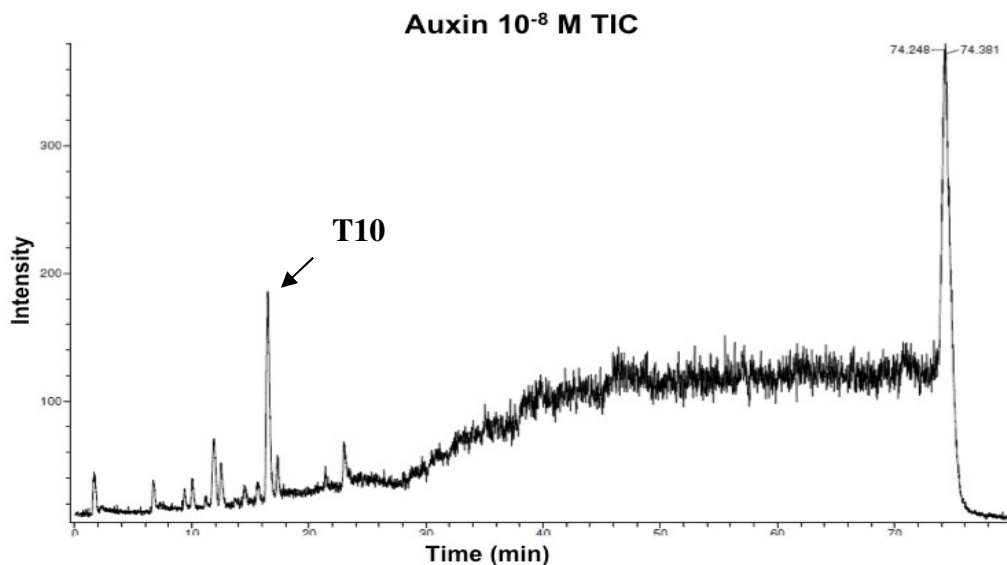
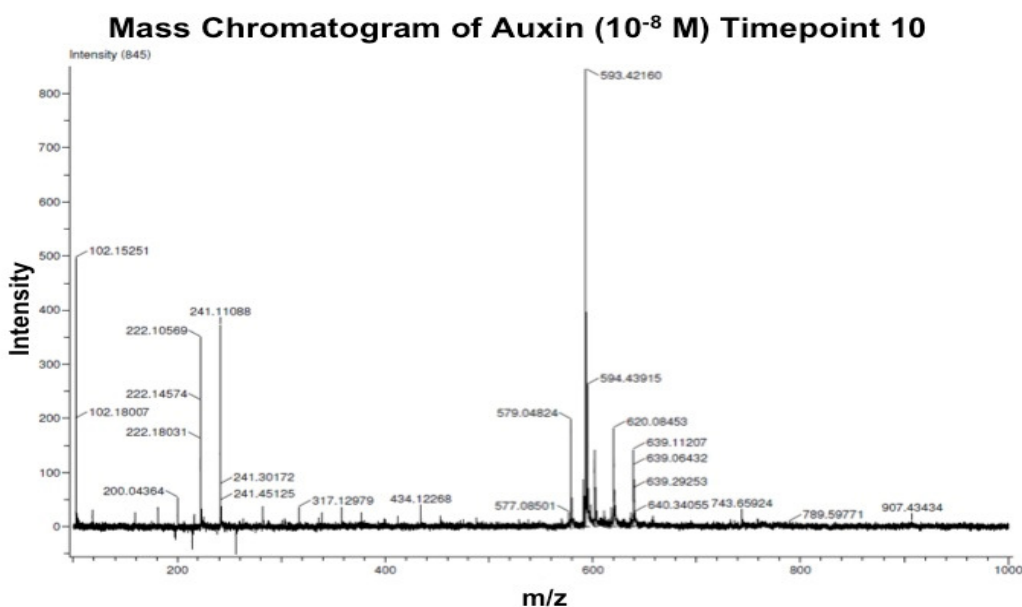


Figure 50: TIC of IAA at  $10^{-8}$  M. Notice the increases at the peak around T10 (16 minutes). The mass chromatogram of the peaks is found in Figure 51.



**Figure 51:** Mass chromatogram of T10 of IAA at  $10^{-8}$ M. The identities of the lipids are shown in Table 25.

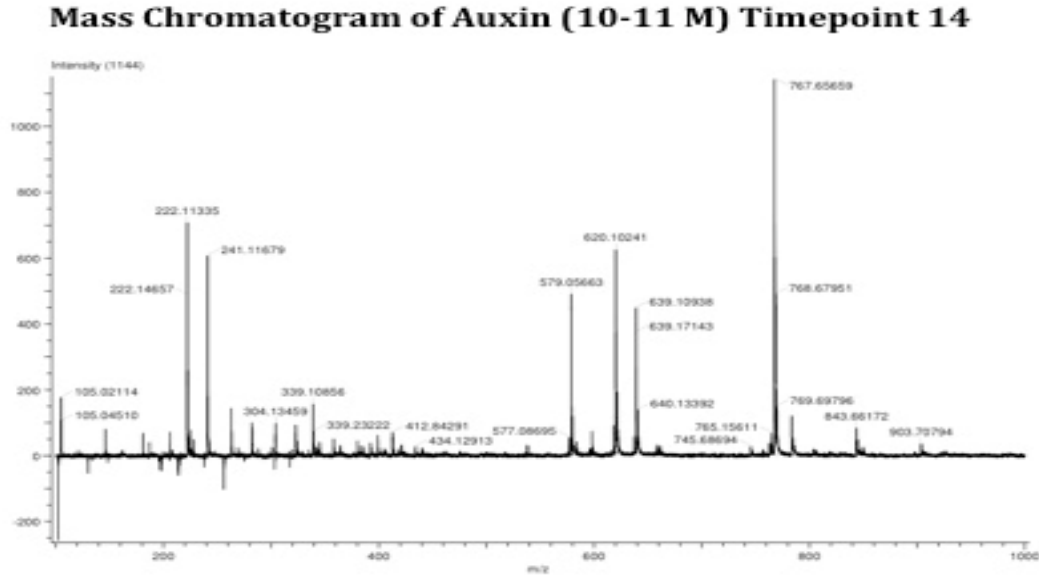
**Table 25:** Compilation of the production of lipids that deviated from controls for IAA at  $10^{-8}$  M.

m/z	Molecule Assignment	Class	Change
222	Dodecatetraenedioic acid	Free Fatty Acids	↑
241	N-(3-oxo-octanoyl)-homoserine lactone	Fatty Amides	↑
593	Diacylglycerol (16:0/18:2)	Diacylglycerides	↓
620	Glycerophosphoinositol (20:4)	Glycerophosolipids	*

#### 4.5.2.3 IAA at $10^{-11}$ M

IAA treatment at  $10^{-11}$  M also showed a noticeable change in the lipid production compared to that in controls. Increased levels of the fatty amide N-(3-oxo-octanoyl)-homoserine lactone and the Glycerophospholipid phosphatidylglycerol were found for the treatment. Glycerophosphocholine (18:0/3:0), glycerophosphoinositol (20:4), eruberin B, and triacylglycerols (16:0/17:1/18:2/17:0/18:1/20:0) were isolated, but not in the control. Mass

chromatogram at timepoint 14 of this treatment had most notable changes in lipid produced, as illustrated in Fig. 52. The identities of the lipids at the peaks in this chromatogram are aggregated in Table 26.



**Figure 52:** Mass chromatogram of timepoint 14 for the treatment of IAA at 10<sup>-11</sup> M. The identities of the lipids are shown in table 26.

**Table 26:** Compilation of the identified lipids produced from a treatment of IAA 10<sup>-11</sup> M that deviated from the controls.

m/z	Molecule Assignment	Class	Change
241	N-(3-oxo-octanoyl)-homoserine lactone	Fatty Amides	↑
579	Glycerophosphocholine (18:0/3:0)	Glycerophosolipids	*
620	Glycerophosphoinositol (20:4)	Glycerophosolipids	*
640	Eruberin B	Flavaonoids	*
767	Phosphatidylglycerol	Glycerophospholipids	↑
843	Triacylglycerols (16:0/17:1/18:2)	Triacylglycerides	*
903	Triacylglycerols (17:0/18:1/20:0)	Triacylglycerides	*

#### 4.5.2.4 Epibrassinolide at $10^{-9}$ M

Treatment of Epibrassinosteroid at  $10^{-9}$  M showed increased production of certain lipids and development of new lipids at timepoint 13, illustrated by a TIC in Fig. 53 and mass chromatogram of T13 peak in Figure 54. All lipid changes for this treatment are presented in Table 27. Increased levels of Dodecatetraenedioic acid, N-(3-oxo-octanoyl)-homoserine lactone, Elmiric Acid, and Triauroylglycerol were found. There were also several lipids that were found that were not present in the control. Heptadecadiynoic acid, Cerotic acid, Glycerophosphocholine, Glycerophosphoinositol, and Chlorophyll a were isolated from the Epibrassinolide treatment but not from the control. These lipids are free fatty acids and glycerophospholipids.

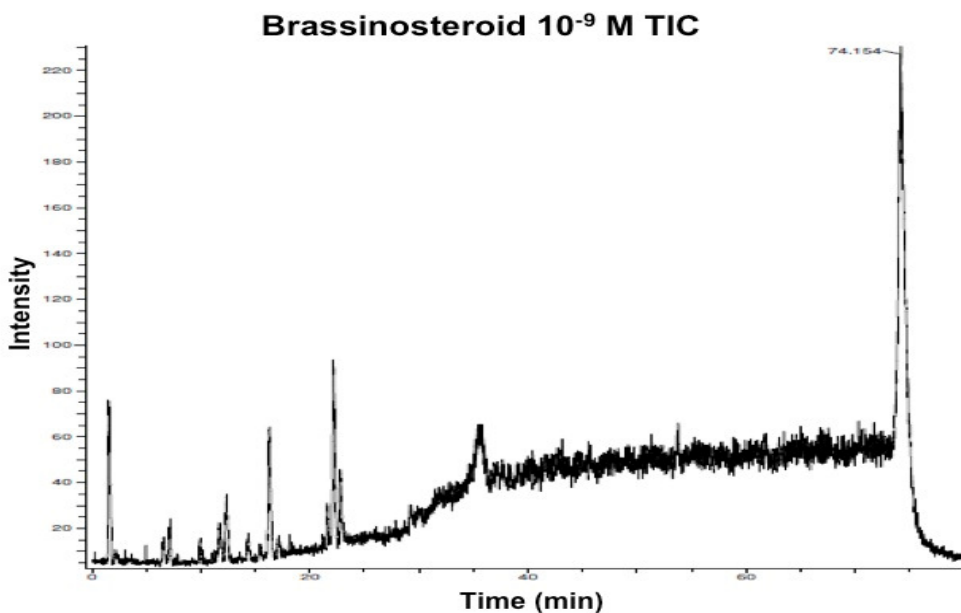
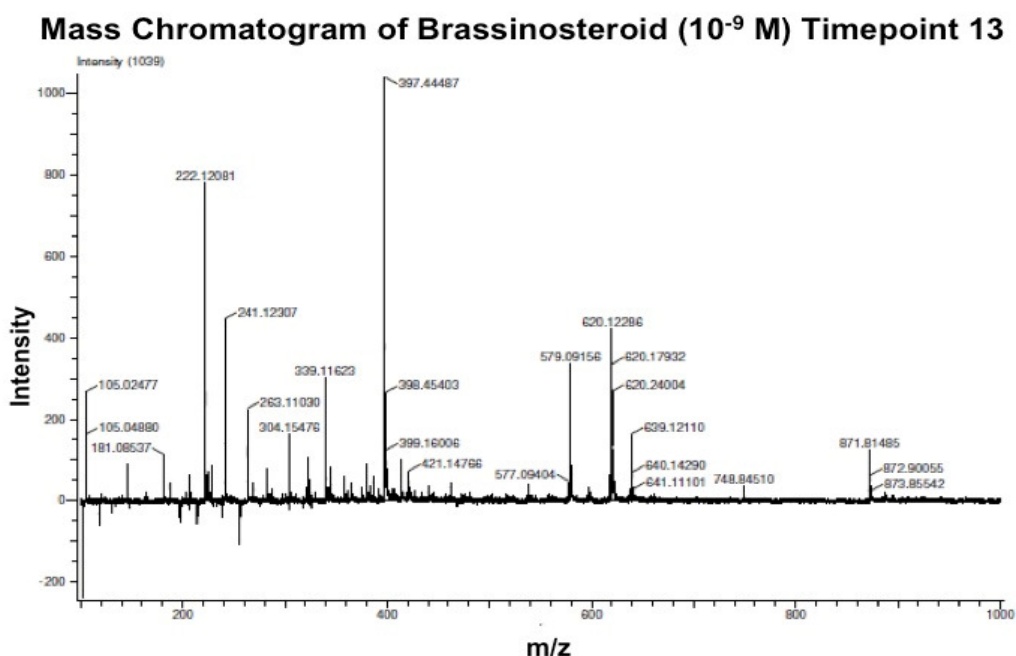


Figure 53: TIC of Epibrassinolide at  $10^{-9}$  M. Noticeable changes in lipids are found at T13. The mass chromatogram of T13 is shown in Figure 54.



**Figure 54:** Mass chromatogram of T13 after a treatment of Epibrassinolide at  $10^{-9}$  M. The identity of the peaks is aggregated in Table 27.

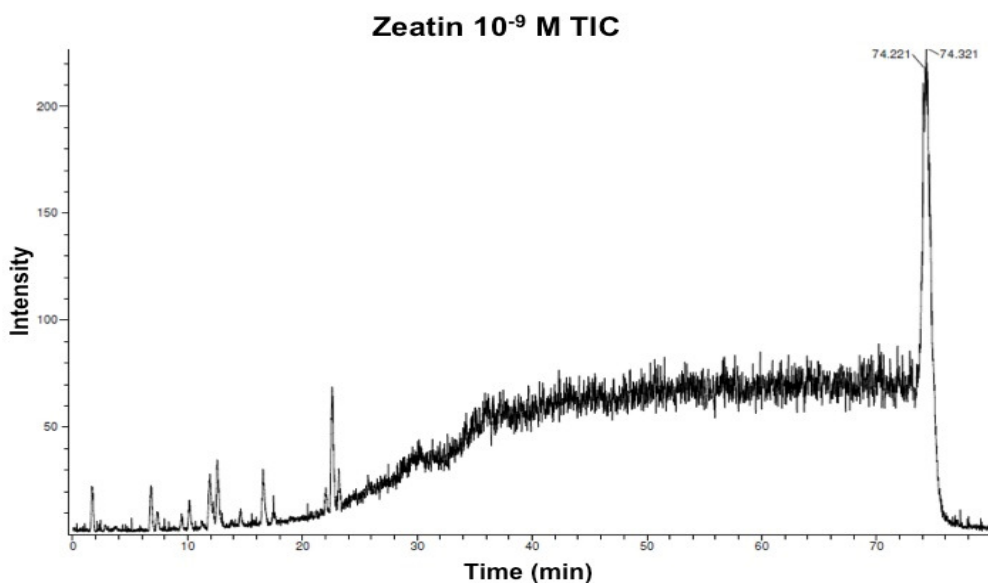
**Table 27:** Compilation of identified lipids from a treatment of Epibrassinolide at  $10^{-9}$  M that deviate from the control.

m/z	Molecule Assignment	Class	Change
222	Dodecatetraenedioic acid	Free Fatty Acids	↑
241	N-(3-oxo-octanoyl)-homoserine lactone	Fatty Amides	↑
263	Heptadecadiynoic acid	Free Fatty Acids	*
339	Elmiric Acid	Fatty Amides	↑
397	Cerotic acid	Free Fatty Acids	*
579	Glycerophosphocholine (18:0/3:0)	Glycerophospholipids	*
620	Glycerophosphoinositol (20:4)	Glycerophospholipids	*
639	Triauroylglycerol(12:0)	Triacylglycerides	↑
871	Chlorophyll a	Pigment	*

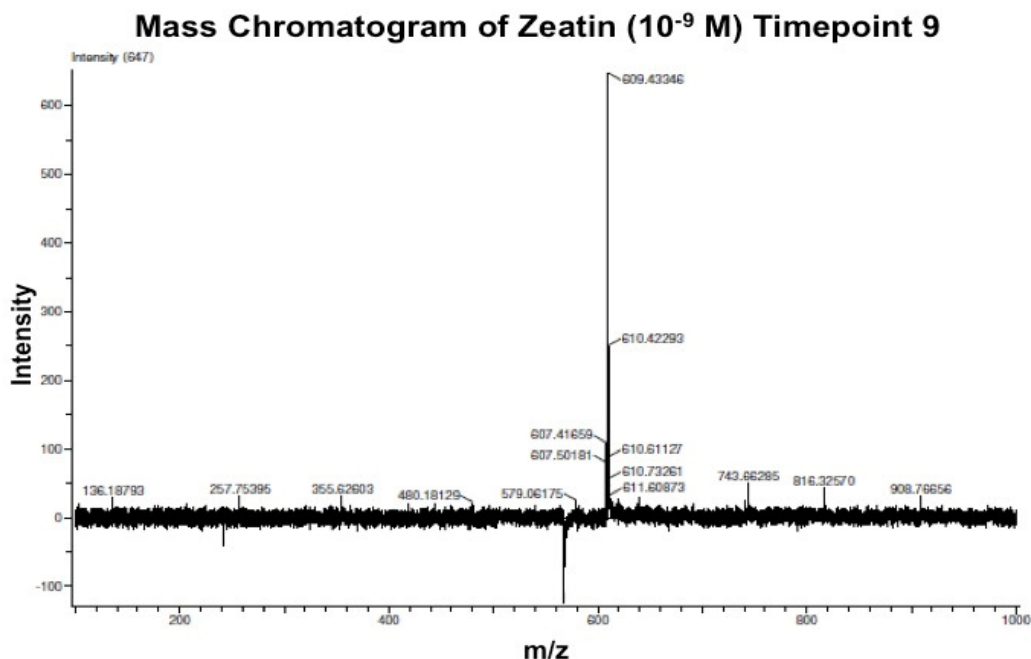
#### 4.5.2.5 Trans-zeatin at $10^{-8}$ M

The Trans-zeatin at  $10^{-8}$  M showed peaks of interest mostly at Timepoint 9, shown in Figs. 55 and 56. The identity of all notable lipids from this treatment are

presented in Table 28. Lipids were found at the m/z values of 609, 743, and 816. M/z value of 609 correlates to diacylglycerol within the larger class of diacylglycerides. This molecule was found at increased levels as compared to the control. M/z value 743 corresponds to monogalactosyl-monoacylglycerol a member of the glycerolipids family. This molecule was also found at increased levels as compared to the control. M/z value of 816 corresponds to glycerophosphocholine which is a member of the glycerophospholipid family and was also found at an increased level.



**Figure 55: TIC of lipids after treatment of Trans-zeatin at 10<sup>-9</sup> M. The lipids at T9 showed a difference in the lipids found in the control. The mass chromatogram of T9 is found in Table 28.**



**Figure 56:** Mass chromatogram of the lipids found at T9 for Trans-Zeatin at  $10^{-9}$  M. The identities of the lipids are presented in Table 28.

**Table 28:** Compilation of the identities of the lipids from a treatment of Trans-zeatin at  $10^{-9}$  M that differed from the control.

m/z	Molecule Assignment	Class	Change
609	Diacylglycerol (16:1)	Diacylglycerides	↑
743	Monogalactosyl-monoacylglycerol (MGMG 32:6)	Glycerolipids	↑
816	Glycerophosphocholine (18:1/20:0)	Glycerophospholipids	↑

#### 4.5.2.6 Zeatin-riboside at $10^{-11}$ M

Treatment of Trans-zeatin-riboside at  $10^{-11}$  M produced notable peaks at timepoint 8, illustrated in Figs. 57 and 58. Additionally, novel lipids were found at 28.2 minutes, shown in Fig. 59, which did not manifest in controls or any other phytohormone treatments. All notable changes in lipids from this treatment are presented in Table 29. Increased levels of the fatty acids dimethylallyl-diphosphate, 1,2-

dipentadecanoyl-sn-glycero-3-phosphate, and GlcCer(d18:0/20:0) were found. There was a decrease in vitexin 2''-acetate, PS(16:0/16:0)[U], C5 isoprenoids, and dimethylallyl-diphosphate. 2E,4E,8E,10E-Dodecatetraenedioic acid, N-(3-oxo-octanoyl)-homoserine lactone, 1-decanoyl-2-tetradecanoyl-sn-glycero-3-phosphoethanolamine, and PI(20:4(5Z,8Z,11Z,14Z)/0:0) were found to be present in the sample despite being absent in the control. Most notably, the new peak identified at 28.8 minutes corresponded to Trilauroyl-glycerol. This triacylglyceride is especially suitable to biodiesel production. It is indicated by \*\* in Table 29, along with glycerophosphoinositol also found concurrently.

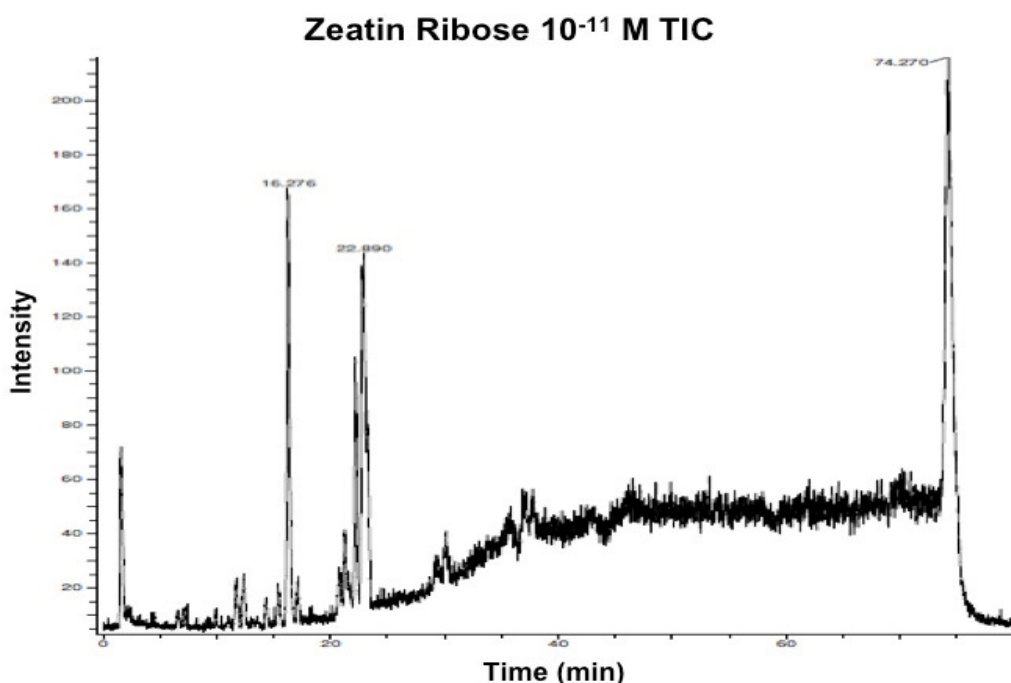


Figure 57: TIC of lipids after a treatment of Trans-zeatin-riboside at  $10^{-11}$  M. The mass chromatograms of T8 and a new peak at 28.2 minutes are found in Table 29 and Figure 59, respectively.

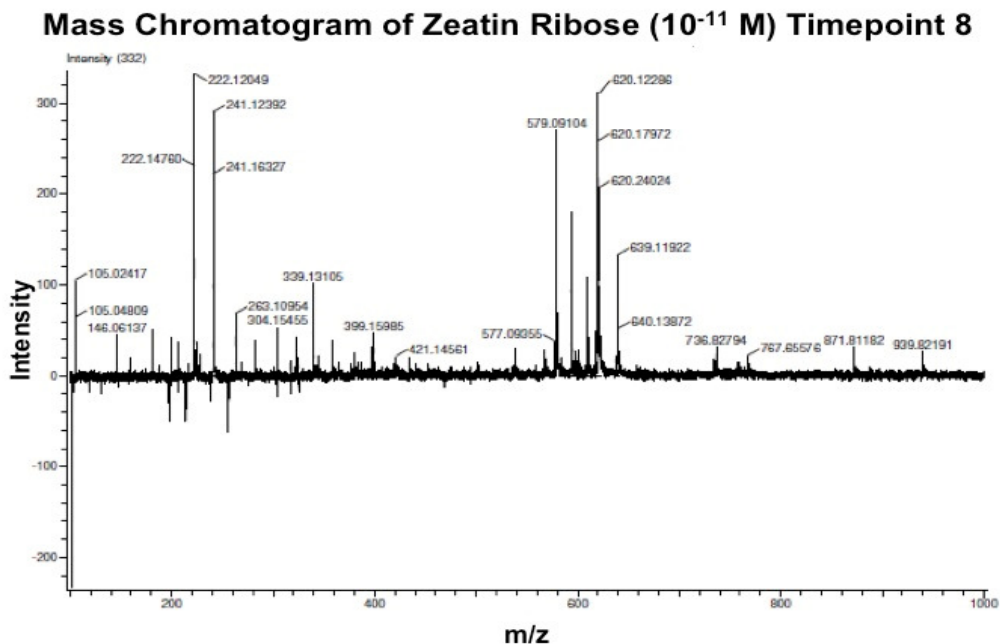


Figure 58: Mass chromatogram of the lipids at T8 after a treatment of Trans-zeatin-riboside at  $10^{-11}$  M. The corresponding lipids are found in Table 29.

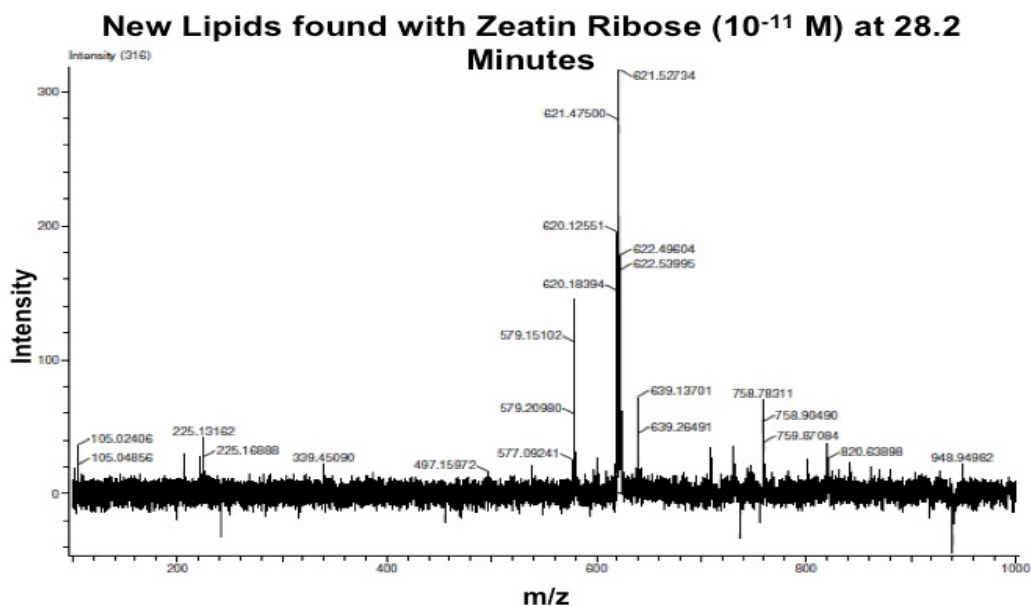


Figure 59: Mass chromatogram of the lipids found at the new peak on the TIC at 28.2 minutes. This peak appeared after a treatment of Trans-zeatin-riboside at  $10^{-11}$  M. The identities of the lipids are listed in Table 29.

**Table 29: Compilation of lipids produced after a treatment of Trans-zeatin-riboside at  $10^{-11}$  M that deviated from the controls. The \*\* indicates previously unreported lipids found at 28.2 minutes.**

m/z	Molecule Assignment	Class	Change
222	2E,4E,8E,10E-Dodecatetraenedioic acid	Free Fatty Acid	*
241	N-(3-oxo-octanoyl)-homoserine lactone	Fatty Amides	*
474	Vitexin 2"-acetate	Flavones and Flavonols	↓
579	1-decanoyl-2-tetradecanoyl-sn-glycero-3-phosphoethanolamine	Glycerophosphoethanolamines	*
620	PI(20:4(5Z,8Z,11Z,14Z)/0:0)	Glycerophosphoinositols	**
639	Trilauroyl-glycerol	Triacylglycerol	**
736	PS(16:0/16:0)[U]	Diacylglycerophosphoserines	↓
247	C5 isoprenoids	Isoprenoid	↓
377	dimethylallyl-diphosphate	Isoprenoid	↓
579	Phthioceranic acid	Branched fatty acids	↑
620	1,2-dipentadecanoyl-sn-glycero-3-phosphate	Diacylglycerophosphates	↑
758	GlcCer(d18:0/20:0)	Neutral glycosphingolipids	↑

## 5. Discussion

### 5.1 Overview

Our primary objective throughout this research has been to find a way to improve the production of algal biofuels, specifically the economic efficiency of lipid production, given our limited resources by focusing on areas to which we could add new information and did require expensive genetic engineering and analysis techniques. From our review of literature, we determined that there are two general possible routes to increasing lipid production and decreasing costs. The first is to modify algal metabolism to induce the algae to grow faster or to produce more lipids. The second is to develop methods of algal processing that are cheaper, faster, and more productive than current methods. We chose *C. reinhardtii* as our experimental model due to its position as a model organism, ease of growth, relative availability, and existing extensive research on it. After this choice, we needed to determine our method of improving the biofuel production of these algae.

Improvement of current algal processing is primarily focused on separating the algae from media, isolating and extracting the lipids, and transesterifying the lipids into usable biodiesel. These techniques are crucial to the production of algal biofuel, and improvements to them increase the viability of biofuels. However, the literature review revealed that attempting to improve aspects of these techniques would be outside the scope of our research. Current techniques are targeted at the large-scale growth of algae and lipid extraction. As we are operating on a much

smaller scale, our techniques are significantly different from those used in a modern industrial setting. We do not possess the resources to replicate the industrial procedures on a small scale. However, if we can find ways to improve production-related qualities of the alga, the findings could be incorporated into the current industrial production techniques. This led to our decision to modulate algal metabolism through the use of phytohormones.

Literature showed that by experimenting with nutrient supplementation, genetically engineering pathways, or bioreactor types, gains could be made in algal lipid production. For our experiments, given our limited time and budget, we decided to pursue experimentation with supplements in the media. Our inspiration to focus on the application of phytohormones came from the hypothesis that it is possible that plants and algae have developed similar biological pathways because they are evolutionarily related. If this is true, then these pathways could be controlled by the same regulators - in our case, phytohormones. There is a possibility that due to evolutionary time distance, the roles of these signals could be different. This possible connection was analyzed through bioinformatics, namely the comparison of *C. reinhardtii* to *A. thaliana* phytohormone signaling pathways. While there were few phytohormone receptors or pathways that matched completely, there was considerable evidence that suggested a connection. Thus, we decided to apply auxins, brassinosteroids, and cytokinins to *C. reinhardtii* to see if there were any effects.

The two primary areas where we sought to see changes were in the growth rates and lipid content of *C. reinhardtii*. We monitored the growth rates through the

use of spectroscopic data collected and the dry mass of the algae. In order to analyze lipid content changes we measured the lipid masses per quantity of algae and then combined this with growth rates to determine the lipid concentration of a culture. Additionally we monitored morphological and visible changes to cell by confocal microscopy and analysed the lipid profiles of the algae under phytohormone treatment by LC-MS. Our experimentation and analysis determined that all the phytohormones had some effect on the growth rate, lipid content, cell morphology, and lipid profiles of *C. reinhardtii*. We found that treatment of Trans-zeatin-riboside yielded the most promising results in these criteria, especially at concentration of  $10^{-11}$  M. Our findings suggest that the fundamental basis of our work is sound, and that phytohormones affect algal metabolism even though their functions are likely markedly different from their late evolutionary successors, plants.

## 5.2 Bioinformatics

To determine whether particular phytohormones would have an effect in *C. reinhardtii*, the genome of this alga was examined to find sequences similar to those proteins involved in the phytohormone signaling pathways in *A. thaliana*. To simplify the analysis, the results were screened by the expect values for the aligned genes; a threshold value of 0.1 was chosen. Matches that met this criterion were further analyzed for homology. In addition, due to the fact that many of the proteins in *C. reinhardtii* have not been annotated, many of the results included hypothetical proteins.

### 5.2.1 Absciscic acid

Previous research on the signal transduction pathway for absciscic acid response has been ambiguous. Candidates for the ABA receptor such as the GCR2 and FCA proteins in *A. thaliana* have been invalidated while the ABA insensitive proteins have been shown to receive phytohormone molecules on the surface of the *A. thaliana* cell membrane, although there is no general consensus on these (Leung, et al., 1994). This ambiguity was reflected in the bioinformatics analysis as no protein involved in the *A. thaliana* phytohormone cascade that was examined demonstrated strong matches with the *C. reinhardtii* genome. Due to this, absciscic acid was not selected for further investigation. It is possible that this pathway diverged differently enough to evolve the functions of absciscic acid we see today in plants.

### 5.2.2 Auxins

Auxins affect the activity of the cell at the transcription level. Auxin response factors (ARFs), which dimerize and bind to DNA to allow transcriptional control were analyzed. However, no close matches with the *C. reinhardtii* genome were observed except for ARF8, ARF6, and ASK2. ARF8 mediates the phytohormone response in *A. thaliana* by expressing auxin regulated genes and, along with ARF6, controls stamen elongation and flower maturation, while ASK2 aids in controlling the mitotic cell cycle (Mattsson, et al., 2003). The LCI1 gene, which was considered to be a strong match with ARF8 in *A. thaliana*, was observed to be involved in the encoding and regulation of a transporter that increases the uptake of CO<sub>2</sub>, but it has

not been characterized biochemically (Ohnishi, et al., 2010). Thus, it is difficult to determine whether the LCII protein is relevant to a possible auxin response mechanism in *C. reinhardtii* or if it contains a conserved transporter domain. In addition, SKP1, a strong match with ASK2, has been shown to be involved in cell division but it is not known whether it is regulated by auxin. Although LCII and SKP1 have not been shown to be directly involved in phytohormone response, previous research regarding the application of auxin to *C. reinhardtii* has demonstrated that *C. reinhardtii* may respond to the phytohormone and does secrete small concentrations of auxin (Jacobs 1951; Jacobs, et al., 1985; Cooke, et al., 2001). Thus, other genes that have not been annotated may be involved in phytohormone response and share homology with the genes involved in phytohormone response in *A. thaliana*. Due to the fact that literature indicates a possible role for auxin and bioinformatics reveal possible homologous proteins found in *C. reinhardtii*, auxins were selected for further analysis.

### **5.2.3 Brassinosteroids**

The signal transduction pathway for brassinosteroids is better known than those of other phytohormones. As indicated in the Results section, the BES1 protein, which encodes a signaling protein that accumulates in the nucleus, and the BZR1 protein, which has a DNA binding domain, had numerous matches in the genome of *C. reinhardtii* with E-values less than .003. However each of these proteins instead

have primary functions related to locomotion in *C. reinhardtii* and may not be associated with the phytohormone response.

Proteins more closely associated with the brassinosteroid response pathway, such as BIN2 and BRI1, elements of the brassinosteroid receptors in plants, did not give any strong matches (Li and Nam 2002). However there is evidence for presence of low concentrations of brassinosteroids in some algal species. The response of *C. reinhardtii* to brassinosteroids has yet to be investigated since minimal research has been conducted regarding the relationship between brassinosteroids and *C. reinhardtii*. Based on this, we chose to pursue investigation of brassinosteroid effects on algae.

#### **5.2.4 Cytokinins**

Cytokinin-like activity has been observed in *C. reinhardtii*; thus it is likely that the alga's genome contains at least a rudimentary signal transduction pathway (Stirk, et al., 2002; Ordog, et al., 2004). While the cytokinin binding receptor CRE1, a histidine kinase in *A. thaliana*, generated some matches with E-values of .024 with the nitrate assimilation regulatory protein (NIT2) and RWP-RK transcription factor, it is likely that they all contain conserved domains common among histidine kinases and associated proteins. Additional matches such as AHK4, active in phytohormone signaling of plants, also matched to *C. reinhardtii* which are involved in unrelated processes. These proteins are, however, involved in diverse functions in *C. reinhardtii*. Due to this possibility, the matches in *C. reinhardtii* cannot be completely

ignored. We chose cytokinins further investigate this possibility. Trans-zeatin and Trans-zeatin-riboside were selected on the basis of their prevalence in the regulation of cell division for *A. thaliana* and due to monetary constraints.

### **5.2.5 Ethylene**

Previous research has demonstrated that ethylene has no role in *C. reinhardtii*. In addition, the bioinformatics analysis did not yield any strong matches. Ethylene, as related to plant signaling, is produced as a gas and can act over larger distances for a multicellular organism (Dugardeyn & Van Der Straeten, 2008). Its practicality is less likely for an organism that lives in water. However, the algal cells may communicate in colonies by an Ethylene-like precursor. In plant cells, this phytohormone does play a growth-inhibiting role and is less valuable to improving biodiesel production from algae (Wray, 1992).

### **5.2.6 Gibberellins**

Previous research has not elucidated the signal transduction pathway for gibberellins in plants. This makes comparison of this phytohormone's signaling pathway to that possible in *C. reinhardtii* very difficult. Proteins that have been proposed to be involved in the pathway did not give any noteworthy matches in our analysis. Thus, it was concluded that gibberellins until further investigation of this phytohormone's signaling in plants was completed, exploring its effects under our monetary constraints would not be cost-effective.

### 5.2.7 Summary

The bioinformatics analysis of the *C. reinhardtii* genome did not give a completely clear indication for phytohormone signal transduction occurring in the alga in a similar fashion to *A. thaliana*. Considering that *A. thaliana* and *C. reinhardtii* are very distantly related, it is difficult to determine whether the two organisms share any homologous proteins in phytohormone response since many proteins that were good matches only shared conserved domains. It is possible that the phytohormone signal transduction pathway precursors in *C. reinhardtii*, if they exist, may involve different proteins or share different overall protein architecture with only necessary underlying conserved domains. Many of the proteins that were strong matches were hypothetical proteins. Since these hypothetical protein genes did not exhibit one-to-one matches with genes from *A. thaliana*, the previously mentioned possibility of different protein architecture could be true. Thus, for a bioinformatics analysis of *C. reinhardtii* to be successful, future research must focus on identifying and characterizing these proteins and completing annotation of other proteins in *C. reinhardtii*.

Based on previous research, it is known that ethylene has no role in algae and that auxins are secreted by some species of algae (Cooke, et al., 2002). In addition, substances similar to cytokinins and brassinosteroids are produced by *C. reinhardtii* under certain conditions (Bajguz & Czerpak, 1996; Stirk, et al., 2002). Despite of the lack of definitive results produced by the bioinformatics analysis, cytokinins and auxins were chosen for the experiments based on the evidence provided by past research that indicates substances similar to auxins and cytokinin are secreted in

algae. Brassinosteroids were chosen because their role and effects on algae, especially in *C. reinhardtii*, have not been fully explored. In addition, the genes BZR1 and BES1 had low E-values of less than 0.01, indicating that there may be some similarities in the receptors.

### **5.3 Phytohormones**

Each treatment of phytohormone had an effect on either growth rate or lipid yield. All of the phytohormones had a positive increase on growth except for T-zeatin. The effects of increased lipid yield were more difficult to measure due to the error in our dry lipid mass data. However, confocal microscopy and LC-MS o verified our suspicions on the effects of phytohormones on lipid yields. Generally, Indole-3-acetic Acid (IAA), an auxin, and Epibrassinolide, a brassinosteroid, increased growth with little effects on the lipid content. Trans-zeatin-riboside, a cytokinin, either increased the growth rate while having a greater effect on increasing lipid content. Based on these observations, a combination of IAA and Trans-Zeatin effects by also increasing both growth rates and lipid content.

#### **5.3.1 Controls**

The control cultures of our experiments were important in establishing baseline levels for growth rates, dry masses of the algae, specific lipid masses of the algae, and lipid concentrations of the cultures. The morphology of the cells and the identities of the lipids found in typical *C. reinhardtii* cells was also compared to

control group cells. These controls were very important in helping analyze the effects of phytohormones on *C. reinhardtii*.

Our algae exhibited a typical growth rate of 0.0226 Abs/day. This corresponds to about  $1.72 \pm 0.01 \times 10^6$  cells/L/day or  $10.4 \pm 0.7$  mg algae/L/day grown in a linear growth phase. Algal growth is documented to occur in logarithmic growth phase. While establishing our growth conditions, we inspected all variable to determine the root cause of the linear growth. The suspected causes of this linear growth rate are scrutinized in our analysis of our methodology below. It is a strong possibility that our cultures were photon flux limited. At this reduced level of light, literature supports algal growth at linear levels (Akimoto, et al., 1997). All algal samples in our study consistently exhibit a linear growth phase. Based on this, our algae did not experience doubling growth rates with or without treatments of phytohormones; therefore, this linear growth rate ubiquitous for all trials makes analysis of the effects of phytohormones on growth rate easier to perform.

Along with growth rates, lipid content was analyzed. The typical specific lipid mass ( $\text{g}_{\text{lipid}}/\text{g}_{\text{algae}}$ ), percentage of lipid by mass of the algae, of the algae was found to be  $60.1 \pm 13.6\%$  for control. A higher number indicates increase in production of lipid content per algal cell. Although this is higher than literature values, this was consistent throughout all of the controls (Sialve, Bernet, & Bernard, 2009). In addition to the specific lipid mass, we calculate the lipid concentration ( $\text{g}_{\text{lipid}}/\text{L}_{\text{volume of solution}}$ ), the production efficiency of a culture. For the controls, the mean lipid concentration was  $0.075 \pm 0.016$  g/L. This value takes into account the growth rate and

the specific weights of the lipids of the algae and provides a more accurate representation of the effects of a phytohormone treatment. Additionally, the confocal microscopy imaging of the control cells showed very few lipid bodies. This is consistent with the study conducted by Wang, et al (Z. T. Wang, et al., 2009).

In order to understand the effects of the phytohormones at the level of cell metabolism, we conducted an analysis of the types of lipids found in *C. reinhardtii*. The analysis allows us to understand how exactly the biological activity of the cell is affected by phytohormones. Previous work in understanding the changes to lipid types under various conditions of stress and environmental factors suggest certain characteristic trends. For example, under increasing temperatures, the quantity of total free fatty acids increases (Akimoto, et al., 1997). To observe such changes, we first examined the types of lipids in the normal cell of *C. reinhardtii*. Using 5 repeats of control samples we identified a set of salient lipids in control cells, presented in Table 22. Among these we found triacylglycerides (TAG), free fatty acids (FFA), and glycerophospholipids (GP). These matched previously documented lipid types from *C. reinhardtii* (Vieler, et al., 2007). These lipids are ubiquitous amongst all eukaryotic cells in functions of energy storage and membrane formation.

In addition to these documented lipids, we also identified diacylglycerides (DG), sphingolipids, aromatic polyketides, and flavonoids. monogalactosyl-monoglycerol (MGMG), and sulfoquinovosyl-monoacylglycerol (SQMG). The DG and sphingolipids again are ubiquitous in eukaryotic cells. The metabolites, aromatic polyketides and flavonoids, are less interesting in the context of biodiesel production

due to their structure of rings. These metabolites would not be involved in transesterification and would remain as side products. As they are hydrocarbons, they would however, burn in a mixture with biodiesel. The MGMG and SQMG are thylakoid membrane lipids expected in organisms containing chloroplasts. However, these two lipids have previously been found as diacylglycerols (i.e MGDG and SQDG) (Vieler, et al., 2007). We suspect that these correspond to documented MGDG and SQDG with one of the fatty acid chains hydrolyzed under our high temperature lipid extraction conditions. The presence of water in the extraction columns at the high temperature would allow such a reaction. Further, the appearance of the monoacylglycerol variants in all 5 controls and experimental samples supports that these products may result from our experimental conditions.

In contrast to the positive matches of lipids from our LC-MS analysis to those documented in literature, we were unable to identify diacylglycerol-*N,N,N*-trimethylhomoserine (DGTS) and digalactosyl-diacylglycerol (DGDG). This could again be a result of hydrolysis reactions under our lipid extraction conditions. These products would appear as the DG that we characterized. The identification of a majority of lipid types and their correspondence to literature findings suggests our LC-MS methodology was successful in separating and resolving a majority of lipids in *C. reinhardtii*.

### 5.3.2 IAA

Though auxins are naturally present in algae, the concentrations at which they are found are minute (less than  $10^{-12}$  M) (Cooke, et al., 2002). We chose to introduce auxins at a range of experimental concentrations between  $10^{-8}$  M and  $10^{-12}$  M to determine its effects on algae. The specific auxin that we utilized for our experiment was indole-3-acetic acid (IAA), the most common auxin found in algae. It is naturally present in different genera of algae and has been studied in algal models (Jacobs, 1951; Jacobs, et al., 1985; Cooke, et al., 2001). Additionally, keeping its economical significance in perspective, IAA's low costs stand to reduce operational costs of algal biodiesel production. Thus, all experiments involving auxins were conducted with IAA.

According to our results, an auxin concentration of  $10^{-11}$  M resulted in the highest increase in growth rate. At this concentration, the growth rate increased by  $52.9 \pm 36.3\%$  relative to control. The minimal growth rate was found at a concentration of  $10^{-9}$  M (Fig. 17). Inhibition at higher concentrations of IAA may echo the inhibitory effects of auxins found in higher plants. At a concentration of  $10^{-8}$  M there is a marked increase in growth rate as compared to  $10^{-9}$  M, although not greater than control (Fig. 17). We noticed this positive result as an overall trend through each of our trials. At such a high concentration of auxin, it is possible that an additional pathway may become activated due to higher ligand binding threshold, which improves the growth rate. The growth increasing effect noted here is not a result of utilizing the IAA as a carbon source because at a concentration of  $10^{-8}$  M,

IAA yields only  $1.2 \times 10^{-6}$  g of carbon material. There is greater possibility of auxin participating in multiple signaling pathways based on its concentration.

The greatest dry mass of the algae exposed to IAA occurred at concentrations of  $10^{-9}$  M and  $10^{-12}$  M, but the increase was only approximately 2% over the control samples. This is not a statistically significant change. The variability among the dry weights of the samples exposed to various phytohormone concentrations was lower when compared to the variability in the identical samples' growth rates. The most effective concentration of auxin at increasing specific lipid mass ( $g_{\text{lipid}}/g_{\text{algae}}$ ) was  $10^{-10}$  M (Fig. 24). But again the standard deviation for this result is large enough to render the effect statistically insignificant. Lipid concentration ( $g_{\text{lipid}}/L_{\text{volume of culture}}$ ) increased negligibly for  $10^{-9}$  M IAA (Fig. 31). Notably at concentration with the fastest growth rate,  $10^{-11}$  M, a decrease of approximately 21% was found. As has been described in the literature review, this strikes at the balance of the growth and lipid metabolism. In increasing growth, lipid production generally decreases and vice versa.

LC-MS and confocal microscopy analyses helped verify the positive impact of auxins on the lipid dry weights of algae. The auxin groups showed the greatest increase in lipid bodies; many (20+) small lipid bodies were spread throughout the cells treated with auxins (Fig. 39) compared to very few in control (Fig. 38). The LC-MS analysis of the auxin treated samples was performed on samples with auxin concentrations ranging from  $10^{-8}$  M to  $10^{-12}$  M. These samples contained all lipids identified in the control samples. Of the 5 auxin concentrations, samples with  $10^{-8}$  M and  $10^{-11}$  M concentrations contained notable changes in quantity of certain lipids or

displayed novel lipids relative to controls. These lipids are presented in Table 25 and 26.

The determination of these relative changes is discussed subsequently in the methodology. The sample with  $10^{-8}$ M auxin showed increases in FFA and DG. These lipids are generally responsible for energy storage in algae (Hu, et al., 2008; Wang, et al., 2009). The stored lipids also play a secondary role of serving as future precursors to membrane lipids. In our growth rate measurements at this auxin concentration, we observed a decrease in growth by 8% relative to controls. This suggests conditions of stress on the algae, which require storage of energy reserves. The FFA and the DG would serve this role of reserve energy. In addition to these lipids, phosphatidylinositol-(C20:4) (PI) was a novel finding for this auxin concentration. We suspect that the PI may be involved in the previously discussed stress signaling.

On the contrary, in the sample with Auxin concentration of  $10^{-11}$ M, we found increases in lipids associated with cell growth and division. Increases in glycerphospholipids (GP) and FFA were observed in this sample. Due to increased growth at this concentration of auxin, a greater amount of cell lipid resources is diverted to lipids necessary for generation of cell membranes. Such lipids include phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol. Additionally, new lipids included specific GPs of a new carbon chain length and saturation (18:0/20:4). Novel triacylglycerides (TAG) as noted in Table 26, relative to control, were also identified. TAGs are normally present for energy storage. We suspect that these TAGs were not present in control samples

because of effects of auxin on cell lipid metabolism. The auxin activates a G-protein-coupled receptor protein to initiate a cascade of signal pathways. This could lead to activation of lipid biosynthesis of particular TAGs. This is supported by the confocal microscopy imaging of auxin-treated samples at  $10^{-11}$  M. These images qualitatively display increases in lipid bodies which store TAGs for energy reserves. This receptor protein also supports the previously hypothesized multiple pathway activation by auxin. Further work can be conducted to illustrate how auxin affects these pathways.

### **5.3.3 Epibrassinolide**

According to our results, Epibrassinolide best increased the growth rate of algae at a concentration of  $10^{-12}$  M, with a statistically significant increase of  $62.7 \pm 28.1\%$  (Fig. 18). The growth rates decreased at higher concentrations of Epibrassinolide, however, none of the treated samples' growth rates fell below the growth rate of the control. This result indicates that Epibrassinolide increases the growth of algae under all tested concentrations. It activates pathways with similar functions as those initiated by auxin to increase growth rates. At higher concentration, however, Epibrassinolide reduced growth rates closer to the level of the controls. We predict that Epibrassinolide plays similar roles in both algae and higher plants – a possible avenue for future experimentation.

Although every treatment involving brassinosteroids resulted in increased growth rates, the dry algal masses of the samples showed a different trend. When exposed to lower concentrations of Epibrassinolide, the dry algal mass of the samples

was lower than the dry algal masses of the controls. At higher concentrations, however, the dry weights approached the level of the control groups. The largest decrease in dry algal mass was approximately 21%, over controls, and observed at the same concentration of  $10^{-12}$  M which yields the greatest increase in growth rate. This result can be attributed to the fact that when *C. reinhardtii* rapidly divides, the cell volume of each resulting cell may not correspond to the number of cells. Due to the increase in growth rate, the cells do not have the same opportunities to produce the cellular products of interest. Therefore, when evaluating the dry mass of algae, brassinosteroids do not prove to be an effective treatment.

Brassinosteroids generally had negligible effects on specific lipid mass. The greatest increase in specific lipid mass occurred at  $10^{-12}$  M. At this concentration, we observed a 30% increase in specific lipid mass over the control group (Fig. 25). However, given the large variation in our results, it is difficult to assert the validity of this finding. An example of the variation can be observed in the trials testing the effects of the  $10^{-12}$  M concentration of Epibrassinolide. The standard deviation of our data suggests negative mass outcomes. This indicates an outlier that results in this large error. Since the error here is propagated from the error of dry lipid masses and dry algal mass, it is magnified by the calculation. Similar to the large standard deviation of specific lipid weight, the lipid concentration also has a large error in its calculation (Fig. 32). Overall, no significant changes between lipid content of controls and the five Epibrassinolide concentrations were found for lipid concentrations (Fig. 32).

A qualitative evaluation of cells exposed to Epibrassinolide using confocal microscopy revealed an increase in lipid body production. Algae treated with brassinosteroids showed a few (<10) lipid bodies in a concentrated region. Treated algal cells also exhibited high levels of non-fluorescent material within the cell. As the autofluorescence comes from chloroplasts, these voids indicate inactivated chloroplasts. That could occur under transition to heterotrophic growth from a carbon source. In this case, it could be the Epibrassinolide; however, this chemical can only provide  $3.36 \times 10^{-6}$  g of carbon material, not enough to be a reliable source of energy. . This void could, alternatively, be a increase in cellular organelles such as the endoplasmic reticulum.

Samples treated with Epibrassinolide across the concentrations of  $10^{-8}$  M to  $10^{-12}$  M were analyzed by LC-MS. All samples showed lipids characterized for the control samples. The  $10^{-9}$  M Epibrassinolide showed notable changes in lipid composition, presented in Table 27. Increases in FFA and TAG were observed. Additionally, we saw development GPs, including glycerophosphocholine and glycerophosphoinositol. Compared in relation to the observed increase in growth rate relative to control, these identified lipids follow the necessary increase of phospholipids needed for development of new cell membranes for cell division. The FFA and TAG provide energy and the GP precursors provide material for the future membrane phospholipids. These findings are consistent with established features of algal cellular metabolism.

### 5.3.4 Trans-Zeatin

According to our data, increasing the concentrations of trans-zeatin decreases *C. reinhardtii*'s growth rate. The highest decrease in growth rate was found at a concentration of  $10^{-9}$  M with a  $47.6 \pm 38.6\%$  decrease as compared to that of the control (Fig. 19). If we were to continue to increase the concentration of trans-zeatin, it is likely that this pattern would continue and the growth rate would continue to decrease. Our data confirms Trans-zeatin acts as an inhibitory signal. This correlated to the role of cytokinins preventing overgrowth in plants by regulating cell division cycles (Riou-Khamlichi, et al., 1999; Tarakhovskaya, et al., 2007).

Trans-zeatin had an interesting effect on algae morphology. Under this treatment, the algal cells were much more clumped together than under any other treatment. Usually, clumping occurs when algae die. Therefore, it is possible that the Trans-zeatin treatment actually resulted in the death of a portion of our cells. The dry algal mass for Trans-zeatin treatments showed negligible differences in mass compared to controls. The greatest decrease was 4% at a concentration of  $10^{-9}$  M and  $10^{-10}$  M (Fig. 19). This suggests that Trans-zeatin increases the individual cell content of the algae. Despite decreasing in growth rate, Trans-zeatin provided positive results for lipid content changes.

We found that at a concentration of  $10^{-9}$  M trans-zeatin yields the highest specific lipid mass increase, but since the increase in production was only about 2%, it is not significant. At higher concentrations,  $10^{-8}$  M, we found that the Trans-zeatin decreased the amount of algal lipid produced by 33% (Fig. 26). The lipid

concentration across the majority of the treatment concentration spectrum also shows negligible changes relative to control. Only at a concentration of  $10^{-8}$  M did we find a significant decrease  $7.4 \pm 36.7\%$  which corresponds to the decrease in specific lipid mass. At this high Trans-zeatin concentration, the decrease in lipid concentration is likely a product of the stress induced on the algal cells. Overall, these results suggest that Trans-zeatin does not play a role in an individual organism's production of lipids, but negatively influences overall growth rates of algal cultures.

The confocal images of Trans-zeatin treated cells showed these effects. A small amount (<10) of rather large lipid bodies were found (Fig. 41). These large lipid bodies can be attributed to the fact that Trans-zeatin decreases the cell growth rate. It appears that the cells are diverting more of their resources to the buildup of intracellular material as opposed to cell division.

LC-MS analysis of samples treated with all concentrations of Trans-zeatin showed the lipids identified from control samples. Notable changes relative to controls were observed for Trans-zeatin-treated samples at  $10^{-8}$  M (Table 28). Increases in Monogalactosyl-monoacylglycerol, DG, and glycerophosphocholine were observed, but no novel lipids were identified. This particular concentration of Trans-zeatin leads to a suppression of the growth rate relative to control.

Additionally, unusual clumping was observed in the sample flasks for this phytohormone. This observation along with confocal images of Trans-zeatin treated cells suggest that stress was induced upon the algae. The DG is, thus, a product of this stress by allowing storage of energy for the cell. The increase in

glycerophosphocholine is likely also a product of the decrease in growth rate of the cells. The rate of cell division is suspected to decrease under this phytohormone. However, growth in *C. reinhardtii* proceeds by an increase in cell size followed by cell replication and division. Trans-zeatin is suspected to stunt the division of these growing cells. Thus, the amount glycerophosphocholine increases as the cell continues to grow without a trigger for cell division. Further work in delineating the exact mechanism of Trans-zeatin regulation would test this conjecture.

### **5.3.5 Trans-zeatin-riboside**

Trans-zeatin-riboside is another increases growth rates in higher plants, but it also has an inhibitory effect. Our results show that growth rate actually increased at concentration  $10^{-9}$  M Trans-zeatin-riboside (Fig. 20). At this concentration there was a  $31.1 \pm 9.4\%$  increase in growth compared to the control. The application of  $10^{-8}$  M showed a slight decrease compared to the optimal growth rate at  $10^{-9}$  M probably because such a large concentration of cytokinin could activate other pathways responsible for directing nutrients to various pathways or has toxic effects on algae.

Algal clumping was also observed during this treatment, but since the growth rates increased, we believe that it is not because algae were dying. Instead we believe that the clumping observed might be a physiological response to the phytohormone. It is known that cytokinins increase cell division which could result in algal cells dividing in a short time span and clumping together. Even though we did not expect

this cytokinin to exhibit stimulatory effects on our algae it still seems that only a stimulatory pathway was activated. Trans-zeatin, the other cytokinin examined, only exhibited an inhibitory pathway.

Treatments with trans-zeatin-riboside increase both the growth rate as well as dry algal mass. The peaks of these two parameters, however, were observed at different concentrations. Trans-zeatin-riboside caused growth to be the fastest at a concentration of  $10^{-9}$  M, but the greatest increase in dry algal mass of  $38.9 \pm 11.9\%$  found at the  $10^{-11}$  M (Fig. 20). Once again, this illustrates the tradeoff between growth rate and final masses. The cell size increases when division does not occur, so lower growth can accompany larger algal cell mass.

In regards to the specific lipid mass, the most effective concentration of Trans-zeatin-riboside was at  $10^{-10}$  M, at which we observed an increase of 3% relative to controls (Fig. 27). However, at a concentration of  $10^{-8}$  M, we noted a decrease in specific lipid mass by about 23%. Due to the variation in our tested concentrations, we cannot ascertain whether Trans-zeatin-riboside directly affects the production of lipids in algae. Similar to Trans-zeatin, Trans-zeatin-riboside mainly affected the growth of the algae. The lipid concentration increases for  $10^{-9}$  M,  $10^{-10}$  M, and  $10^{-11}$  M Trans-zeatin-riboside by approximately 40-44% relative to control (Fig. 34). This increase is slightly lower than that by an IAA & Trans-zeatin-riboside combination; however, the variance associated with this calculation is lower. Additionally, growth under this treatment is faster. Notably,  $10^{-11}$  M displays the highest increase in growth based on dry algal mass. Since a decrease in specific lipid mass at  $10^{-11}$  M was

negligible compared to control, the increase in growth rate contributed primarily to the increase in lipid concentration. Though the lipid content per cell did not change, the overall lipid content of the culture was higher compared to control. This yield is optimal for biodiesel production and is the most important metric. Based on these observations increases in growth did not compromise lipid content of algal cells, contrary to previous work in nutrient manipulation. This means the production of the final product benefits from both growth rate and total lipid content.

Confocal imaging of Trans-zeatin-riboside treated algal cell shows an equivalent number (~5) of lipid bodies (Fig. 43). However, compared to control cells, these lipid bodies are much larger (Fig. 52). An interesting morphological characteristic is that there appear to be many small black voids within the cell. The circular shapes of these voids were present in all of the images taken of Trans-zeatin-riboside treated samples. These voids are likely to be many small vesicles. Determining the nature of these vesicles could reveal further insights into the effects of the Trans-zeatin-riboside on *C. reinhardtii*.

The LC-MS analysis of Trans-zeatin-riboside treated samples was conducted with concentrations over the range of  $10^{-8}$ M to  $10^{-12}$ M. Notable changes in features of identified lipids were observed at  $10^{-11}$ M, as presented in Table 29. Increases in several types of FFA and DG were found. These findings, as established with previous phytohormones, suggest an increase in storage of energy in the form of lipids. However, as opposed the decrease in growth seen in Trans-zeatin, Trans-zeatin-riboside increases growth relative to controls. Thus, we suspect the cell does

not divert all of its lipid resources to storage, as suggested by the generally negligible changes in specific lipid mass of Trans-zeatin-riboside treated cells. These lines of evidence point to regulation of alternate pathways by Trans-zeatin-riboside, compared to those used by Trans-Zeatin. As a result lipid profiles are consistent with increased cell division and faster turnover of lipids. These include increases in GPs and new detection of sphingolipids. These lipid types are necessary for generation of cell membranes at the higher growth rates. Additionally, isoprenoids, which serve as precursors for antioxidative pigments such as carotenoids of chloroplasts, were found to decrease relative to controls (Thompson, 1996). We suspect the induction of higher rates of cell division depletes these precursors in order to produce the metabolites necessary in basal cell metabolism. Overall, the lipid profile is consistent with cellular metabolic processes under increased growth.

### **5.3.6 IAA & Trans-zeatin**

A combination of IAA and Trans-zeatin had an interesting effect on the growth rates and lipid yields of *C. reinhardtii*. The resulting effects appeared to significantly differ from the characteristics of both the individual treatments of IAA and Trans-zeatin. In terms of growth rate, the algae had two optimal treatments to raise increase growth rates: a treatment of  $10^{-11}$  M of IAA and  $10^{-10}$  M of Trans-zeatin and a treatment of  $10^{-11}$  M of IAA and  $10^{-12}$  M of Trans-zeatin. Both of these combination treatments increased the growth rates by approximately 47% and 45%,

respectively (Fig. 21). There were no significant changes, however, to dry algal mass relative to control (Fig. 21)

Notably, Trans-zeatin at  $10^{-10}$  M and  $10^{-12}$  M alone showed no increase in growth rate. Also, treatments of Trans-zeatin and IAA treatments separately only show minor increases in dry algal mass. However, when *C. reinhardtii* is exposed to a combination of IAA and Trans-zeatin, it shows a greater increase in growth rate and dry algal masses. The presence of two optimal concentrations for this mixed phytohormone treatment reveals that IAA and Trans-zeatin work by mutually regulating pathways. The concentrations of each of these phytohormones have great effects on how they regulate those specific pathways. This is very similar to how auxins and cytokinins in plants regulate multiple different metabolic pathways related to cell division. Small changes in their concentrations can lead to either overgrowth or stunted growth in plants.

The majority of our results indicated a decrease in the specific lipid masses of *C. reinhardtii* (Fig. 28). The only exception to this observation was found at a concentration IAA at  $10^{-11}$  M and Trans-zeatin at  $10^{-9}$  M, at which point there was a 0.1% increase – a negligible change. The IAA-only trials did not exhibit a clear trend for the effects of auxin on specific lipid mass in algae and Trans-zeatin-only trials did not show a clear decrease in specific lipid mass. The decrease in specific lipid mass may then be attributed to cross effects on metabolic pathways. Also, the decrease in specific lipid mass might be the tradeoff the cell must make to divide faster. The lipid concentrations for this combination treatment show significant decreases at all

concentrations except at  $10^{-11}$ M auxin and  $10^{-9}$ M trans-zeatin; for example,  $10^{-11}$ M IAA and  $10^{-10}$ M Trans-zeatin showed a  $28.5 \pm 39.8\%$  decrease (Fig. 35)

LC-MS and confocal microscopy experiments were not run on the mixed treatment algae due to budget constraints. Without complete analysis by these techniques we cannot conclude this combination treatment's effects with complete certainty. Use of these techniques could add to the findings of specific lipid mass and lipid concentrations by showing decreases in lipid types relative to controls. Additionally, noting changes in cellular morphology features would allow visualization of the specific metabolism of *C. reinhardtii*.

### **5.3.7 IAA & Trans-zeatin-riboside**

The combination of IAA and Trans-zeatin-riboside also exhibited mutual effects on the growth and lipid content of *C. reinhardtii*. Like the previous combination phytohormone treatment, a mixture of IAA and Trans-zeatin-riboside resulted in effects that differed from their individual treatments. Individually, IAA and Trans-zeatin-riboside, both increased growth rates by  $52.9 \pm 36.3\%$  and  $31.1 \pm 9.4\%$  respectively. Together, they only increased growth rates by  $11.5 \pm 2.3\%$  at a concentration of  $10^{-11}$  M auxin and  $10^{-12}$  M trans-zeatin-riboside (Fig. 22). It is difficult to determine the reasons for these results since it is counterintuitive to see that while the individual treatments drastically increase the growth rates, the mixture of phytohormones increased the growth rates to a lesser degree. It is possible that other pathways exist to prevent over reproduction of the cells. These pathways could

be activated in response to increased levels of auxin and/or cytokinins. The mixed treatment yielded the greatest increase in dry algal mass of  $1.4 \pm 0.4\%$  when treated at  $10^{-11}\text{M}$  IAA and  $10^{-11}\text{M}$  Trans-zeatin-riboside. This increase was negligible when considering the errors in our measurements.

The combination of IAA and Trans-zeatin-riboside phytohormones showed a significant increase in the production of lipids. At a concentration of  $10^{-11}\text{M}$  IAA and  $10^{-10}\text{M}$  Trans-zeatin-riboside, we observed approximately a 44% increase in specific lipid mass (Fig. 29). The lipid production levels tapered off at the other tested concentration levels of Trans-zeatin-riboside with IAA constant at  $10^{-11}\text{M}$ . Notably, none of the levels indicated a decrease in lipid production relative to controls. Of particular interest is that Trans-zeatin-riboside, individually, did not increase lipid production but rather increased growth rates. The combination of IAA and Trans-zeatin-riboside significantly increased specific lipid mass while only slightly increasing the growth rate of the algae. As a result the lipid concentration increased by approximately  $47.6 \pm 54.5\%$ , which is nearly equivalent to the greatest change of  $44.6 \pm 42.1\%$  for the Trans-zeatin-riboside treatment (Fig. 36). Due to the greater variance in this calculation with respect to the same calculation for Trans-zeatin-riboside treatment, we recommend the latter as a favorable choice for biodiesel production.

Unfortunately, due to budget constraints, we were unable to perform confocal microscopy and LC-MS analysis, which further limits our confidence in the lipid content changes reported by the quantitative measurements. Due to the errors of our

data, visualizing the changes in quantity of lipid bodies would help further explain the underlying effects for lipid concentrations. Additionally, determining the types of lipids would be important towards assessing this treatment's practicality for biodiesel production.

## **5.4 Methodology Analysis**

### **5.4.1 Growth Rates**

In assessing the validity of these findings, the limitations of our methodology need to be addressed. Though we designed our growth and measurement techniques based on established methods in the literature, we found some unexpected features, especially in the growth of the algae. According to the literature, *C. reinhardtii* typically grows at a rate of 1.5 doublings per day (Akimoto et al. 1997). This is characteristic of exponential growth. Our algal system, however, maintained a linear growth rate. We suspect that certain growth conditions may be responsible for the observed linear growth. First, the algae were grown in minimal media without a source of carbon. Thus, the algae relied solely on CO<sub>2</sub> supplied from the air. Though we used a shaker table to aerate the algal flasks, the possibly insufficient amount of CO<sub>2</sub> available may not have allowed the algae to reach their maximum growth potential. Pumping higher concentrations of CO<sub>2</sub> into the growth flasks could eliminate this growth pattern; however, this was beyond our means.

Though our design may limit the total concentration of CO<sub>2</sub> available in the system, the CO<sub>2</sub> is fully accessible from the environment. We use cotton stoppers to establish axenic growth conditions. The combination of the material of the stopper and the use shaker tables allows CO<sub>2</sub> and gases to fully diffuse into the solution. This implies that the generally small changes between growth rates of various cultures are unlikely to be constrained by the CO<sub>2</sub> levels. However, the growth system may be limited by the levels of light provided.

Light is essential to the activation of chloroplast photosystems necessary for photosynthesis and, thus, further growth of the organism. In our design, we chose to use the minimal level of light in order to maintain the basal exponential growth rates. We measured these light levels in units of lux; however, a more applicable measurement of the light level would be photon flux density. Lux is a measurement of the light falling over a given area as perceived by human eyes, while photon flux density measures the actual amount of photons from that light source that strike the subject. As mentioned, we chose 2 klux as the minimal light level; however, it is possible that this level of light did not provide high enough level of photon flux through the system. Thus, the algae grown our system may be limited in their growth by the minimal level of light provided. Adding growth increasing substances under such experimental conditions may limit the observance of actual increases in the growth rate. Additional resources, primarily light, would be required to enact the actual response from these growth increasing signals.

The linear growth was initially a salient feature of our algae. In order to recreate exponential growth as documented in the work of others, we explored various conditions, including light levels, temperature, and alternative inoculation procedures within our monetary limitations. The linear growth pattern, however, remained a constant feature for the algae. Our experiments were broken into 4 repetitions per growth period for a particular phytohormone treatment. As we did 8 total repetitions, the second set of 4 were performed after a month's time. This choice may lend to some of the variability through uncontrollable environmental factors that could change. At the same time, by separating a repeat by this extensive period we can better capture the natural variability of this biological organism. Thus, all findings here originate from algal cultures exhibiting a linear growth. The uncontrolled variables inherent in our particular algal culture system should not invalidate our results. They remain constant through all trials and do not interfere with our analysis of changes to the criteria we examined in the algae.

The linear growth rates actually help in analysis of our data by allowing for clear association between algae growth per unit time and UV absorbance at 670 nm as determined by a point-slope formula from spectrophotometric data. However, the wavelength used to measure algal cell number changes in our study is still in debate. There are currently two different proponents of accurately perform this measurement. One possibility is to use 670nm as the wavelength of choice. Chlorophyll absorbs light at 680nm, thus assuming the chlorophyll content is constant within our experimental conditions and measurement at this wavelength would simultaneously

indicate the number of cells in solution. Alternatively, a measurement at 550nm avoids possible correlations of cell quantity with chlorophyll quantity. This method relies solely on the scattering of transmitted light by the number and size of cells in solution. As we chose to use the former wavelength for growth measurements, it is plausible that our growth rates reflect metabolic changes in the chlorophyll content under influence of phytohormones. For example, Indole-3-acetic acid (IAA) induces changes in nitrogen metabolism. It has been shown that chlorophyll pigment levels are proportional to the nitrogen levels available to an algal cell. If the IAA can influence the nitrogen levels available to the cells, the chlorophyll levels will change will the number and sizes of cells may not. Thus, under such a possibility, the measurement of growth at 670nm would suggest changes in growth though no actual changes to the number of cells occurred. As we use IAA in our study, these suspected effects may compromise the validity of our results for IAA induced changes in growth rate. If similar are also occurring under treatments of the other experimental phytohormones, then by measuring at the 670nm wavelength, our findings of growth are highly influenced by measurement of actual chlorophyll content changes. In observing our pellets of dry algae, we did not observe any noticeable visual differences in intensity of color between various samples with the IAA group and across other treatments.

Additionally, IAA breaks down over time under light. This results in visible change in the color of the compound. We did not observe this change at the low quantities we used in our study. We determined the absorption wavelength of this

degradation product and found that it did not confound our measurements at 670nm. Since we sought to observe the immediate and short-term effects of IAA after algal cells are first exposed to this chemical, this degradation was not of a major concern. For future work, if algae were engineered to inherently regulate and produce an auxin, ideally one would model the effects of an auxin that does not break down under light and can have a longer lasting presence in the external environment of the algal cells.

It is important to keep in mind that these algae are biological systems that cannot be controlled with complete certainty; therefore, differences between trials will occur. Although we cannot conclude whether these results can be applied to algae growing in exponential pattern, our findings support positive effects of phytohormones on the growth rate of *C. reinhardtii*.

Beyond the measurement of growth rates, other procedures may lend to error in our measurements. After growth of an algal sample for 10 days, we measured the total dry mass of the algal samples. The algae were centrifuged and the excess supernatant was discarded. After lyophilization the masses were measured. We suspect that losses due to algal cells remaining in the supernatant were minimal. Care was taken to avoid rupture of cells due to excessive force under centrifugation without compromising the separation and collection of the algal cells from media. Generally, the dry masses trend with spectrophotometric measurements of growth. However, due to the greater variability and previously mentioned concerns with the spectrophotometric measurement of growth rates, we rely more on dry algal masses

as representation so growth. These differences can be attributed to the fact that the spectrophotometer measures cell density as opposed to cell contents. The cell density of the cultures refers to actual number of algae within the solution. The total cell contents of the algae include the lipids, proteins, and organelles within the cell. The data suggests that the differences in cell content play a larger role in determining final mass as opposed to cell density.

In general, it appeared that the phytohormones increased the growth rates of the algae. The logical next step was to identify how the phytohormones impacted lipid production rates. Based on our review of literature on phytohormones, we anticipated that the lipid content of algae would increase without causing deleterious effects upon the algae's growth rates. Due to experimental constraints of our culture system, we were unable to perform analyses on large volumes of algal cultures. This limiting factor made it difficult to precisely quantify lipid concentration with high confidence due to the small sample size.

#### **5.4.2 Lipid Analysis**

Our procedure for extraction of lipids exhibits slight losses. The Soxhlet extraction is up to 90% effective using solvents hexane and isopropanol at an even ratio of 50-50 (Guckert et al 1981). The thimbles used to hold algae in the extraction columns also have inherent inefficiency. According to the manufacturer's specifications (Wyvern Scientific Inc.), the thimbles used during the extraction process retain about 10% of the lipid yields. This resulted in an extraction efficiency

of about 81% for our samples. In order to make valid comparisons across different samples, we normalized our lipid dry masses by factoring in each sample's algal mass in the calculation of lipid concentration. This reduced the degree of variance observed in final lipid accumulation. Although the inefficiency of our extraction apparatus reduced the lipid dry masses, the types of lipids and their relative concentrations should be unaffected.

The result from the confocal microscopy imaging provides an alternative to exact quantification of lipid content of cells. However, certain issues affect the resolution of this technique. The Nile Red dye used for visualization of lipids fluoresces brightly only when in a relatively hydrophobic environment. This limits visualization of lipids to only those found in lipid bodies, which are enclosed by a hydrophobic membrane. Though this can result in incomplete visualization, this characteristic of Nile Red is actually fairly useful because lipids found in lipid bodies are generally only neutral lipids. Neutral lipids are the most important lipids in the production of biofuels because they are most easily transesterified into biofuels. This characteristic also accounts for why the entire cell does not fluoresce even though lipids are clearly prevalent in the plasma membrane and membranes of various organelles. In order to improve the confocal microscopy analysis, the algae should have been analyzed through a wider range of time. For example, being able to track the changes in cellular morphology from the beginning of treatment can reveal when lipid bodies are being produced. Also, determining the changes in cellular

morphology after our 10 days of treatment would help us fully characterize the full effects on the cellular life cycle of *C. reinhardtii*.

The characterization of the lipids found in the control and phytohormone treated algae was performed via LC-MS procedure derived from Plante et al (2006). In our implementation of this procedure we encountered a few sources of ambiguity in the identification of the lipids. This made quantification of the lipids impossible due to uncontrollable properties of the mobile phase solvent. Acetonitrile was chosen as a solvent due to its ability to separate with high resolution. However, due to the absorbance of UV light between 190 - 400 nm for Acetonitrile, a solvent ramp was produced in the absorbance data. This made identification of peaks in the LC-MS chromatogram very difficult. Thus, peaks of lesser intensity could not be identified because they were masked by the solvent ramp. These peaks would be less interesting because they would indicate lower concentration lipids. We sought to analyze the larger effects of phytohormone treatments on lipid metabolism. The peaks of greater intensity than the solvent background were, thus, analyzed. As a result, our analysis may not detail the complete array of lipids in the *C. reinhardtii*.

Simultaneously, we examined the total ion current as a secondary marker for identification of lipids. Using this, we were able to detect lipids in addition to those derived from absorbance measurement. However, similarly to absorbance chromatograms, the ion current chromatograms displayed abundant amounts of noise. The primary reason for this noise is the residual cell products in the samples from the lipid extraction process. This noise also contributes to a lack of a clearly defined

baseline. Without a clear baseline, quantifying the intensity of peaks on the mass spectrum lacks accuracy. As a result of this feature of the chromatograms, comparisons between control and phytohormone-treated samples could only provide a relative comparison of intensity, as the calculation of exact amounts carries a great degree of uncertainty. Further, the software paired with our LC-MS does not calculate integrated peak areas. This contributes to the inability to perform exact quantification lipid amounts after separation.

In analyzing the mass spectrum, we found additional ambiguity in ascertaining the exact structures of the lipids. Difficulties in selecting peaks imply that the precision of the identified  $m/z$  values for lipids may not actually be reliable and a high-resolution identification was not possible. Using these highly precise  $m/z$  values and searching the Nature Lipidomics Lipid Map database and AOCS Lipid Library, yielded no direct matches. We did not use standards in our study and could not verify whether these  $m/z$  values were indeed accurate and precise. By slightly relaxing our search parameters for  $m/z$  values, we were able to identify several isomers of particular lipids. Many of the lipids identified in this manner corresponded to previously reported lipids in *C. reinhardtii*. Therefore, we believe that the relaxed search parameters did not detract from the validity of the original  $m/z$  values. Ideally, we would have used the highly precise  $m/z$  values; however, without standards for calibration and verification, this was not possible. Based on evidence we see in our study, future work could incorporate standards of lipids we have identified that also match those previously reported.

In our results, we do not present all possible isomers and variations, such as length of carbon chains, the saturation, and functional groups. We present only a single isomer of the possible lipids at each m/z value. In certain instances, the matches in the databases indicated alternative structure within  $\pm 0.5$  m/z of identified peaks. These matches were from various different classes of lipids. There is possibility of overlapping m/z peaks for our samples. Two lipids of corresponding masses would thus not be resolved under our methodology. NMR and IR analysis or use of a different solvent along with lipid standards would resolve of these discrepancies.

## 5.5 Economic Analysis

There are many costs associated with producing algal biodiesel. The main contributors are the cost of the media, the phytohormones, the materials, and the energy used for growth and extraction. Table 30 lists the various costs of these inputs within our experimental design for each 150mL experimental culture. For our purposes, the cost of the thimbles was highest due to our need to keep each treatment separate. In the industrial setting, if thimbles were to be used, one thimble could accommodate more algae by at least a factor of 10. Thus to truly asses the cost of our experimental scheme, the cost of the thimble can be ignored.

**Table 30: Costs of growing algae and extracting lipid for our experimental scale.**

<b>Input</b>	<b>Cost per Sample of 150 mL (\$)</b>
Media + Phytohormone	0.02
Extraction Solvents	1.17
Thimble	3.16

Centrifugation	0.02
Lyophilization	0.36
Electricity for Lighting	0.21
<b>Total Cost</b>	<b>4.94</b>
<b>Total Cost without Thimble</b>	<b>1.78</b>

If the costs mentioned above were the cost to produce biodiesel industrially it would cost somewhere in the range of \$5000 to produce one liter of biodiesel (or equivalently \$20000 to produce one gallon of biodiesel) which would not be economically competitive with gasoline. However, the experiments did not take advantage of any economies of scale, where running large-scale facilities would drive down the cost of production.

Currently, several algal biofuel startups have the capability to produce biofuel from algae on a large scale, at a cost of roughly \$33 per gallon before other cost saving measures (Lee 2010). These measures include selling spent biomass as feedstock and building near existing factories for a cheap source of sterile CO<sub>2</sub> and heat (<http://www.worldwatch.org/node/5391>). Taking these factors into account, biodiesel prices can be reduced to the range of \$3.50 per gallon. However, because biodiesel has lower energy content than gasoline, this still translates to a cost of \$150 per barrel of gasoline equivalent, well above the 2011 costs of oil at \$100 per barrel. Also, these industrial prices do not incorporate cost reductions possible by the improvements to algal growth and lipid content seen in our experiment. In the case of Trans-zeatin-riboside, the weight percentage of lipids was determined to be approximately 47% relative to controls. If this gain could be replicated in industrial-scale production, the final cost of biodiesel could be cut significantly.

Another factor that cannot be ignored is algal biodiesel cost subsidy from the US government. An increase in biodiesel use would not only decrease our dependency on foreign nations, but it would also drastically improve our environmental impact. Therefore, it is likely that with enough pressure, the U.S. government could be convinced to offer subsidies to companies producing alternative energy, including those producing algal biodiesel. Currently, oil production is one of the most heavily subsidized industries in the US. The U.S. government offers large tax breaks to oil companies in order to lower the cost of finding and producing oil. According to a 2005 study by the Congressional Budget Office, capital investments such as oil field leases and drilling equipment are taxed at an effective rate of 9 percent, significantly lower than the overall rate of 25 percent at which businesses are generally taxed (Kocieniewski, 2010). This tax break encourages domestic companies to provide the U.S. with oil and decreases American dependency on foreign oil. The U.S. government also currently subsidizes ethanol, offering a tax credit of 45-cents per gallon and a 54-cent tariff. This action indicates the willingness of the government to offer economic incentives for the use of alternative energy. If algal biodiesel were to show promise as an energy source with the potential to substantially replace oil, the U.S. government could halt the subsidization of oil production and instead allocate the same funds towards subsidizing algal biofuel.

In the end, the most likely scenario is that a combination of factors will have to contribute cost reductions to the production process before the increased use of algal biodiesel. Between the rising cost of oil, decreases in algal biodiesel costs, and

government subsidies, there is a real potential for algal biodiesel to become economically viable in the near future.

## 5.6 Summary

The application of phytohormones has an effect on growth rates and lipid yields. Some phytohormones, such as auxins, increase growth rates while others such as trans-zeatin-riboside increase final lipid concentrations. This results in a trade-off between increased growth and increased lipid production based on the choice of phytohormone(s), because increased growth rates can lead to stunted lipid production and vice versa. Therefore the best phytohormone combination is the one that addresses each of these to the best level. In the end, the combination of auxin and trans-zeatin-riboside phytohormones showed the most significant increase in the production of lipids without a loss of growth rates. At a concentration of trans-zeatin-riboside at  $10^{-10}$ M and auxin at  $10^{-9}$ M, we observed a 43% increase in specific lipid mass, with lipid production levels tapering off at the other tested concentration levels. It is important to observe that none of the levels indicated a decrease in lipid production, merely a lower increase. Of particular interest is that individually, auxin and trans-zeatin-riboside did not increase the specific lipid weight, but rather increased growth rates. However, the combination of these phytohormones significantly increased lipid concentrations while only slightly increasing the growth rate of the algae.

The auxin and trans-zeatin-riboside mixture showed the greatest increase in lipid concentration for our solutions. The final concentration of lipid in the mixed treatment of  $10^{-11}$  M auxin and  $10^{-9}$  M trans-zeatin-riboside was  $0.107 \pm 0.039$  g lipid/mL culture, a 22.4% increase in the concentration over the control culture that only yielded 0.083 g lipid/mL culture. This increased concentration was a result of both an increase in growth rates and of lipid production. Although there were higher increases in growth rates and higher increases in lipid yields from other phytohormone treatments, this particular treatment yielded the best balance between growth and lipid production. The auxin and trans-zeatin-riboside mixture provided the best results for an overall increase in lipid yield, and is a promising direction for future research.

## 6. Conclusion

This work began with an idea of linking the regulation of growth and metabolism between algae and plants. In the context of biofuel production, we wanted to investigate whether these phytohormones could increase growth rates and lipid contents of algae. In order to address this leap across evolutionary distance between plants and algae, we began by exploring homology between *A. thaliana* and *C. reinhardtii*. As both are model organisms in their respective fields, we performed a bioinformatics analysis using these two organisms to link known phytohormone pathways of plants to pathways in algae.

This analysis revealed generally weak evidence for homology of the signaling pathways between the organisms. There are a few main issues with our choice of *A. thaliana* as a comparison. *A. thaliana* and *C. reinhardtii* diverged from each other approximately 1 billion years ago. This evolutionary time span of divergence is similar to that of humans and fungi. At this scale, numerous differences are highly likely to have originated. As higher plants continued to evolve, they had to develop more complex signaling methods to direct differentiation in plant tissues. This is the primary function of phytohormones. Even though the algae are single celled organisms, we believe that complex signaling between algal cells may take place. Algae have been found to thrive in a multitude of harsh environments requiring extensive signaling. Even though there are obvious faults associated with our choice

of organisms to compare, we were still able to find homologous regions in genes that we suspect are involved in phytohormone signaling.

Functions of many of these homologous genes have not been elucidated yet. Some matched genes produce hypothetical proteins whose functions have not been fully characterized. To supplement these findings, we reviewed studies which had shown evidence of phytohormone action in algae. Due to time constraints, however, we were unable to complete a thorough bioinformatics analysis. With additional time, we would have chosen a different primary organisms and involved additional organisms for this comparative analysis. Another possible improvement would be able to further analyze some of the matches. For example we found a number of conserved histidine kinase proteins. We should investigate whether these proteins had cytokinin binding domains attached to them. If they did, then that would suggest that they were components of the cytokinin receptor. As time goes on and bioinformatics becomes more ubiquitous as an investigative tool, the level of information available for this analysis will only increase, allowing future researchers to further our findings.

Ultimately, our bioinformatics analysis provided some support for our hypothesis. We found a number of conserved domains involved in phytohormone signaling which proves a certain level of homology between *A. thaliana* and *C. reinhardtii*. Our work would have been strengthened with a more thorough bioinformatics analysis, but the time and skills necessary to complete such an analysis were outside the scope of our undergraduate project. As a result of our analysis in conjunction with literature evidence, we had possible phytohormones to test. We

pushed forward to empirically investigate effects of indole-3-acetic acid (IAA), Epibrassinolide, Trans-zeatin, and Trans-zeatin-riboside on the growth and lipid content of algae. Based on preliminary evidence from these tests, we additionally pursued testing combinations of IAA & Trans-zeatin and IAA & Trans-zeatin-riboside. For each phytohormone we tested concentrations between  $10^{-12}$  M and  $10^{-8}$  M. At these concentrations, we sought to eliminate possible toxic effects of these chemicals and avoid initiating heterotrophic metabolism with these chemicals as the carbon source. Measurements of growth were performed by spectrophotometrically determined growth rates and dry algal mass of cultures. Measurements of lipids were assessed by mass of lipids per gram of algae. Additionally, we calculated the lipid concentration of a culture. This definitive measure simultaneously yields the production capacity and rate of lipids in a culture and is most informative of possible benefits to biodiesel production. The former measurements analyzed individually and then collectively, reveal the effects of the tested phytohormones.

Our most notable result from treatment of all the tested phytohormones was with Trans-zeatin-riboside. This phytohormone yielded an increase growth rate  $31.1 \pm 9.4\%$  over controls at the concentration of  $10^{-9}$  M. At concentration of  $10^{-11}$  M, it yielded a corresponding dry algal mass change of  $38.9 \pm 11.9\%$ . Due to the previously discussed possible sources of error for the growth rate calculation, we rely on the change in dry algal mass as an accurate indicator of effects on growth of *C. reinhardtii*. Thus, we recommend Trans-zeatin-riboside as the phytohormone of choice to improve growth. It should be noted that although Epibrassinolide did show

a greater increase in the measured growth rate at  $62.7 \pm 28.1\%$ , its corresponding dry algal mass change was negligible relative to controls. This suggests that the growth rate may not accurately reflect the actual growth of alga. In evaluating lipid content changes, Trans-zeatin-riboside again shows the greatest increase lipid concentration. Trans-zeatin-riboside at the concentration of  $10^{-11}$  M yielded a  $44.6 \pm 42.1\%$  increase in the lipid concentration of a culture. A similar increase, though slightly lower, was also noted at  $10^{-9}$  M which corresponds to the concentration at which growth rates showed the greatest increase. This correspondence indicates that the phytohormone effects increase along a consistent dose response curve. These findings support that Trans-zeatin-riboside has effects on the metabolism of *C. reinhardtii* and has applications in a biodiesel production system.

The validity of these findings could be further strengthened by implementing methodological changes discussed previously. In our experimental system, all environmental variables are kept constant and axenic culture conditions were attempted as much as possible. The only variable that changes between treatment groups and controls is the type and quantity of phytohormone. Therefore, the effects revealed by our measurements are a result of treatment with the respective phytohormone. However, particular measurements we performed cannot distinguish between primary effects of phytohormone to directly initiate cell signaling or regulatory changes and secondary effects that are consequences of an intermediary process that phytohormones may trigger. After reevaluating the techniques behind

measurements and calculations we performed, we find that the overall changes can be linked to phytohormones only through collective assessment of the respective results.

The growth rates were measured at 670nm as opposed to at 550nm. Due to this choice in wavelength, we are measuring number of cells as a function of chlorophyll content of cells. It is possible that phytohormones we tested affect the synthesis of chlorophyll pigments with single cells. This would imply that chlorophyll content changes, while the number of cells may remain constant. This effect on chlorophyll could possibly be at the root of the discrepancies between measurements of spectrophotometrically determined growth rate at 670nm and growth rate determined by mass. If this effect does indeed occur, then spectrophotometry techniques could be highly suited for investigation of metabolism effects if various wavelengths could be attributed to specific contents or metabolites. Due to these possible confounds, we do not rely heavily on the measurement of growth from this spectrophotometrically derived growth rate. Instead, we give more weight to the measurement of growth as final dry algal mass of a sample

The dry algal masses reflect changes in total number of cells and their sizes. Thus, if the rate of cell doublings were to increase, the increased final amount of cells would be directly reflected in changes in dry algal masses for treatments relative to controls. We find that in some phytohormone treatments, the dry algal masses do not deviate significantly from those of controls. This indicates that those particular treatments do not produce primary or secondary effects to yield growth changes. The treatments that do indeed show changes relative to controls can, as result, be linked to

actual effects of the respective phytohormone. Additionally, these highly distinguishable changes in dry algal mass were observed under the possible constraints on light we introduced due our experimental design. This indicates that this flaw in growth conditions did not restrict observation of phytohormone effects. Greater changes in growth could have possibly been realized under greater photon flux across cultures.

Having assessed changes in growth, we wanted to determine effects of phytohormones on cell metabolism. The measurement of lipid concentration begins this assessment by quantifying changes to the lipid content of cultures. This calculation inherently accounts for dry algal masses and, intuitively, could only show its multiplier effect on the lipid content of a culture. However, in comparing the specific lipid masses to the corresponding treatment's dry algal mass, one can see the two generally trend together. This indicates that the changes in lipid content of cells at least in part contribute to the changes observed to the biomass measured by dry algal mass. Therefore, the reported changes in lipid concentrations reflect actual effects of phytohormones on the metabolic processes of the cell.

Analyzed individually and then collectively, these three quantitative measurements cannot distinguish between primary and secondary effects of the respective phytohormones. In terms of growth, we cannot ascertain whether the phytohormone has directly acted on a signaling pathway or has initiated a non-specific cellular response. Despite this, we do find increases in biomass of cultures. The biofuel production process places high value on improving biomass yields. Doing

so implies that more algae are available from which to extract lipids for biodiesel. The phytohormone treatment of Trans-zeatin-riboside, a cytokinin, achieves this improvement in the total biomass.

Similar to the growth measurements, the lipid measurements cannot distinguish between primary or secondary effects of phytohormones to bring about the observed changes. The observed increases in lipid contents could be due to a direct effect on another component of cell metabolism that then shifts lipid metabolism. Further evidence of the means of phytohormone action can be delineated from intracellular changes. Sensitive techniques such as confocal imaging and LC-MS were used to try to differentiate the mode of phytohoromone action.

Using Nile Red dye with confocal imaging, we were able to identify lipid bodies in sample cells. As this dye binds only to regions of hydrophobicity, we were limited to visualizing only lipid bodies. Additionally, we were unable to quantify the lipid bodies in the samples due to limits on our time. Freeware software, such as ImageJ (NIH), can process images for closely approximating visual changes. For our purposes, qualitative analysis suffices. In comparison of the treatment samples and controls, striking differences in lipid bodies between the two groups are observed. Generally, all phytohormone treatments significantly increase lipid bodies in cells relative to control. The addition of phytohormones has influence on the metabolism of cells, especially for lipids.

As with our previous measurements it remains unclear whether the phytohormones are directly acting on signaling or regulation pathways of metabolism

or the effects are through a secondary process. Treatments of Trans-zeatin-riboside and Epibrassinolide, particularly, reveal areas that diminish in autofluorescence from chloroplasts. It is plausible that these phytohormones are actually inducing a stress response in the algae that results in inactivation of chloroplasts and storage of energy as lipids of these lipid bodies. Similar effects of increased inactive chloroplasts are seen when a mixotrophic alga is grown heterotrophically. Due to available fixed carbon, the chloroplasts are less necessary and the cell favors utilizing available carbon sources. This effect cannot be distinguished from the collected measurements. The confocal imaging, however, does illustrate that all phytohormone treatments dramatically increase the number of lipid bodies, suggesting that direct effects may occur.

Examining lipids closely using LC-MS reveals increases and decreases in particular types of lipids and new lipid types, reported relative to controls. Paired with confocal imaging, this LC-MS analysis indicates that metabolic changes do indeed occur under treatments of the phytohormones. As we previously stated, however, we had to relax our search parameters when identifying raw  $m/z$  values from the high-resolution output of LC-MS due to resolution reduction from our selected solvent. Even under these conditions, many of the lipids we identify correspond directly to those previously documented from algae under similar analysis of *C. reinhardtii*. Thus, the reported changes in lipid types under our relaxed search parameters carry validity. The effects of phytohormones, regardless of the possible intermediary process, do result in conversion of lipid types to desirable diacylglycerides or

triacylglycerides or increased synthesis of new lipids. Additionally, the artifact of a solvent front does not compromise our results. The solvent front masks lipids of lower concentrations; whereas, we are interested in larger changes in the categorized lipids types. These observations corroborate the evidence of increased evidence lipid concentrations for Trans-zeatin-riboside. Actual changes in the metabolism of the cell are observed under treatment of this phytohormone.

On the whole, our measurements do confirm phytohormone effects on growth and lipid metabolism of *C. reinhardtii*. Though our experimental design and measurements could be more robust with considerations detailed in this critique, additional techniques drawn from fields of biochemistry and molecular biology would be necessary to characterize the exact mechanism of phytohormone actions. For our purposes, we sought to determine effects of phytohormones in the context of biodiesel production. To this end, improving the efficiency of the biodiesel production process begins with increasing the quantity of lipids from a culture and how quickly that can be repeated. Our findings indicate an increase in the biomass of cultures under treatment of phytohormone, particularly Trans-zeatin-riboside. a cytokinin.

Additionally, there are changes in the metabolism of lipids leading to greater quantity desirable lipids being produced, particularly for Trans-zeatin-riboside. Taken together, this implies both an increase in lipid product and rate at which it can be produced relative to controls. This suggests that treatment of phytohormones is capable of bypassing the compromise between growth rates and lipid content that has

plagued previous work in improving biofuel yields from algae. The most promising manipulation of algae, thus far, has been conducted by manipulating the nutrients given to the algae. However, this manipulation generally results in increases to one of the efficiency factors, either growth rates or lipid metabolism. The mechanism of Trans-zeatin-riboside action, on the other hand, can increase both factors. Before implementing this, however, characterizing this exact mechanism is important to the eventual application at an industrial level.

Future work must extend upon these findings by determining whether the phytohormone affects growth and metabolic process directly and indirectly. Our bioinformatics analysis has not conclusively established homology between phytohormone pathway genes. Further the evolutionary distance between *C. reinhardtii* and multicellular plants may imply a lack of functional proteins that can directly interact with phytohormones. The next best possibility of phytohormone action may be a stress response. Thus, next steps can be taken by comparing gene expression of *C. reinhardtii* with that produced by other known stressors. Additionally, gene expression can be analyzed for genes known to be involved in stress signaling pathways. Alternatively, molecular biology techniques can be used to determine proteins activated directly by phytohormones. Findings from this future work could then be efficiently translated to biodiesel production by genetic engineering to artificially activate genes that respond to the phytohormones. In this manner costs of phytohormones can be converted to one-time costs of engineering the algae.

Beyond this investigation, other aspects of this complete process can be explored as well. We began testing combinations of phytohormones based on our preliminary results. There is some evidence in our measurements of additional simultaneous increases in growth rates and lipid contents, in the case of the IAA & Trans-zeatin combination. As an example, comparing the dry algal masses of IAA and Trans-zeatin individually and then in combination, interesting effects are noted. IAA alone showed negligible change, while Trans-zeatin decreases the dry algal mass. Added together, however, they have an effect of increasing the dry algal mass. This suggests possibility of interlinked processes that can be further explored. Additionally, as we noted maximum increases in spectrophotometric growth rates from Epibrassinolide, a combination of this hormone with Trans-zeatin-riboside should be examined.

Secondly, the method of identification and quantification of lipid classes must be perfected by combining the LC-MS with analysis via NMR and IR spectroscopy. This would eliminate the ambiguity in identification of the exact lipid molecules present in algal samples. High-resolution identification of lipids in algae would then be possible and provide a global view of algal metabolism. Third, the application of phytohormone must be scaled up for mass production. It is possible that the changes we observed at the experimental scale may not contribute practically at the industrial level. Lastly, costs of needed materials must be reduced and the process must be streamlined through the use of better instrumentation and tested with industrial scale growth methodologies. Investigation of these recommended areas could lead to

development of a renewable and cost-effective liquid fuel that can eventually replace petroleum fuels.

Past research in algal biofuel has centered on lowering its production costs through genetic modifications or optimizing the growth conditions to increase the rate at which the algae grows and produces lipids, the component from which biodiesel is derived. However, due to time and monetary constraints, our research focused on a novel way of increasing the economic viability of producing biodiesels from algae by treating cultures with phytohormones. Our findings suggest algal biodiesel has potential to serve as the alternative to current fossil fuel derived energy paradigms. We hope our work make a small contribution to this pressing sector of our political and societal economies.

Looking back at our experience in conducting this research under the Gemstone Program at the University of Maryland, we have all learned extensively about the techniques and processes of designing and analyzing experiments. Our experience working together has been an excellent exercise in the benefits and troubles with team work. We have learned how to divide work among a group of fourteen people coming from diverse academic backgrounds and how to assign the right tasks to the right people. We have learned to come together and solve some immense challenges to our work along the way. Having little prior knowledge of this field, we have, under the guidance of our mentor, designed the experiments of this study by: identifying a problem, evaluating the extent of the problem, understanding

the impact of a solution, researching protocols, devising adapted and novel protocols, and analyzing collected data. In hindsight of this completed process, there are several improvements we could now make to the design of this study to improve.

First, by looking at a phylogenetic tree during our literature review, we may have been able to pick a better candidate organism that is closer to *A. thaliana* than was *C. reinhardtii* or a plant that was closer to *C. reinhardtii* than was *A. thaliana*. The closer relationship would have yielded better understanding of homology between phytohormone signaling pathways possible in algae as compared to those in plants. Second, we should have contacted more experts in the field early into our design process, so that we would have been able to gather more up-to-date knowledge about the research and relevant experimental techniques. Finally, we could have begun our data analysis sooner during the experimental process. If the growth trials had been run simultaneously, as mentioned, we would have naturally had more time to thoroughly examine and analyze our data.

Beyond these lessons, the most important skill learned during this process was the importance of time and budget management, which has been critical during the various parts of the extended experimentation processes. Though we have had our share of management and scholastic problems throughout this process, the quality of the firsthand experience gained has been invaluable in helping us undergraduates grasp the rigor of the scientific community.

## 7. Appendix

### A.1 Data from Growth Trials

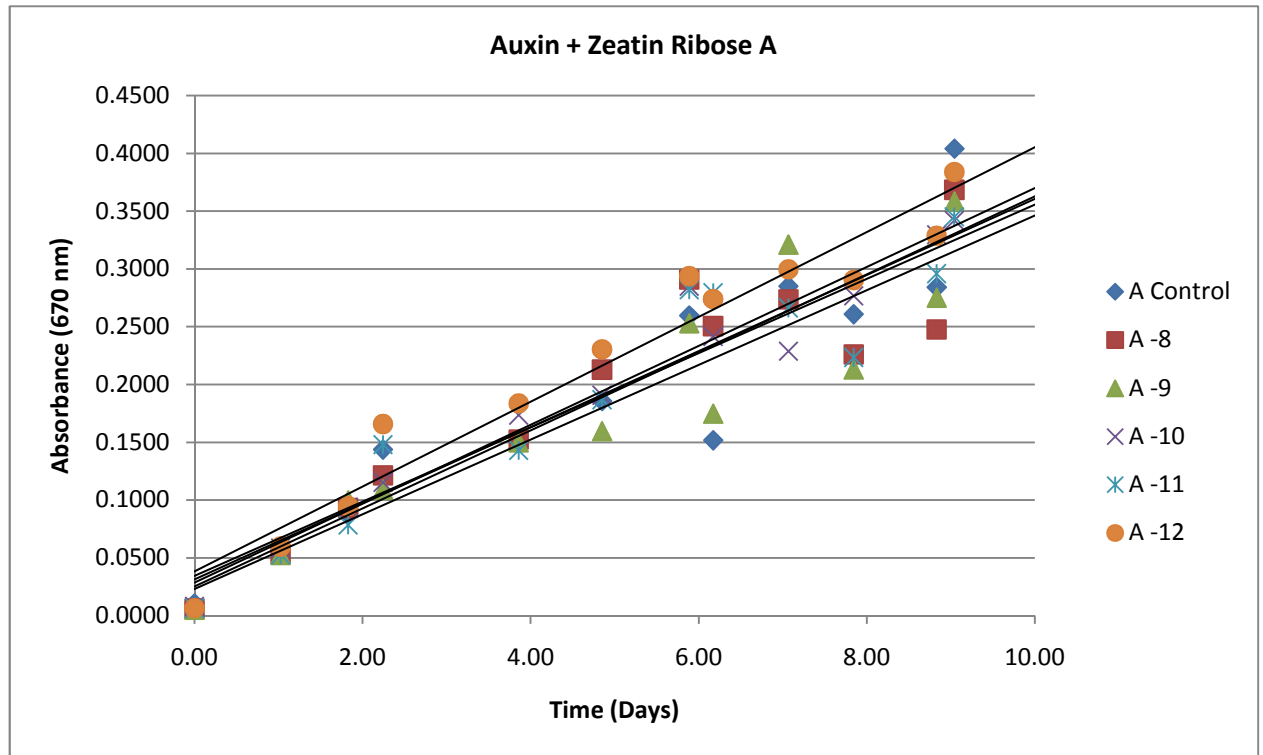


Figure 60: An example of the growth data for one concentration of auxin for one trial over the span of ten days

### A.2 Linear Regression

Table 31: This is an example of Absorbance readings for a control sample that are then used to calculate the growth rate.

Time Elapsed (Days)	A Control
0.00	0.010606
1.02	0.059986
1.83	0.088496
2.24	0.143687
3.86	0.150201
4.85	0.185565
5.89	0.259671

<b>Time Elapsed (Days)</b>	<b>A Control</b>
6.17	0.151704
7.07	0.285089
7.84	0.260834
8.83	0.284181
9.04	0.404050

Linear regression analysis of the data was performed by Microsoft Excel 2007 functions: =LINEST()

Slope data was averaged across all trials of the same concentration of the same phytohormone through the following function:  
=AVERAGE()

Error was calculated through the standard deviation using the following function:  
=STDEV()

### **A.3 Algal Mass**

The algae were then lyophilized and the dried product was massed. The masses measured in trials for the same concentration of the same phytohormone were measured and their experimental uncertainty was calculated through the standard deviation as in section A.2.

### **A.4 Lipid Extraction**

The dried algae underwent a Soxhlet extraction method and lipid yield was calculated by measuring the mass change. The average mass of lipid extracted was taken with the standard deviation as the experimental uncertainty as in section A.2.

### **A.5 Specific Mass of Lipid**

The specific mass of lipid was calculated by dividing the mass of lipid extracted by the mass of algae used in the extraction. The error was propagated through the following equation:

$$E = V \sqrt{\left(\frac{a}{A}\right)^2 + \left(\frac{b}{B}\right)^2}$$

Where V is the value calculated, a is the error in mass of algae, A is the mass of algae, b is the error in mass of lipid, and B is the mass of lipid.

## A.6 Lipid Concentration

The lipid concentration was defined as the mass of lipid generated per liter of media every 10 day period. It was calculated through the following equation:

$$\text{lipid concentration} = \left(\frac{M_T}{M_E}\right)(M_L) \div (V_C)$$

As described in the methodology,  $M_T$  is the total mass of algae,  $M_E$  is the mass of algae used in the extraction,  $M_L$  is the mass of lipid extracted, and  $V_C$  is the volume of the culture, which in our experiment was 0.150 L.

## A.7 M-File for T-test

```
%% Load the .mat file called t-test arrays.
%% Growth mass
for i=1:5
    [h_growth_aux(i) p_growth_aux(i)]=ttest2(growth_con,growth_aux(:,i),[],'left');
    [h_growth_brass(i) p_growth_brass(i)]=ttest2(growth_con,growth_brass(:,i),[],'left');
    [h_growth_zea(i) p_growth_zea(i)]=ttest2(growth_con,growth_zea(:,i),[],'left');
    [h_growth_zr(i) p_growth_zr(i)]=ttest2(growth_con,growth_zr(:,i),[],'left');
    [h_growth_aux_zea(i) p_growth_aux_zea(i)]=ttest2(growth_con,growth_aux_zea(:,i),[],'left');
    [h_growth_aux_zr(i) p_growth_aux_zr(i)]=ttest2(growth_con,growth_aux_zr(:,i),[],'left');
end
%% Extraction Mass
for i=1:5
    [h_ex_aux(i) p_ex_aux(i)]=ttest2(ex_con,ex_aux(:,i),[],'left');
    [h_ex_brass(i) p_ex_brass(i)]=ttest2(ex_con,ex_brass(:,i),[],'left');
    [h_ex_zea(i) p_ex_zea(i)]=ttest2(ex_con,ex_zea(:,i),[],'left');
    [h_ex_zr(i) p_ex_zr(i)]=ttest2(ex_con,ex_zr(:,i),[],'left');
    [h_ex_aux_zea(i) p_ex_aux_zea(i)]=ttest2(ex_con,ex_aux_zea(:,i),[],'left');
    [h_ex_aux_zr(i) p_ex_aux_zr(i)]=ttest2(ex_con,ex_aux_zr(:,i),[],'left');
end
%% Lipid Mass
for i=1:5
```

```

[h_lip_aux(i) p_lip_aux(i)]=ttest2(lip_con,lip_aux(:,i),[],'left');
[h_lip_brass(i) p_lip_brass(i)]=ttest2(lip_con,lip_brass(:,i),[],'left');
[h_lip_zea(i) p_lip_zea(i)]=ttest2(lip_con,lip_zea(:,i),[],'left');
[h_lip_zr(i) p_lip_zr(i)]=ttest2(lip_con,lip_zr(:,i),[],'left');
[h_lip_aux_zea(i) p_lip_aux_zea(i)]=ttest2(lip_con,lip_aux_zea(:,i),[],'left');
[h_lip_aux_zr(i) p_lip_aux_zr(i)]=ttest2(lip_con,lip_aux_zr(:,i),[],'left');

end
%% Growth Rate
for i=1:5
    [h_rate_aux(i) p_rate_aux(i)]=ttest2(rate_con,rate_aux(:,i),[],'left');
    [h_rate_brass(i) p_rate_brass(i)]=ttest2(rate_con,rate_brass(:,i),[],'left');
    [h_rate_zea(i) p_rate_zea(i)]=ttest2(rate_con,rate_zea(:,i),[],'left');
    [h_rate_zr(i) p_rate_zr(i)]=ttest2(rate_con,rate_zr(:,i),[],'left');
    [h_rate_aux_zea(i) p_rate_aux_zea(i)]=ttest2(rate_con,rate_aux_zea(:,i),[],'left');
    [h_rate_aux_zr(i) p_rate_aux_zr(i)]=ttest2(rate_con,rate_aux_zr(:,i),[],'left');

end

```

## A.8 T-test results for all treatments and all metrics

```

>> p_growth_zr
p_growth_zr =
    0.0894    0.0170    0.1514    0.0016    0.1628

>> p_rate_zr
p_rate_zr =
    0.0020    0.0000    0.0001    0.0096    0.2169

>> p_growth_aux_zr
p_growth_aux_zr =
    0.0001    0.0003    0.0024    0.0000    0.0014

>> p_ex_aux_zr
p_ex_aux_zr =
    0.0909    0.1881    0.5037    0.0101    0.0889

>> p_ex_aux_zea
p_ex_aux_zea =
Columns 1 through 3
    0.0139    0.0113    0.0126
Columns 4 through 5
    0.0185    0.0026

```

```

>> p_growth_aux_zea
p_growth_aux_zea =
  Columns 1 through 3
    0.0015  0.0038  0.0004
  Columns 4 through 5
    0.0145  0.0001

>> p_rate_aux_zr
p_rate_aux_zr =
  Columns 1 through 4
    0.0275  0.0190  0.0432  0.0370
  Column 5
    0.0037

>> p_lip_zr
p_lip_zr =
  Columns 1 through 3
    0.1146  0.0959  0.0030
  Columns 4 through 5
    0.0221  0.0077

p_growth_aux =
    0.8765
    0.5705
    0.9342
    0.7190
    0.5549

p_ex_aux =
    0.8462  0.8493  0.9198  0.7764  0.9725

p_lip_aux =
    0.5832  0.2279  0.1819  0.6209  0.7762

p_rate_aux =
    0.9620  0.9998  0.9618  0.1645  0.9873

p_growth_brass =
    0.8786  0.9248  0.9987  0.9981  0.9945

p_ex_brass =
    0.8074  0.7938  0.8887  0.7699  0.8917

```

p\_lip\_brass =  
0.9751 0.9145 0.8564 0.8327 0.7922

p\_rate\_brass =  
0.9840 0.9779 0.8604 0.9875 0.5781

p\_growth\_zea =  
0.8724 0.9309 0.9380 0.8232 0.6694

p\_ex\_zea =  
0.2265 0.4802 0.4380 0.6026 0.7902

p\_lip\_zea =  
0.9366 0.3706 0.3349 0.7069 0.8078

p\_rate\_zea =  
0.9944 0.9962 0.9549 0.8347 0.6963

p\_growth\_zr =  
0.0894 0.0170 0.1514 0.0016 0.1628

p\_ex\_zr =  
0.1507 0.5050 0.2405 0.1832 0.1093

p\_lip\_zr =  
0.1146 0.0959 0.0030 0.0221 0.0077

p\_rate\_zr =  
0.0020 0.0000 0.0001 0.0096 0.2169

p\_growth\_aux\_zea =  
0.0015 0.0038 0.0004 0.0145 0.0001

p\_ex\_aux\_zea =  
0.0139 0.0113 0.0126 0.0185 0.0026

p\_lip\_aux\_zea =  
0.9737 0.7479 0.9991 0.9845 0.8972

p\_rate\_aux\_zea =  
0.6933 0.6885 0.8464 0.0943 0.7053

```
p_growth_aux_zr =
    0.0001  0.0003  0.0024  0.0000  0.0014
```

```
p_ex_aux_zr =
    0.0909  0.1881  0.5037  0.0101  0.0889
```

```
p_lip_aux_zr =
    0.3537  0.1142  0.3883  0.1974  0.5394
```

```
p_rate_aux_zr =
    0.0275  0.0190  0.0432  0.0370  0.0037
```

## A.9 ANOVA sample code for calculation

```
%ANOVA/MULTIPLE COMPARISON
```

```
close all
clear all
diary on
fid = fopen('no_applications.txt','w');
```

```
% Growth Rates
```

```
ex_aux_zea_c = [0.016      0.019  0.021  0.017];
ex_aux_zea_8 = [0.015      0.024  0.013  0.017];
ex_aux_zea_9 = [0.020 0.015  0.002  0.017];
ex_aux_zea_10 = [0.017      0.013  0.019  0.02];
ex_aux_zea_11 = [0.018      0.016  0.018  0.015];
ex_aux_zea_12 = [0.016      0.024  0.02   0.016];
%*****
```

```
Data = [ex_aux_zea_c, ex_aux_zea_8, ex_aux_zea_9, ex_aux_zea_10,
ex_aux_zea_11, ex_aux_zea_12 ];
Makes = {'ex_aux_zea_c','ex_aux_zea_c','ex_aux_zea_c','ex_aux_zea_c',...
'ex_aux_zea_8','ex_aux_zea_8','ex_aux_zea_8','ex_aux_zea_8',...
'ex_aux_zea_9','ex_aux_zea_9','ex_aux_zea_9','ex_aux_zea_9',...
'ex_aux_zea_10','ex_aux_zea_10','ex_aux_zea_10','ex_aux_zea_10',...
'ex_aux_zea_11','ex_aux_zea_11','ex_aux_zea_11','ex_aux_zea_11',...
'ex_aux_zea_12','ex_aux_zea_12','ex_aux_zea_12','ex_aux_zea_12'};
```

```
[p,a,s] = anova1(Data,Makes)
```

```
fprintf(fid,'p (Number of Applications, procedure: 1-way ANOVA) = %9.5f \n\n',p);
```

```

%% Tukey-Kramer.
%From the above, you should be able to tell if there is a difference between growth
rates at the
%95% confidence level. If the growth rates turn out to be different, i.e., p<0.05, use
%multiple comparisons using the Tukey-Kramer procedure below.
%Otherwise, they are not statistically different and it's done.

%*****
if p<0.05
figure
[c,m,h,nms] = multcompare(s,'ctype','tukey-kramer','alpha',.05)
temp1=['=' '=' '='];
temp2=[1 2 3];
[nms num2cell(temp1) num2cell(temp2)]
disp('Pairwise intervals are: ')
c %This gives the pairwise intervals along with the mean difference
end
diary off
fclose(fid)
%You should be able to see which value is different (at the 95% confidence level)
%from above.

```

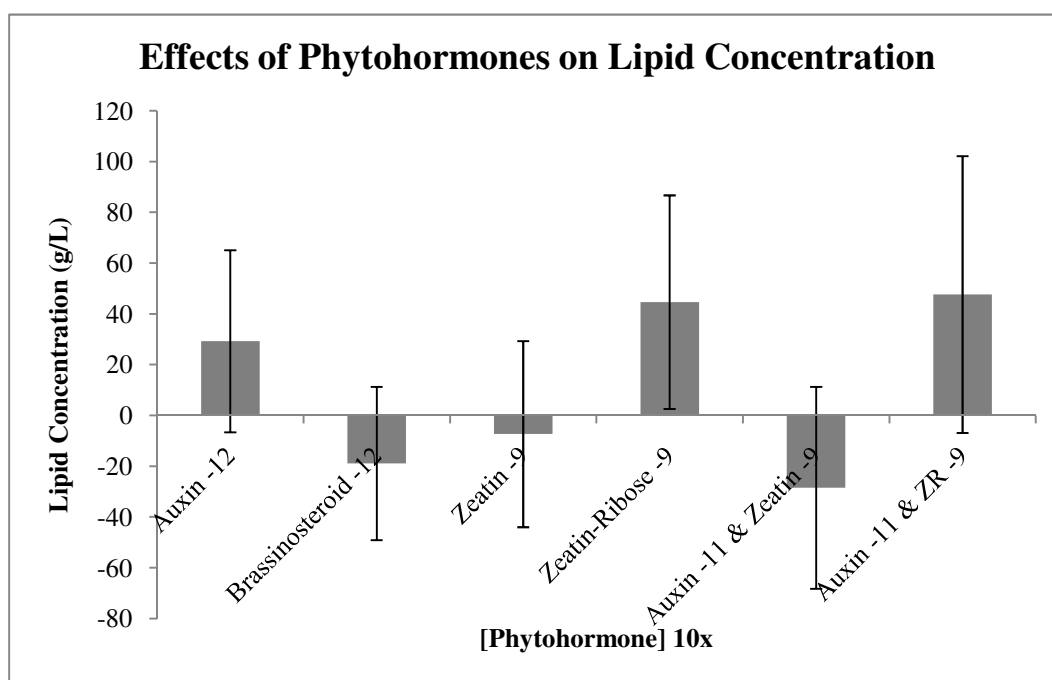
## A.10 ANOVA p-values from all treatments and all metrics

Phytohormone and Metric	P-value	Statistically significant groups based on 10 <sup>x</sup> M Treatment
Auxin: growth rate	0.0064	11 is statistically higher than 9
Auxin: mass of growth	0.84	
Auxin: mass of extracted algae	0.79	
Brassinosteroid: growth rate	0.0122	12 is statistically higher than 11 and CONTROL
Brassinosteroid: mass of growth	0.41	
Brassinosteroid: mass of extracted algae	0.999	
Zeatin: growth rate	0.0168	Control is statistically higher than 8 and 9

Phytohormone and Metric	P-value	Statistically significant groups based on 10 <sup>x</sup> M Treatment
Zeatin: mass of growth	0.64	
Zeatin: mass of extracted algae	0.93	
Zeatin-ribose: growth rate	0.484	
Zeatin-ribose: mass of growth	0.17	
Zeatin-ribose: mass of extracted algae	0.48	
Auxin and Zeatin: growth rate	0.0214	10 and 12 are statistically higher than 9
Auxin and Zeatin: mass of growth	0.478	
Auxin and Zeatin: mass of extracted algae	0.606	
Auxin and Zeatin-ribose: growth rate	0.558	
Auxin and Zeatin-ribose: mass of growth	0.7288	
Auxin and Zeatin-ribose: mass of extracted algae	0.4027	
^^ Growth Metrics    Lipid Metrics v v		
Auxin: mass of extracted lipids	0.53	
Auxin: lipid mass/total mass	0.9	
Brassinosteroid: mass of extracted lipids	0.73	
Brassinosteroid: lipid mass/total mass	0.47	
Zeatin: mass of extracted lipids	0.28	
Zeatin: lipid mass/total mass	0.79	
Zeatin-ribose: mass of extracted lipids	0.13	

Phytohormone and Metric	P-value	Statistically significant groups based on 10 <sup>x</sup> M Treatment
Zeatin-ribose: lipid mass/total mass	0.0125	9 is statistically higher than 8 and 12
Auxin and Zeatin: mass of extracted lipids	0.5577	
Auxin and Zeatin-ribose: mass of extracted lipids	0.8457	

### A.11 Comparison of changes in lipid concentration of treatments



**Figure 61: Changes in lipid concentrations under treatments of phytohormones relative to controls. Though the errors in the calculations are large, the effects are supported by confocal images of lipid bodies. Trans-zeatin-riboside shows the most consistent and effective increase of  $44.6 \pm 42.1\%$ . Support for effects of combination of IAA & Trans-zeatin-riboside is weaker due to larger errors and unavailable confocal imaging and LC-MS analysis. This combination should be investigated in future work.**

**Table 32: Percent changes in lipid concentration for phytohormone treatments.**

<b>Phytohormone(s) Treatment</b>	<b>Lipid Concentration Change relative to controls (%)</b>
<b>IAA</b>	$29.2 \pm 35.8$
<b>Epibrassinolide</b>	$-18.9 \pm 30.2$
<b>Trans-zeatin</b>	$-7.4 \pm 36.7$
<b>Trans-zeatin-riboside</b>	<b><math>44.6 \pm 42.1</math></b>
<b>IAA + Trans-zeatin</b>	$-28.5 \pm 39.8$
<b>IAA + Trans-zeatin- riboside</b>	$47.6 \pm 54.5$

## 8. Glossary

**Aliquot:** A portion of a total amount of a solution

**Abscission:** The shedding of leaves in mature plants

**Autotrophy:** the ability to be self-sustained by producing food from inorganic compounds that are oxidized directly without sunlight to yield energy

**Basic Local Alignment Search Tool for Proteins (BLASTP):** Compares presence of query amino acid sequences to the genome of an organism to find close matches

**Bit Scores:** A number that gives an indication of how statistically significant the gene alignment of two proteins is; the higher the score, the better the alignment

**Biomass:** Quantity of biological material available for use

**Combustion:** a process in which a substance reacts with oxygen to give heat and light

**Comparative genomics:** The study of relating gene sequences between organisms

**Concentration:** A relative amount of substance within another substance

**Cytotoxic:** Toxic to cells

**Diterpenoid/Sesquiterpenoid:** Naturally occurring polycyclic molecular structures

**E-Value:** A statistical measure of match between queried sequence of genome to database genome; this value indicates the probability of a random sequence also making an identical alignment with the match.

**Hemocytometer:** A device originally designed for the counting of blood cells that is now also used to count other types of cells as well as other microscopic particles.

**High Performance Liquid Chromatography (HPLC):** A type of column chromatography used to identify and quantify compounds.

**HPLC-Electrospray Ionization-Time of Flight-Mass Spectrometry (HPLC-ESI-TOF-MS):** A analytical technique which pairs an HPLC to mass spectrometry to determine the exact chemical composition of a mixture. The ESI refers to the

injection method of samples into the MS. The TOF refers to the mode of identification by the MS.

**Homologous:** Similar in function or characteristics between two organisms

**Hormone:** An intercellular chemical messenger

**Hydrophilic:** Soluble in water

**Intracellular receptors:** Receptors located within a cell

**Lipophilic:** Soluble in lipids

**Lyophilization:** Also called freeze-drying, this is a dehydration process typically used to preserve a perishable material or make the material more convenient for transport.

**Ortholog:** either of two or more homologous gene sequences found in different species

**Permeability:** the property of something that can be pervaded by a liquid (as by osmosis or diffusion)

**Petri Dish:** A shallow dish used to grow microorganisms

**Phosphorylation:** the process of transferring a phosphate group from a donor to an acceptor; often catalyzed by enzymes

**Photobioreactor:** a bioreactor which incorporates some type of light source to provide photonic energy input into the reactor.

**Phycology:** Study of algae

**Phytohormone:** A plant hormone

**Transesterification:** the process of exchanging the alcohol group of an ester compound with a smaller alcohol in order to produce a single hydrocarbon chain from a compound containing multiple such chains; catalyzed by the addition of an acid or base

**Transformation:** the genetic alteration of a cell resulting from the uptake, genomic alteration, and expression of foreign genetic material (DNA)

**Transmembrane protein channels:** Proteins embedded in the cell membrane that facilitate the exchange of materials across the membrane

**Slurry:** to transport a mixture of a liquid and a non-soluble compound

**Spectrophotometry:** A quantitative analysis procedure to determine concentrations of solutions through the absorption of monochromatic light

**Stomata:** Pores located on the surfaces of plants that allow the exchange of compound with the plant's environment

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