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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: auxin, brassinolide, trans-zeatin, and trans-zeatin-riboside. These were systematically introduced to algae at a range of concentrations. Auxin, brassinolide, and trans-zeatin-riboside increased algal growth rates at concentrations of 10^{-8} M to 10^{-12} M. A combination of auxin with trans-zeatin-riboside yielded an increase growth rate of 11%, a specific lipid mass increase of 51%, and, most notably, a lipid concentration increase of 33%. We did not find statistical significance ($n = 8$; $p > 0.05$) in the above changes due biological variance and human error. However, these findings are consistent in each trial. Therefore, we believe that a treatment of auxin at 10^{-11} M and trans-zeatin-riboside at 10^{-9} M yield practical benefits to biodiesel production.

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APPLICATION OF SELECTED PHYTOHORMONES ON *C. REINHARDTII*
METABOLISM

By

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requirements for the Gemstone Program

2011

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Dedication

We would like to dedicate this thesis to the University of Maryland, for helping us to grow and learn 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, as will be discussed later.

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 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 the 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 metabolites 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. 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 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 posited 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 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. 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

The research conducted by Genes to Fuels was guided by the following research 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* similarly 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 crude oil products. Traditionally, these have included fossil fuels such as coal, natural gas, and petroleum. However, a major problem associated with fossil fuels is their non-renewability. Currently world energy needs are met by fossil fuels, 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 and 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 (EIA Energy Outlook 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.

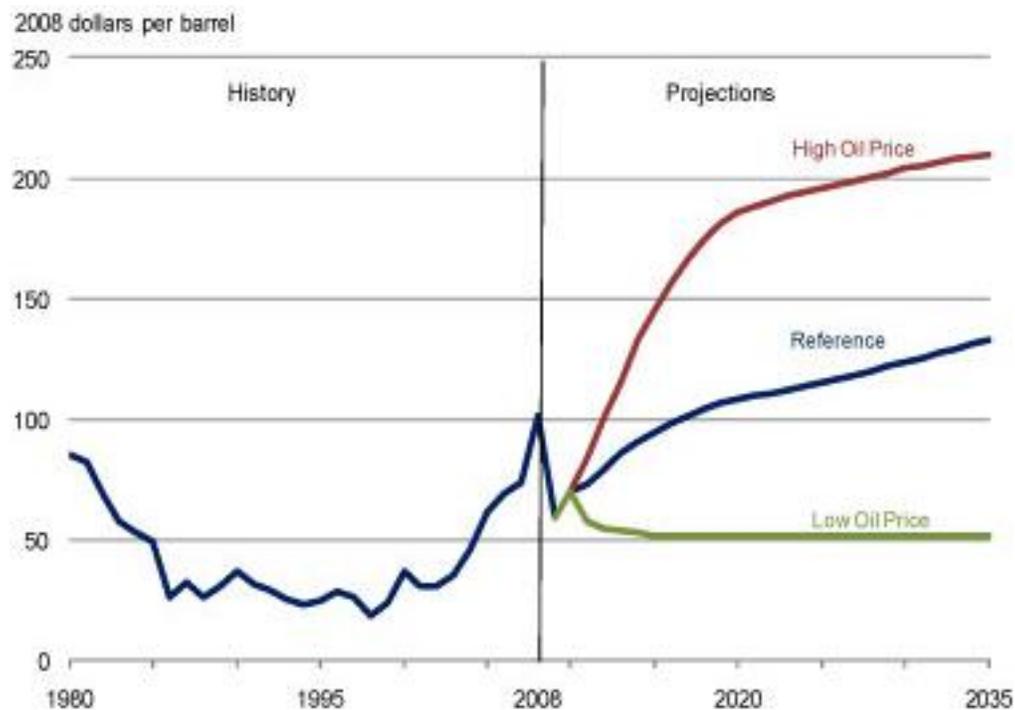


Figure 1: Projected gaps in worldwide crude oil prices based on variable supply-demand conditions reflecting 2008 and 2009 periods (Source: EIA 2010)

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 (World Energy Council 2004). 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, down 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 (EIA 2010a). 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).

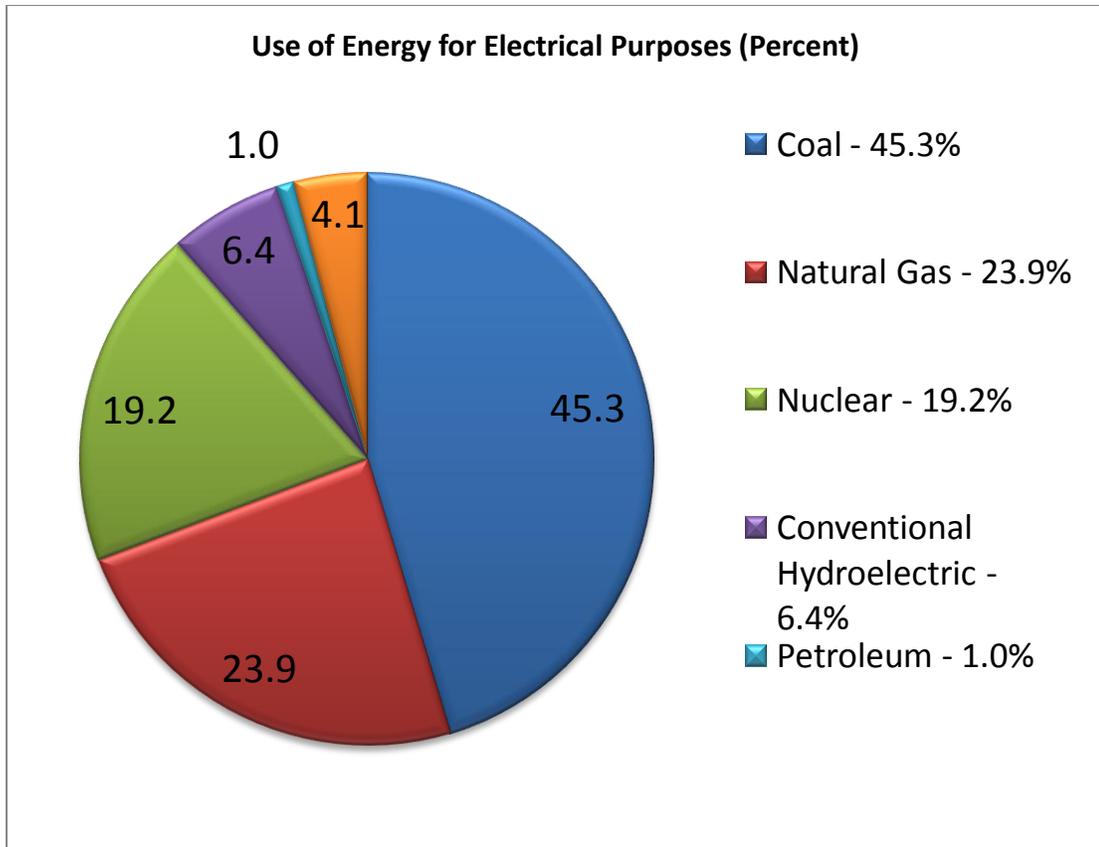


Figure 2: Conventional use of energy for electrical purposes (Note: values do not total to 100%)

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 (Conti , Administration 2011). 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)

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

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 and 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 (EIA 2009). 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, however, results in greater operational and maintenance costs of the facility (Denny and O'Malley 2009). Cyclical operation increases mechanical stress on moving parts of the turbines leading to increased rate of component failure and higher maintenance costs. As a result, given high initial capital costs, electricity production from tidal

energy proves very expensive in the near future (Denny and 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 depreciation is compounded by the expected increases in waste management costs for radioactive material (Du, Parsons and 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 (JOSKOW et al. 2009, Du et al. 2009). By changing the initial capital costs to be equivalent with other power plants, the levelized cost of nuclear power is 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 and Trevors 2010). Their apprehension stems from dramatic advances required in plant designs and construction 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 a waste-free and safe renewable energy. Sunlight is an abundant and essentially limitless form of energy which has been harnessed through the use of solar photovoltaic (SPV) systems. SPV systems are currently an attractive technology for alternative energy generation due to their ability to produce renewable electricity. It offers flexibility in increasing production volume at low capital costs and negligible maintenance costs (Tiwari and 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 = 2.9×10^{11} kWh; 98.6 quads = 2.9×10^{13} kWh] of solar power each year which is only a small fraction of the 46,700 quads

[1.4×10^{16} kWh] that fall on the contiguous 48 states annually. Of these 98.6 quads consumed annually, only 0.08 quads [2.3×10^{11} kWh] are actually converted to electrical energy (Dresselhaus and 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, balance of 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 (Yang, 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. Solving many of the problems associated with solar energy must observe the variability of sunlight with the time of day, weather conditions, and seasons

(Dresselhaus and 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 these efforts possible, reductions in costs of wind technology 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 and 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 with these credits (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 and 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 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 et al. 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 and 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 et al. 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, beyond the higher cost and lower energy output than gasoline, ethanol is mostly incompatible with the current US fuel distribution infrastructure. It is unsuited for transportation through extant pipelines because of its corrosive properties (Yacobucci, Schnepf and 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 and 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 CO₂ 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 contained in 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). Yet 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 and 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 and 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 with promising results (Xiong et al. 2008, Li, Xu and Wu 2007b). 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 of the transesterification process (Fukada et al. 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 microalgae may be best source for biodiesel because of its high lipid content (Hossain et al. 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 microalgae 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 microalgae can exceed 80% of the biomass dry weight, microalgae have the potential for high lipid productivities (Chisti 2007). Refer to Table 3 for doubling times of some common algal species.

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 day⁻¹ (Griffith and Harrison 2009). See Table 2 for the doubling time of some common algal species. Note that these doubling times are under growth in the algae's optimal conditions. 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 (Chisti 2007, Carioca et al. 2009). 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

Algal Species	Growth Rate (day-1)	Growth Conditions
<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

<i>C. reinhardtii</i>	21%
<i>C. vulgaris</i>	14-22%
<i>B. braunii</i>	26.8%
<i>C. protothecoides</i>	50-57%

(Source: Lee et al. 1998; Sialve et al. 2009; Xiong et al. 2008)

2.1.4.7 Economics of Algal Biodiesel

A reduction in the cost of algal biofuel production can be brought about in three ways: increasing the growth rates, increasing lipid yields of the microalgae, resulting in increased lipid production per unit of biomass, and focusing on decreasing the energy requirements of the production process, particularly the harvesting and extraction phases. Several methods of cost reduction via increased growth rates and lipid yields have been targeted for near-future research. Genetic

engineering of algal strains is one of the most promising fields of research to increase the metabolic production of lipids. However, this is a highly expensive option.

Alternative research has focused on improving nutrient supplementation and oxidative stress in order to increase lipid yields. This solution proves to be more affordable than that of genetic engineering.

Costs can be cut in other facets of algal biodiesel production as well.

Improvements can be made in the design of photobioreactors, biomass harvesting techniques, and downstream processing technology. 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 cost of many of the inputs (i.e. sunlight, water, etc.), the post-growth procedures are extremely costly. These post-growth steps, necessary for preparation of lipids for conversion to biofuel, contribute the majority of the costs associated with the process (Wijffels 2010). The biomass must be harvested, the lipids extracted, and the remaining cell components recovered. 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).

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. (Li, Du and Liu 2008a). By being able to produce more lipids, at a faster rate per area used for algal cultivation, will significantly improve the cost-effectiveness of this alternative energy form. See Table 5 for a summary of the important factors in alternative energy.

Table 5: Checklist of ideal criteria for alternative energy

	Portable through pipelines	Compatible with existing engines	Renewable	Competes with food supply
Petroleum	Yes	Yes	No	No
Hydroelectric	No	No	Yes	No
Nuclear	No	No	Yes	No
Solar	No	No	Yes	No
Wind	No	No	Yes	No
Ethanol	No	Yes	Yes	Yes
Crop biodiesel	Yes	Yes	Yes	Yes
Algal biodiesel	Yes	Yes	Yes	No

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 reproductive rates, measured as specific growth rate (μ) 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 O₂. 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.

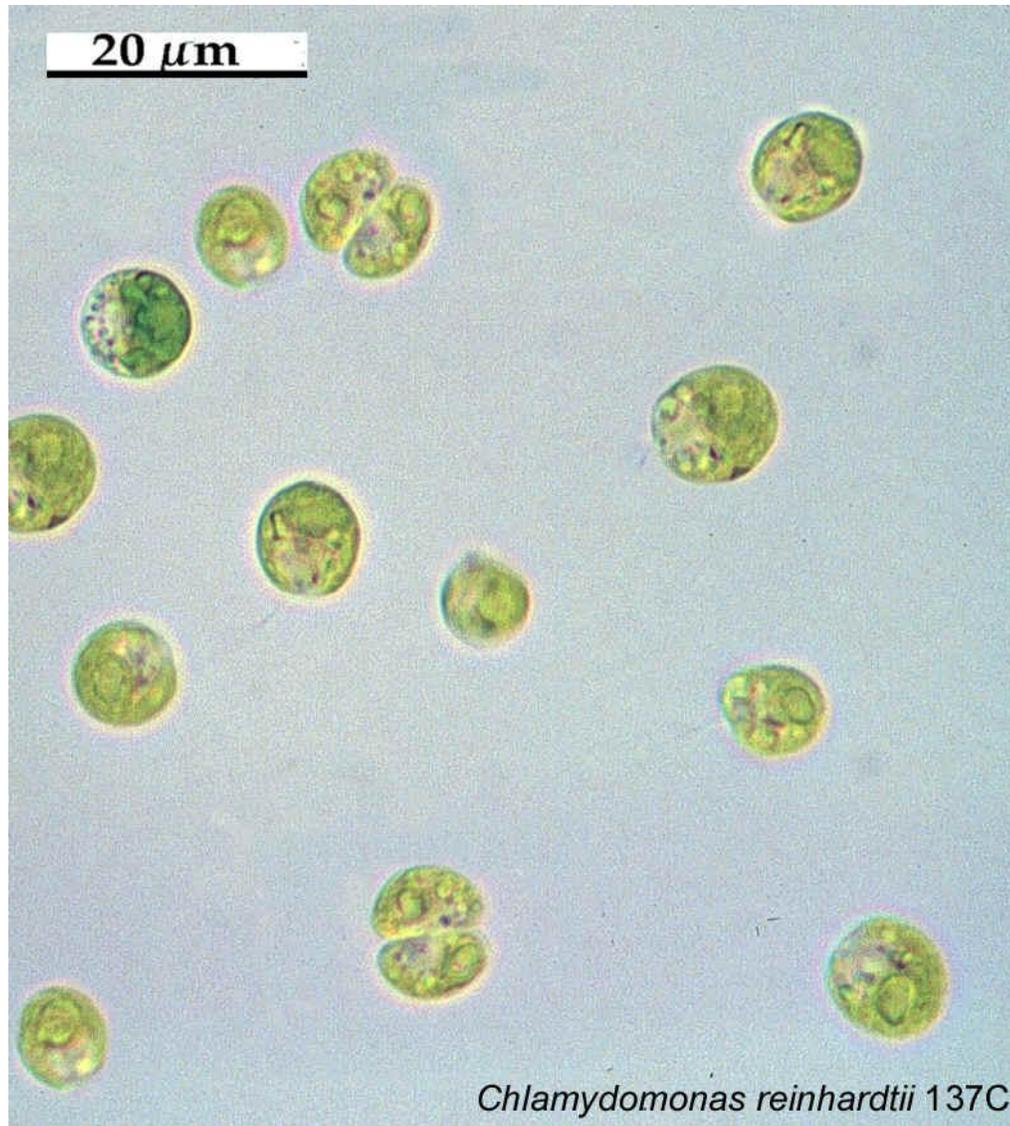


Figure 3: Light microscopy images of *C. reinhardtii* (Source: <http://www.uniprot.org/taxonomy/3055>)

Additionally, *C. reinhardtii*, like other algae, can produce lipids which can be converted to biodiesel through a transesterification reaction. (Thompson, 1996; Chisti 2007). This reaction uses a nucleophilic group, such as methanol, to interact with the carbonyl group of the fatty acid esters of the lipid. This proceeds to the separation of the fatty acid esters from the attached glycerol group. 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 and Largeau, 2005). Instead, they must undergo a more complicated hydrocracking procedure to transform the triterpenes into octane, kerosene, or diesel (Hillen et al 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 lead 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 (NR Boyle, JA Morgan 2009). *C. reinhardtii* is a facultative heterotroph, meaning

that while it undergoes photosynthesis to produce energy, it can also synthesize chlorophyll and photosystem components using acetate in the absence of light (El-Sheekh 1993). Normally, *C. reinhardtii* grows photoautotrophically using its photosynthetic apparatus to harvest light to create electrons and eventually produce NADPH and ATP. These molecules allow the cell to function and fix carbon via the Calvin Cycle (Rochaix et al. 2002). *C. reinhardtii* grown heterotrophically on only acetate, however, has been shown to produce a lower biomass yield, stressing the importance of photon absorption to carbon fixation. *C. reinhardtii* has also been demonstrated to grow mixotrophically, which utilizes photosynthesis and carbon molecules in culture medium. Please 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 (NR Boyle, JA 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 near symmetry. 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 (Reikhof et al. 2005).

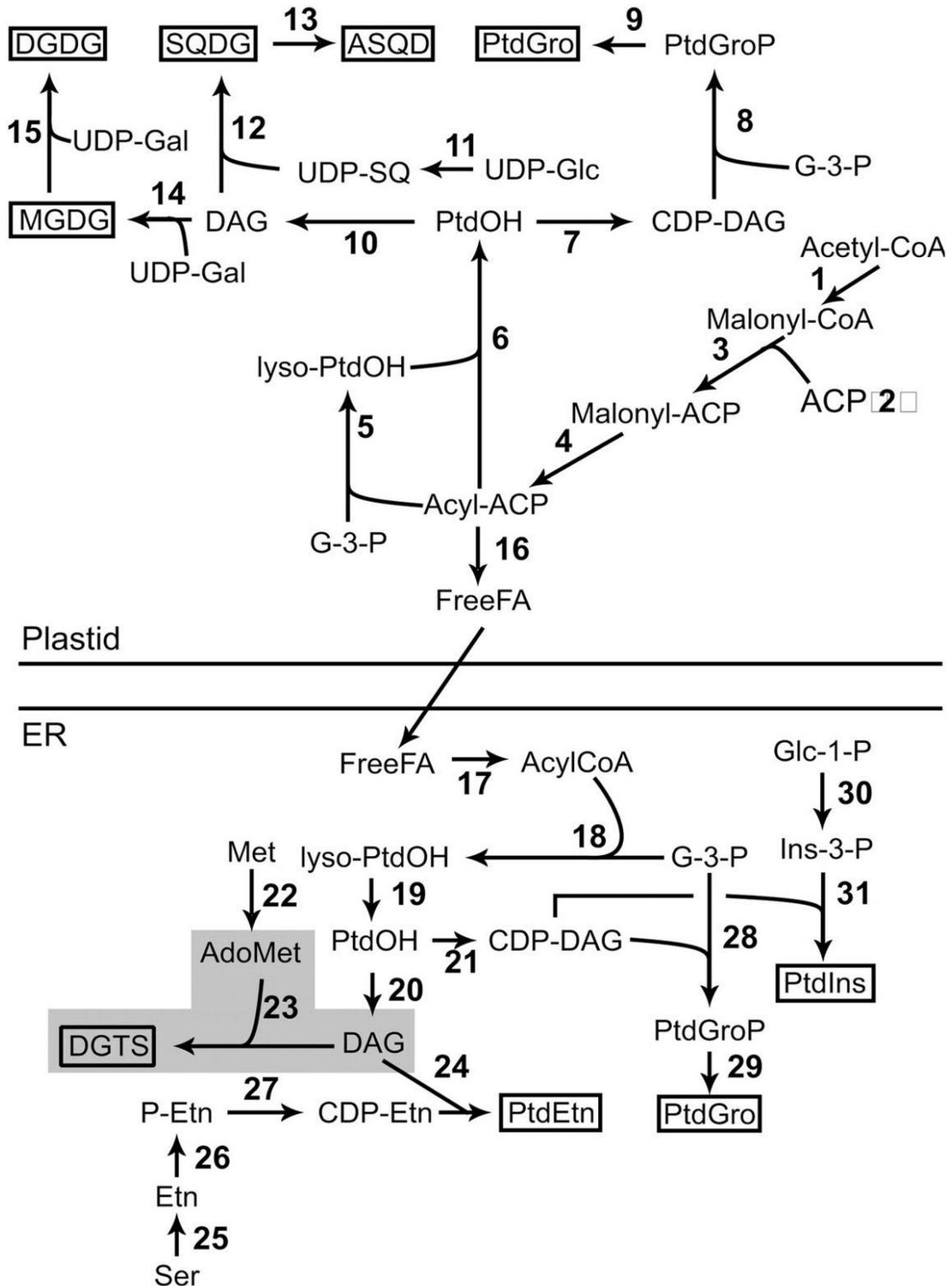


Figure 4: Known and Hypothesized pathways for biosynthesis of lipids in *C. reinhardtii*. The lipids of interest in algae are DATA, diacylglycerol; DGDG, digalactosyldiacylglycerol; DGTS,

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 choice organism for algal study is its short life cycle and generation time (Levine and 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 (Levine and Ebersold 1960; Donnan et al. 1985). This wide range of specific growth rates is attributed to the ability of *C. reinhardtii* to undergo both sexual and asexual reproduction (Levine and Ebersold 1960; Cragie and Cavalier-Smith 1982). 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 and 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 (Cragie and Cavalier-Smith 1982; Spudich 1980; Oldenhof and Zachleder 2007).

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% CO₂,

and agitating cultures over 1 L in total volume (Levine and Ebersold 1960). Under normal photosynthetic growth, *C. reinhardtii* will reproduce to a final cell concentration of 2×10^7 cells/mL (Levine and Ebersold 1960). In the absence of light, *C. reinhardtii* can sustain normal growth using carbon sources such as sodium acetate added to minimal media (Levine and 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 (Oldehof and Zachleder 2007; 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 (Bassi et al. 1992). The minimum photosynthetic capacity of *C. reinhardtii* is 0.103 O₂ 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 dissipating 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 dissipation of incoming light by the naturally forming clumps of algae (Melis et al. 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 et al. 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 (Beckman et al. 2009). This can increase the photosynthetic efficiency of algae by reducing the absorbed 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. Optimally, in room temperature and indirect sunlight conditions, *C. reinhardtii* has a specific

growth rate of 1.50 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 reaction of any type of lipids found in the algal cell. These lipids fall into three primary types: neutral lipids, glycolipids, and polar (i.e. acidic) lipids (Guckert et al 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 et al 2006).

C. reinhardtii contains four major glycerolipids: Monogalactosyl-diacylglycerol (MGDG), digalactosyl-diacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), and glycerophospholipids (GP) (Sato et al. 2000; Wang et al. 2009; Vieler et al 2007). 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 absolutely vital for the photosynthetic apparatus. With respect to lipids, it also contains a single fatty acid chain that can be used in biodiesel production. (Vieler et al 2007).

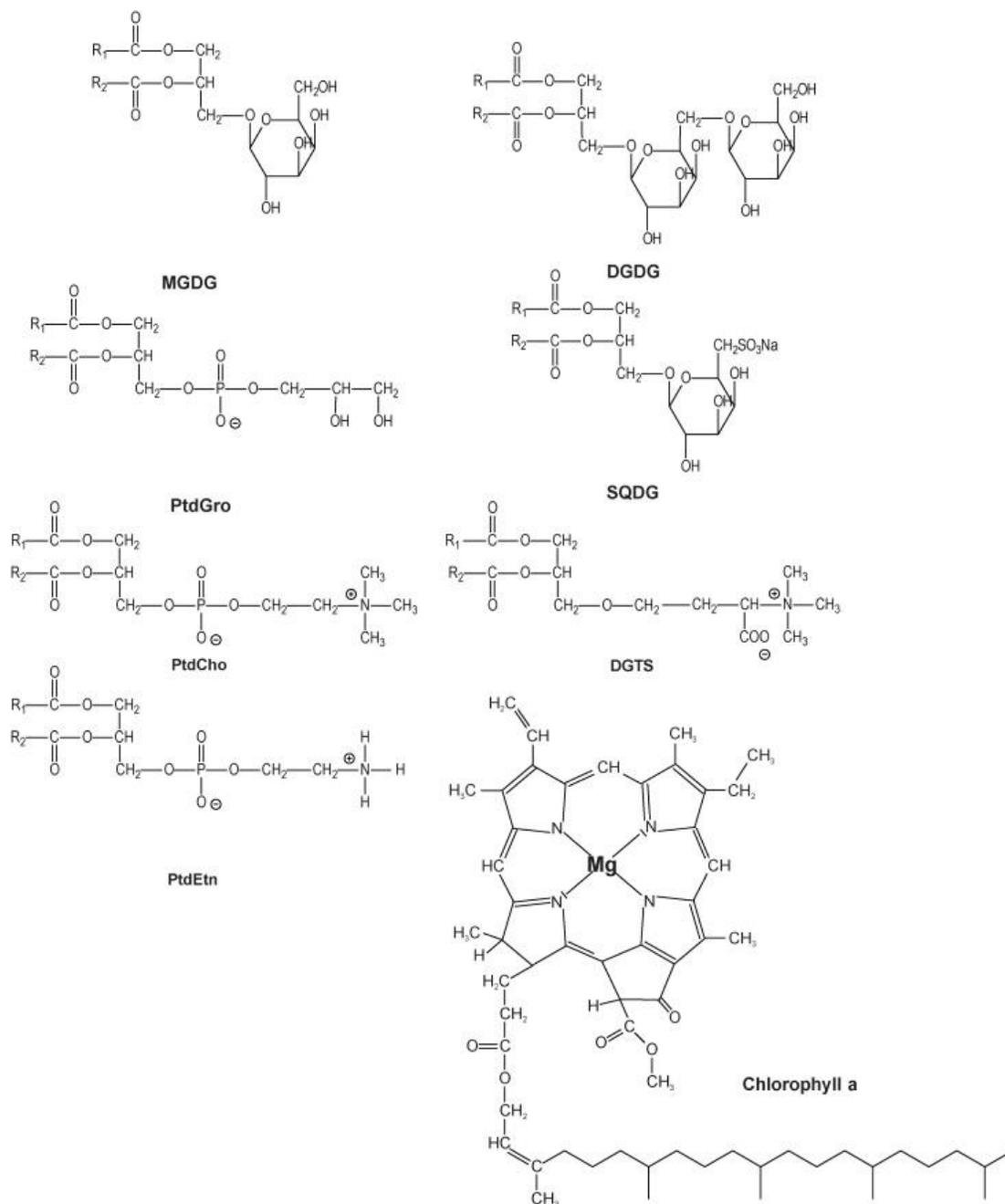


Figure 5: Structures of the most important lipids in *C. reinhardtii*. The abbreviations are as follows – MGDG: Monogalactosyl-diacylglycerol, DGDG: digalactosyl-diacylglycerol, SQDG: sulfoquinovosyldiacylglycerol, PtdGro: phosphatidylglycerol, PtdCho: phosphatidylcholine, PtdEtn: phosphatidylethanolamine,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

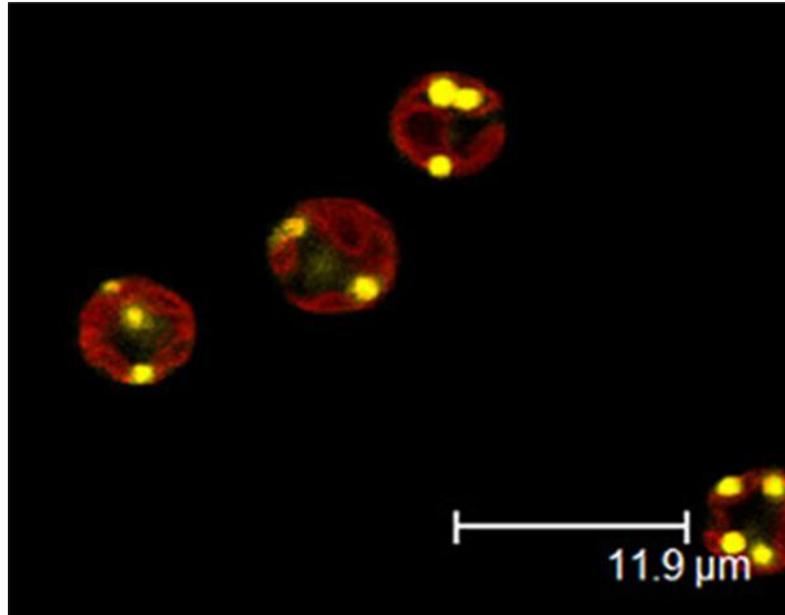


Figure 6: Confocal microscopy survey of lipid bodies in *C. reinhardtii*. Red is the autofluorescence of chlorophyll and yellow is fluorescence of lipid bodies stained with Nile Red dye. These algae represent wild-type *C. reinhardtii*. Source: Wang et al 2009, © Eukaryotic cell, 2009.

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 2007). 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.

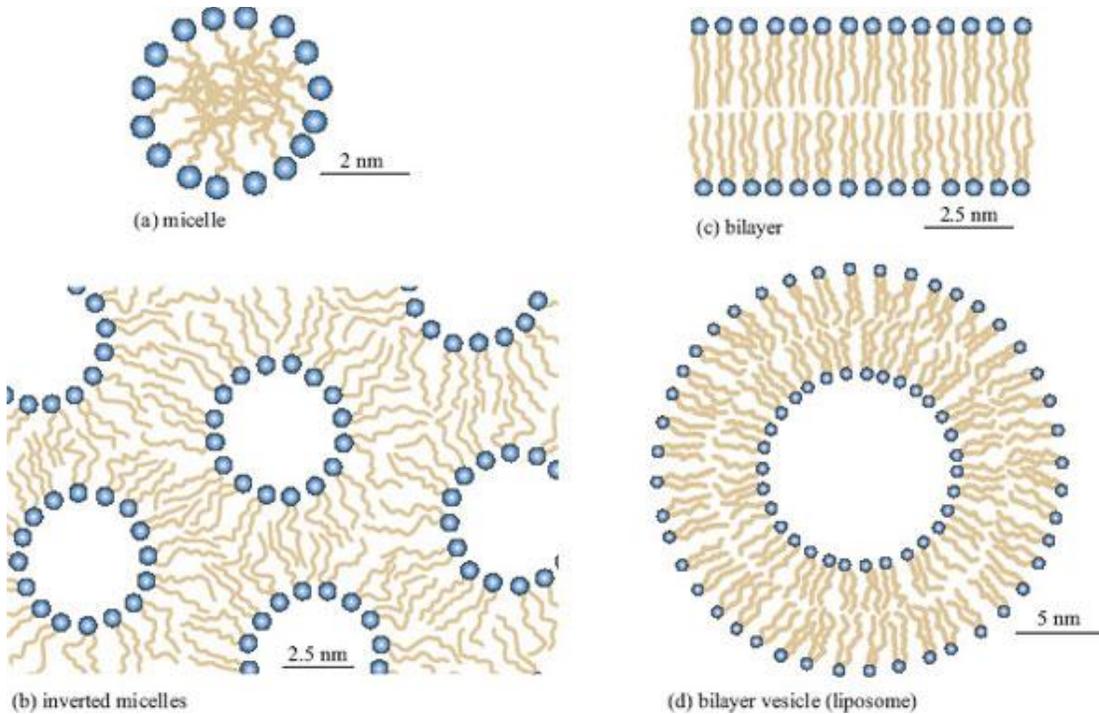


Figure 7: The organization structures for lipid aggregation occurs as (a) monolayer micelles, (b) inverted micelles, (c) lipid bilayers, and (d) bilayer fused vesicles. The lipid bilayers are most prominent in cell membranes and bilayer vesicles are features of cellular organelles. Source: <http://openlearn.open.ac.uk>

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 (Christie WW year). 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 deprivation of phosphorus, SQDG is produced in greater quantity, and under deprivation of sulfur, GP are more abundant. In terms of biodiesel production, these lipids can be utilized. These lipids may not be the optimal choice for biodiesel 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 carbon chains, are energy rich molecules. Algae, however, store energy primarily as starch and other carbohydrates. Storing energy in the form of lipids occurs as triacylglycerides, neutral lipids (Hu et al. 2008). Under conditions of nutrient deprivation, however, lipids may also contribute as energy reserves by being stored as lipid bodies. *Neochloris oleoabundans*, as an example, shows 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 states. Notably, 25% of these lipids are of the C18:1 type, optimal for biodiesel production (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 (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 catabolize 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. This lipid molecules yield 3 long hydrocarbon chains compared to the 2 produced from all other lipid types. A secondary benefit of using these lipids would be simpler refinement which need not isolate the functional groups separated from the lipids. However, triacylglycerides do 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 existing signal transduction pathways, contributing to the regulation of these processes. Phosphatidylinositol groups on lipids are important binding sites for many signaling proteins. More specifically, studies reveal lipids help in mediating stress-related signal transduction. In one study, the algae *Chlamydomonas moewusii* was subjected to osmotic stress and it was shown that quantity of phospholipids increased in response to this stress (Guschina and 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 (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. Further, they have a higher ignition delay resulting from a lower cetane number than unsaturated chain lipids. 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. 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 et al. 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)

Properties	Saturated Chains	Unsaturated Chains
Energy Yield	^a Higher	Lower
Viscosity	Higher	^a Lower
Cetane Number	Lower	^a Higher
Cloud Point	Higher	^a Lower
Pour Point	Higher	^a Lower
Oxidative Stability	^a Higher	Lower

Accounting for these properties of fatty acid chains of lipids, the ideal lipid composition is C18:1. This is a fatty acid chain of 18 carbon atoms with a single carbon double bond. The biodiesel produced from this type of lipid provides the best combination of energy yield, combustion quality, storage life, and usage 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 algae growth and lipid production. Since these processes are interlinked through ensuring survival of the algal cell, both processes are dynamically related. Past and current investigation into improving lipid production for biodiesel from algae has focused on capitalizing

on this relationship. Researchers have explored the effects of inducing stress on the algae through environmental factors and observing the changes to biomass productivity and lipid yields. Though these changes can provide benefits to biodiesel production, their major problem is the many compromises that must be made between the two variables.

2.2.5.1 Light

The flux of light across 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 (Norman et al.). As illustrated in Table 6, light between 3 and 12 k-lux (i.e. ranging from an increase from indirect sunlight to direct sunlight) can produce a range of growth rates between 0.60 and 4.50 day⁻¹ 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 m²/s [2.96 k-lux], the specific growth rate for *C. reinhardtii* can be 1.15 day⁻¹, while under a higher intensity light with 325 μmol photons per m²/s [24 k-lux], growth has been observed to increase to 1.55 day⁻¹ (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⁻¹ to 2.64 day⁻¹ (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.

Growth at high intensity light is made further impractical by the reduction in lipid yields. The fatty acid content of *C. reinhardtii* also changes with respect to light intensity, as illustrated in Table 7. At low light conditions, fatty acids total 57.0mg/g, consistent with cells exposed to very high intensity of light. At more optimal light conditions around 6 k-lux, 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 k-lux.

Efficiency of light utilization, however, can be reduced by clumping of algae and oxidative damage to their photosystems. Clumping is a major problem in dissipation of light available to algae (Melis 2006, Melis 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 and Adams 2003, Ringlstetter, Kaiser and 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*

Light Intensity (k-lux)	^aApproximate growth rate, μ (day⁻¹)	^aTotal cell yield (g/L)	^aTotal fatty acid content (mg/g)
3	0.60	0.38	57.0
6	1.50	1.11	77.4
9	3.50	2.23	76.4
12	4.50	2.64	61.9

^aAkimoto 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⁻¹ relative to a temperature range of 15 to 35°C, as listed in Table 7 (Akimoto et al. 1997). Though this range yields dramatic improvements to the speed of biodiesel generation, maintaining growth conditions above or below ambient room 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⁻¹ (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 and 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. Temperature 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*

Temperature	^a Specific Growth	^a Cell yield (g/L)	^a Total free fatty
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(constant light, 6 k-lux)	Rate, μ (day ⁻¹)		acids (mg/g)
15°C	0.79	0.90	72.0
20°C	1.31	0.96	72.1
25°C	1.50	1.11	77.4
30°C	1.39	1.06	81.1
35°C	1.23	0.97	84.1

^aAkimoto et al 1997

2.2.5.3 Salt

A third important environmental factor is the salt content of the algal growth media. Halotolerance levels, the ability to survive in media of high salinity, differ from one algal strain to the next and determine 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 microalga *Dunaliella maritima* 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 and 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 difference between lipid yields is 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 in the form of nitrates such as ammonia. Nitrogen is essential for protein synthesis; therefore, a steady supply of nitrates 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 (Li et al. 2008b). Comparable results were produced by another study analyzing the alga *Chlorella protothecoides* (Xiong et al. 2008). Similar results were also observed with *Nannochloris oculata* and *C. vulgaris* (Luque de Castro and 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.

In another nitrogen-deprivation study, *C. vulgaris* and *C. emersonii* were grown in a low nitrate medium. Both algae showed increased lipid yields in the low nitrate medium, but biomass productivity was lower than that of the algae grown in control medium (Illman, Scragg and 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 et al. 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 (Sato et al. 2000, Hu et al. 2008).

Table 9: Summary of nutrient effects on algae

Nutrient	Concentration relative to optimal	Effect on growth	Effect on lipid yields
Nitrogen	High	Increase	Overall Decrease
Nitrogen	Low	Decrease	Overall Increase
Phosphorus	Low	-	Decrease GP
Sulfur	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. A method to create similar effects as seen in these environmental manipulation studies, without the cost of compromises, is needed. This study investigates one such possibility. 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 (Li et al. 2007a). They not only regulate the rate of growth exhibited by plants 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, the evolutionary precursors to land plants, are under investigation. 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 and Trewavas 1991).

Although this topic has not yet been extensively examined, particularly in relation to algal biodiesel production, studies illustrating relations 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 et al. 2000). Similarly, abscisic acid and the brassinosteroid family of phytohormones have also been linked to biotic and abiotic stress response signaling (Krishna 2003, Tsavkelova et al. 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 and Mardis 2008). As mentioned previously, *C. reinhardtii* may not contain the highest lipid content among algal species. Nonetheless, it makes for an ideal candidate for this research. *C. reinhardtii* is considered 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 and 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). This comparison has revealed a high degree of homology between the two organisms. 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 (Merchant et al. 2007, Melis 2006). A large portion of these families code for kinases, guanylyl cyclases, and adenylyl cyclases, which are involved in second messaging

systems. Many of these families also share roles in photosynthetic process 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 the work of Kalanon and McFadden, chloroplast protein translocation complexes were compared between *A. thaliana* and *C. reinhardtii* (Kalanon and 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 et al. 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, Min and Lefebvre 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 (Proschold, Harris and Coleman 2005, Lefebvre and Silflow 1999).

The use of these techniques allows comparisons between extant plant phytohormone pathways and their 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

The exploration of phytohormone signaling mechanisms has produced several lines of findings. 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-part system with a histidine protein kinase receptor plant (Hwang and 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 et al. 2000, Nam and 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 activity, 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 Abscisic Acid

Abscisic 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 and 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. As a result, ABA levels are found to increase significantly during seed maturation and during periods of environmental stress (Xiong et al. 2008). ABA also plays a role in the storage of some cellular products (Salisbury and Ross 1992).

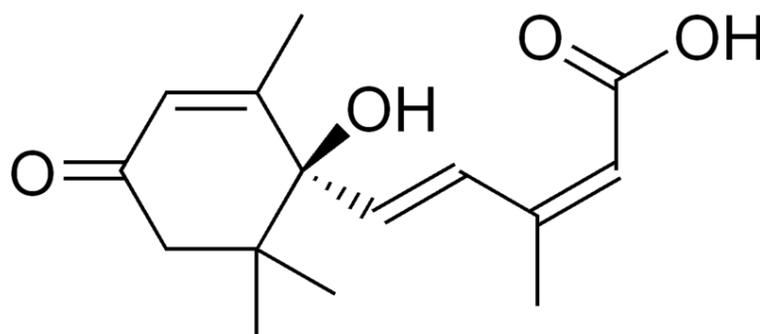


Figure 8: The abscisic acid molecule, which aids in the response of plants to biotic and abiotic stresses in the environment

In *A. thaliana*, ABA biosynthesis is controlled by several genes, which are upregulated by salinity, dehydration, and other osmotic factor (Nambara and Marion-Poll 2005, Eckardt 2002). 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 (Xiong et al. 2008, Seo et al. 2006). 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 (Guo et al. 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 et al. 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 and Assmann 2009), and a receptor-like protein kinase RPK1 (Hong et al. 1997), have been associated with ABA signaling. However, given the complex roles of environmental variables in ABA signaling, the findings of these receptors may be found to be invalid as well. Analyzing ABA signaling homologies of known receptors across species may therefore yield invalid results.

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 (Leung, Merlot and Giraudat 1997, Himmelbach, Yang and Grill 2003). 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 and Marion-Poll 2005, Yoshida et al. 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 (Galston and Purves 1960, Woodward and Bartel 2005, Bartel 1997). Plants can synthesize their own auxins from tryptophan or indole-3-butyric acid, or they can obtain them from their surroundings (Hagen, Uhrhammer and Guilfoyle 1988, Schneider and 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 and Purves 1960, Woodward and 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 and Purves 1960). Auxins cause these profound changes due to their activity at the transcriptional level (Himanen et al. 2002, Brummell and Hall 1987). Their effects can be observed as early as 3 minutes after binding to cellular phytohormone receptors (Hagen et al. 1988, Brummell and Hall 1987). 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.

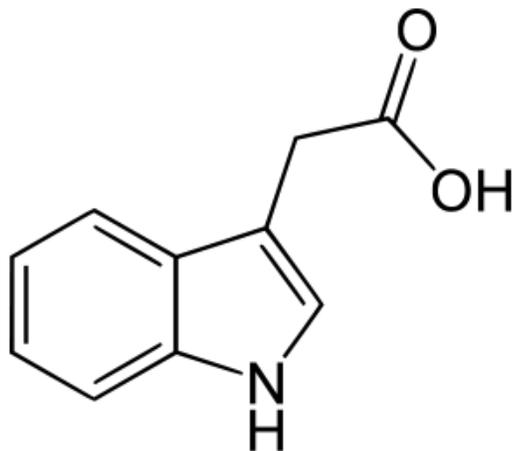


Figure 9: Indole-3-acetic acid, a member of the auxin class of phytohormones, which are responsible for growth processes in plants

Auxins are one of the few families of phytohormones that are naturally secreted in algae (Jacobs 1951, Jacobs 1985). 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 (Jacobs 1951, Jacobs 1985, Overbeek 1940). In certain algae of the *Chlorophyceae* class, low concentrations of IAA actually stimulate an inhibitory effect on growth, while high concentrations have proven toxic (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* (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 (Jacobs 1951) Lau et al. 2009). 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 (Jacobs 1951).

Auxin has been traced through plant lineages, and its evolutionary pattern suggests a link between *Charyophytes* (water-plants) and *Bryophytes* (land-plants) (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 (Clouse and Sasse 1998b, Kepinski and 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 T1R1 protein receptor as a component of the SCFT1R1 complex (Kepinski and Leyser 2005, Dharmasiri, Dharmasiri and Estelle 2005, Tan et al. 2007). T1R1-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 T1R1 to bind promiscuously to various IAA analogues (Tan et al. 2007). Binding of auxin to T1R1 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 G₁ to S phase transition and by regulating transcription of CDK inhibitory protein KRP2 and ARFs (Himanen et al. 2002, Liscum and Reed 2002).

More than 20 ARFs have been identified in the *A. thaliana* genome, serving various functions for auxin responses (Liscum and 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 and Reed 2002). Many of the ARFs bind to ARFAT motifs and promote downstream auxin dependent responses involved in cell growth (Ulmasov et al. 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 (Clouse and Sasse 1998a, 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 (Hu et al. 2008, Clouse and Sasse 1998a). 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 (Clouse and Sasse 1998a). Applied at nM to μ 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 especially responsive to this phytohormone.

Brassinosteroids appear to promote cell division by upregulating the transcription of the gene *CycD3*, which is also used by cytokinins to activate cell division (Hu et al. 2008). There is also evidence that brassinosteroids play an important part in the way plants respond to various stresses, due to its role in rapid plant growth (Mandava 1988). Furthermore, responses to biotic and abiotic stresses, such as pathogens, heat, salt, and drought, may be mediated by brassinosteroids in conjunction with other phytohormones (Mandava 1988, Krishna 2003). The pathways explaining these responses are yet to be fully elucidated in plant models. Current evidence points to the upregulation of gene expression for brassinosteroid intermediates.

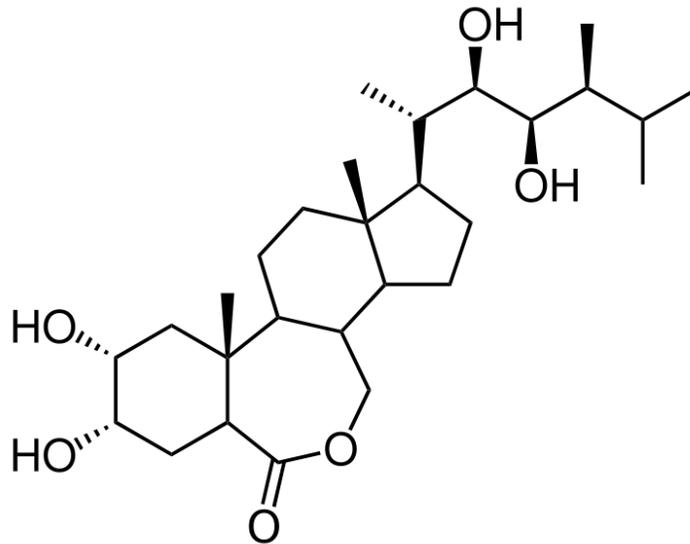


Figure 10: Brassinolide, a member of the brassinosteroid family of phytohormones

Effects of brassinosteroids observed in plant models have been mirrored in algae. *C. vulgaris* has been noted to produce brassinosteroids endogenously as necessary signaling elements when grown under conditions of light (Tsavkelova et al. 2006). Homologs of these genes may be found in *C. reinhardtii* as well (see the section on Brassinosteroids). There is also evidence of brassinosteroid-induced changes in the metabolism of *C. vulgaris* (Bajguz and 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 and 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 (Clouse and Sasse

1998a). 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 will 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 (Tarakhovskaya, Maslov and Shishova 2007, Riou-Khamlichi et al. 1999). 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 et al. 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 and 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 and 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 microalgae (Stirk et al. 2002, Ördög et al. 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.

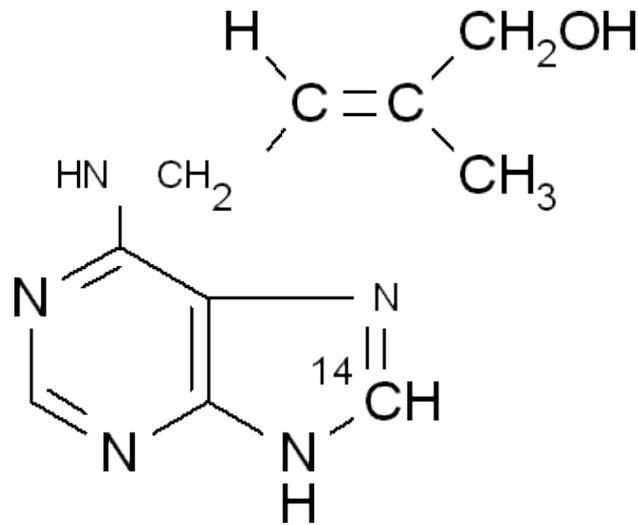


Figure 11: Trans-zeatin, a member of the cytokinin class

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 part of the signal cascade (Yamada et al. 2001, Hwang and Sheen 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 and 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 (see the section on cytokinins). The effect of increased growth from cytokinins is a product of the activation of these regulators of the cell division cycle and differentiation (Sheen 2001, Riou-Khamlichi et al. 1999). Cytokinins increase transcription activation of a cyclin D3, involved with the G₁-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 and 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 algae *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. 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 and 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.

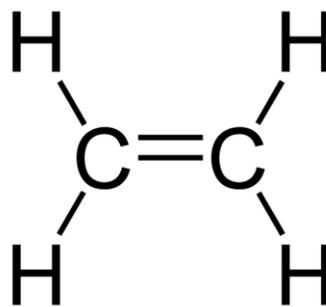


Figure 12: Ethylene, a gaseous phytohormone which aids in the growth and development of many land plants

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 (Guo and Ecker 2004, Hua and 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) (Guo and Ecker 2004). These transcription factors aid in the growth response to ethylene. Ethylene receptor, ETR1, is located on the endoplasmic reticulum (Chen et al. 2002). This receptor also 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 (Wang, Li and Ecker 2002). Evidence suggests that CTR1 acts similarly to MAPK kinases. Therefore, a MAPKK cascade may be involved with ethylene signaling as well (Wang et al. 2002).

Evidence for the use of ethylene in algal species is limited. These findings are very specific and 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 allow seed germination (Nakajima et al. 2006). Some gibberellin biosynthetic enzymes, such as GA 3 β -hydroxylase, are coded by sGA3ox1. GA 3 β -hydroxylase converts GA20 to bioactive GA1 (Figure 13) using the CaMV-35S promoter. Application of Gibberellin caused cells to increase in size (Gonai et al. 2004). Little evidence for endogenous gibberellin activity has been observed in green algae. Jennings (1968) observed increased growth of algae in response to gibberellin effects (Jennings 1968). In *C. reinhardtii*, gibberellin activity has not been demonstrated (Kato, Purves and Phinney 1962).

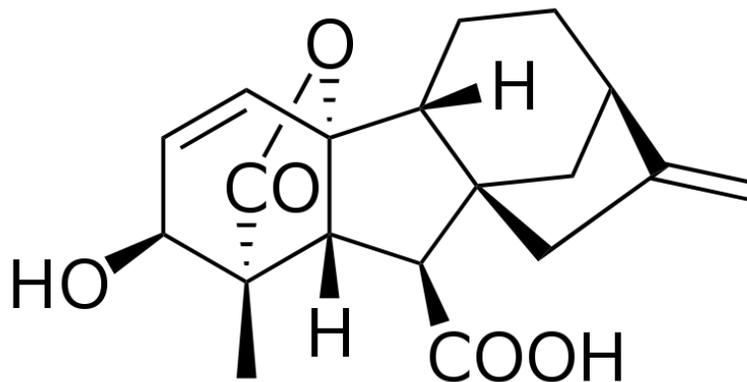


Figure 13: Gibberellin 1 (GA1) is a member of the gibberellin class of phytohormones

Characterization of gibberellin signaling components has not yielded many significant results. 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 (Vandenbussche et al. 2007; Ueguchi-Tanaka et al. 2005). Downstream repressor proteins, DELLA, are therefore inactivated to yield transcription of cell growth genes under the presence of gibberellins. 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 innovations which may serve as 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. 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 synthesis 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 finding 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 LCMS and confocal microscopy 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.

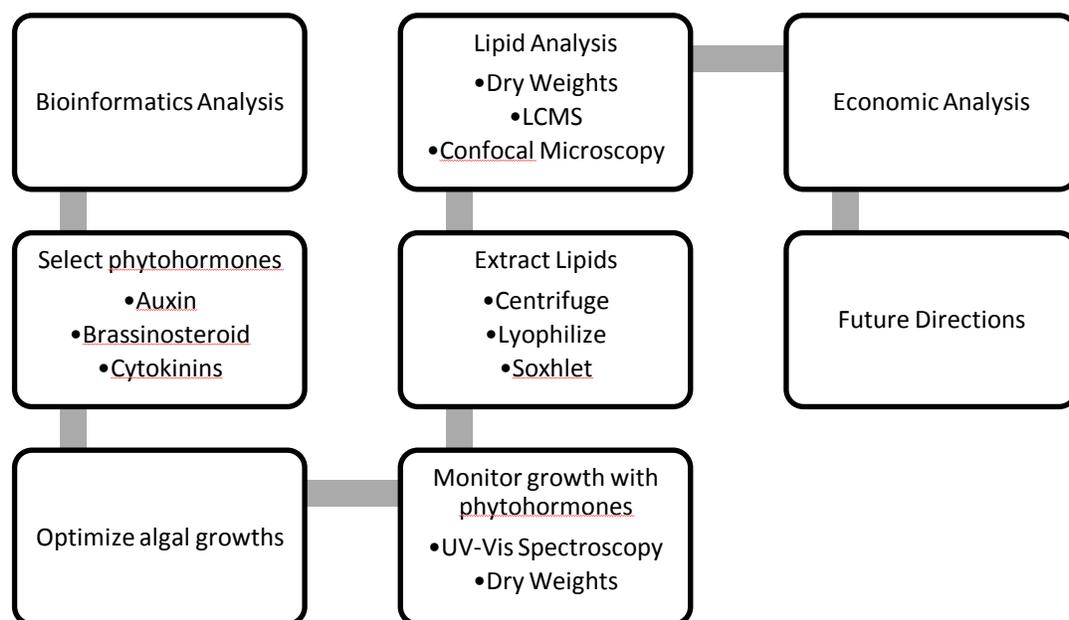


Figure 14: Flow chart depicting the basic outline of the methods

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 determined. The gene sequences of the most common proteins and receptors involved in each pathway were obtained from Arabidopsis Information Resource (www.arabidopsis.org) (Table 10). 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		
Total	9	6	9	12	7	6

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 CO₂.

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 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 any 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 FeSO₄ and EDTA. The solution was brought to a boil. EDTA and FeSO₄ 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 ⁻¹)	Hutner's Trace Elements	Concentration (mg/mL)
NH ₄ Cl	0.05 g	EDTA Disodium Salt, Dihydrate@	66.6
MgSO ₄ ·7H ₂ O	0.02 g	ZnSO ₄ ·7H ₂ O	29.33
CaCl ₂ ·2H ₂ O	0.01 g	H ₃ BO ₃	15.2
K ₂ HPO ₄	0.072 g	MnCl ₂ ·4H ₂ O	6.8
KH ₂ PO ₄	0.036 g	FeSO ₄ ·7H ₂ O	6.66
Hutner's Trace Elements	1 mL	CoCl ₂ ·6H ₂ O	2.13

3.2.3 Addition of Phytohormones

The phytohormones tested in this study were indole-3-acetic acid, 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 mixture of phytohormones in a range of concentrations from 10⁻⁸ to 10⁻¹². This high end of this range was chosen after initial tests showed that concentrations of

phytohormones over 10^{-6} generally killed the algae. The low end of this range was chosen after the literature review showed that *C. reinhardtii* actually produces auxin and brassinosteroids at concentrations around 10^{-13} (Overbeck, 1940). These cultures were otherwise grown in the conditions as above. The initial 4 phytohormone treatments were indole-acetic acid, epibrassinolide, trans-zeatin, and zeatin. An additional two phytohormone treatments were conducted after analyzing the initial data sets. These treatments were a combination of indole-acetic acid and trans-zeatin and a combination of indole-acetic acid and trans-zeatin-riboside (Table 13). These mixed 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.

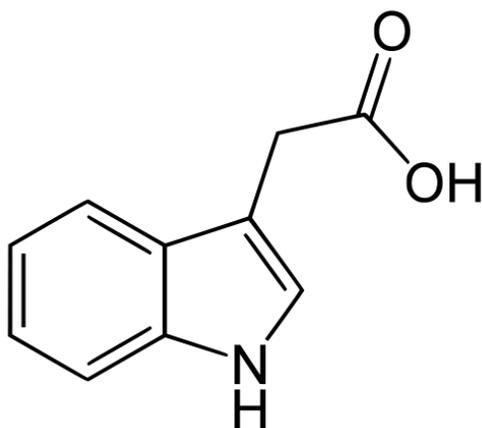
Each trial consisted of a set of 5 cultures with concentrations of phytohormones for each order of magnitude ranging from 10^{-8} to 10^{-12} M. These solutions were created from 1 mM stock solutions of phytohormones dissolved in ethanol. The ethanol was used to help dissolve the phytohormones. In order 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.

Table 12: List of the phytohormones and their properties

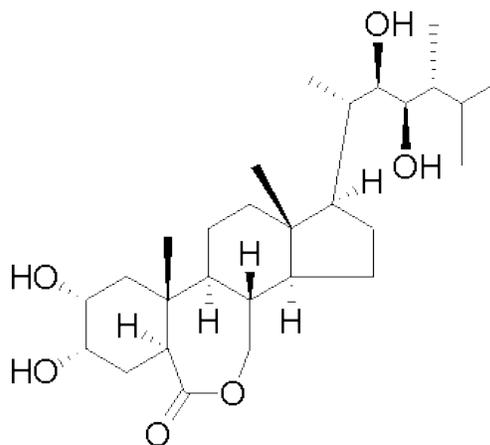
Phytohormone	Category	Formula	Molecular Weight	Catalog Number
3-Indolacetic Acid	Auxin	C ₁₀ H ₉ NO ₂	175.18	I2886
Epibrassinolide	Brassinosteroid	C ₂₈ H ₄₈ O ₆	480.68	E1641
Trans-Zeatin	Cytokinin	C ₁₀ H ₁₃ N ₅ O	219.24	Z0876
Trans-Zeatin-Riboside	Cytokinin	C ₁₅ H ₂₁ N ₅ O ₅	351.36	Z0375

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
Auxin	10 ⁻¹² - 10 ⁻⁸		8
Brassinosteroid	10 ⁻¹² - 10 ⁻⁸		8
Zeatin	10 ⁻¹² - 10 ⁻⁸		8
Zeatin-Riboside	10 ⁻¹² - 10 ⁻⁸		8
Auxin	Zeatin	10 ⁻¹¹ 10 ⁻¹² - 10 ⁻⁸	4
Auxin	Zeatin-Riboside	10 ⁻¹¹ 10 ⁻¹² - 10 ⁻⁸	4



Auxin: 3-Indolacetic Acid



Brassinosteroid: Epibrassinolide

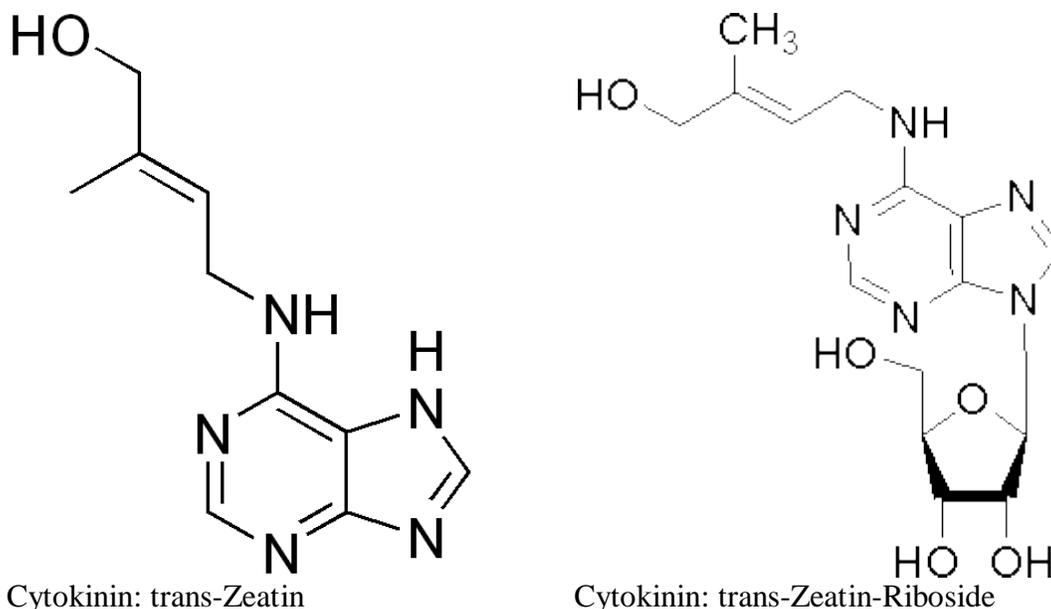


Figure 15: The molecular structures of the phytohormones are shown above (Chemdraw Citation)

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 by the Beckman Avanti J-25I Centrifuge (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.

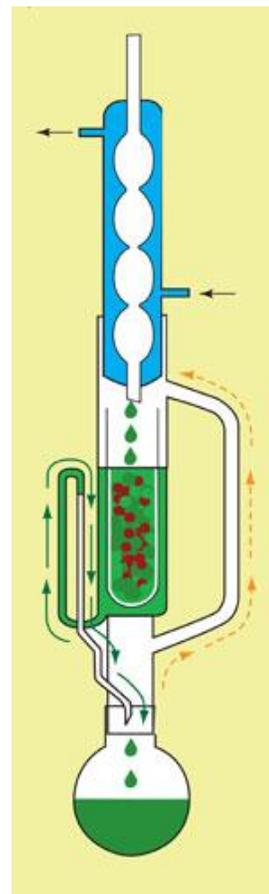
The resuspension of the cell pellet allowed for more even drying and easier handling of the dry algae during the Soxhlet protocol. A rotary evaporator could be used in place of the lyophilizer for the same results (Jiang, 1975). The mass of the dry pellet (M_T) was determined by taking the difference 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 (Guckert, 1981). 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 subtracting 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 10 mL hexane and 10 mL isopropanol. This mixture was chosen in order to be able to extract greatest amounts and types of lipids (Manirakiza, 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.

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 solvent containing the lipids after Soxhlet extraction was transferred to scintillation vials and then evaporated using a N_2 evaporator until dry. The tips of the evaporator were constantly adjusted to be 1 cm away from the top of the solution. The airspeed of the evaporator was adjusted to ensure 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_L) 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 solution.

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

$$M_T = \text{total mass of algae growth } (g)$$

$$M_E = \text{mass of algae used for extraction } (g)$$

$$M_L = \text{mass of extracted lipids } (g)$$

$$V_C = \text{volume of culture } (g)$$

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 liquid chromatography – electrospray ionization – time of flight (LC-ESI-TOF) setup.

The dry lipid extracts were resuspended in isopropanol before LC/MS analysis. A gradient for the 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 B and ramped up to 100% over the course of 30 minutes. This final condition was maintained for 40 minutes, after which a ten minute flushing period occurred. The flow rate for the experiments was adjusted to 0.25 mL/min. The UV/Vis spectrometer on the Agilent 1100 HPLC was set to monitor absorbances at 220 nm. The sample size of each trial was 10 μ L (Veiler, 2007). Originally the gradient used was 0% Mobile Phase B to 100% Mobile Phase B over a period of 30 minutes, however, most of the lipids did not come out until the very end, so the initial concentration and time span of Mobile Phase B needed to be increased. This yielded a better resolution for the lipid separation. The downside to using this acetonitrile mobile phase is that the acetonitrile has a small absorbance around the same wavelengths of the lipids detected by the HPLC. Although this interference eliminates 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, 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 spectrums 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 wavelength were respectively 488 nm and 540 nm. Chlorophyll also excited at 488 nm, but the emission spectrum of chlorophyll was determined to be insignificant in comparison to the emission spectrum of the Nile Red. The resulting data was used to qualitatively determine whether more or less lipids were created by the algae. Morphological changes including size and shape were also determined from the resulting images.

4. Results

4.1 Genomic Analysis

We used the BLAST tool to compare the genomes of *A. thaliana* and *C. reinhardtii*. We searched for specific genes whose receptors were most commonly found in *A. thaliana*. While evaluating the results of the search, we examined the E-values and bit scores for each receptor. A low E-value (0.01 or lower) indicates that the results are more significant and 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 our data to find which phytohormones could possibly induce changes in growth rate or lipid yield.

4.1.1 Abscisic Acid

Gene AB11 generated a match with a hypothetical protein 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. Moreover, the genes from *C. reinhardtii* did not have good scores in either area. The genes involved in the abscisic acid response for *A. thaliana* generated matches predominantly with hypothetical proteins.

Previous research on the signal transduction pathway for abscisic 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; Riano-Pachon et al. 2008). 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. Only hypothetical proteins displayed matches with the proteins in *A. thaliana*. Due to this, abscisic acid was not selected for further investigation.

Table 14: Genomic analysis results for matches of genes involved in abscisic acid recognition between *C. reinhardtii* and *A. thaliana*

Gene Name	# of Matches	Lowest E-Value	Highest Bit Score	Matches w/E-value <.1
GCR2	7	3.5	33.7	0
ABI3	5	9.8	31.9	0
FCA	10+	1.4	37.4	0
ABI2	0	N/A	N/A	0
RPK1	0	N/A	N/A	0
GTG1	2	4.0	33.7	0
ABI1	4	.009	42.8	1
GTG2	2	.89	37.4	0
RPK1	2	4.0	33.7	0

4.1.2 Auxins

Similar to the results for abscisic acid, the matches for the genes involved in auxin response in *A. thaliana* were mostly hypothetical proteins. However, the auxin response factors (ARFs), which dimerize and bind to DNA to allow transcriptional control in *A. thaliana*, provided some matches of interest in *C. reinhardtii*. Genes ARF8 and ASK2 provided strong matches 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 CO₂-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. In addition, the SKP1 protein in *C. reinhardtii*, which serves as an ubiquitin ligase, had an E-value on the order of 10⁻⁴⁸ and a maximal identity of 84%, indicating possible homology.

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 CO₂, 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. 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 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 (Jacobs 1951; Jacobs et al. 1985; Cooke et al. 2001). 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 matches of genes involved in auxin recognition between *C. reinhardtii* and *A. thaliana*

Gene Name	# of Matches	Lowest E-Value	Highest Bit Score	Matches w/E-value <.1
ABP1	1	3.6	33.7	0
TIR1	1	6.5	33.7	0
KIP2	5	2.1	33.7	0
ARF1	0	N/A	N/A	0
ARF19	4	2.9	35.6	0
ARF8	10+	.003	44.6	6
ARF6	9	.70	37.4	0
CYCD3	1	2.7	33.7	0
ATCUL1	6	5e-16	87.8	1
SKP1	4	2.3	33.7	0
RBX1	1	3.3	33.7	0
UBC3	1	1e-17	91.5	1
UBC4	0	N/A	N/A	0
UBC5	1	2e-8	60.8	1
ASK2	10+	5e-48	192	8
ASK9	4	.25	35.6	0
ASK11	3	4e-24	111	1

4.1.3 Brassinosteroids

Proteins involved in the brassinosteroid response in *A. thaliana* provided minimal results except for the BRZ1 and BES1 proteins. 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). Genes BZR1 and BES1 provided matches with low E-values of less than 0.01, high bit scores, and maximal identities near 100%.

The signal transduction pathway for brassinosteroids is better known 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, which is encoded by the *bin2* gene (Li and Nam 2002). When brassinosteroids bind on the membrane, BIN2 is inactivated by an unknown mechanism, which allows hypophosphorylated nuclear proteins such as Brassinazole resistant (BZR1) to suppress transcription (He et al. 2002). 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, including ZMP1 and VPS34, has functions related to locomotion and are not associated with the phytohormone response, indicating that the proteins only share conserved domains. In addition, since BZR1 and BES1 are not proteins critical to the brassinosteroid response, the strong matches were not entirely

significant. Proteins more closely associated with the brassinosteroid response pathway, such as BIN2 and BRI1, did not give any strong matches. Despite the lack of matches to justify the use of brassinosteroids in the experiments, they were chosen to serve as a possible negative control. In addition, the response of *C. reinhardtii* to brassinosteroids can be more fully investigated since minimal research has been conducted regarding the relationship between brassinosteroids and *C. reinhardtii*.

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

Gene Name	# of Matches	Lowest E-Value	Highest Bit Score	Matches w/E-value <.1
BZR1	10+	7e-05	89.1	10+
BRI1	6	.69	37.4	0
BIN2	1	5.6	33.7	0
BES1	10+	.002	44.6	9
DET2	0	N/A	N/A	0
DWF4	4	9.7	33.7	0

4.1.4 Cytokinins

Proteins associated with the cytokinin response in *A. thaliana* generated some strong matches in *C. reinhardtii*. Some strong matches, such as the RWP-RK transcription factor and the nitrate assimilation regulatory protein (with E-values of .069 and .024, respectively, and maximal identities of 100%), were generated by the histidine kinase CRE1, a cytokinin-binding receptor in *A. thaliana*, but since both are also classified as histidine kinases, it is likely that they only share conserved domains rather than similar functions. On the other hand, many of the other proteins tested generated mostly hypothetical proteins.

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; 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 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. Zeatin and zeatin-ribose were selected on the basis of their prevalence in the regulation of cell division for *A. thaliana*.

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

Gene Name	# of Matches	Lowest E-Value	Highest Bit Score	Matches w/E-value <.1
CRE1	10+	.069	41.0	1
AHK4	10+	.069	41.0	1
AP2	3	5.0	33.7	0
ARR	0	N/A	N/A	0
CYCD3	1	2.7	33.7	0
AHK5	6	9.6	33.7	0
ARR5	0	N/A	N/A	0
ARR2	5	.047	41.0	1

ARR19	1	4.5	33.7	0
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4.1.5 Ethylene

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

Previous research has demonstrated that ethylene has no role in *C. reinhardtii*. In addition, the bioinformatics analysis did not yield any strong matches. Most of the weak matches consisted of hypothetical proteins, indicating that ethylene was not a good candidate for further research.

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

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

4.1.6 Gibberellins

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

further investigated. The GID1 protein, an F-box protein that is critical to the response of *A. thaliana* to gibberellins, did not produce any matches.

Previous research has not elucidated the signal transduction pathway for gibberellins. 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). However, the GID1 protein did not yield any matches. In addition, other proteins that have been proposed to be involved in the pathway did not give any noteworthy matches. Thus, it was concluded that gibberellins were not likely to initiate a response in *C. reinhardtii*.

Table 19: Genomic analysis results for matches of genes involved in gibberellin recognition between *C. reinhardtii* and *A. thaliana*

Gene Name	# of Matches	Lowest E-Value	Highest Bit Score	Matches w/E-value <.1
GID1	1	4.6	33.7	0
GAI	3	4.3	33.7	0
RGA	8	1.3	35.6	2
RGL2	8	.34	37.4	0
RGL3	6	.33	37.4	0
RGL1	1	3.7	33.7	0

4.1.7 Overall

Most of the proteins that matched with the proteins involved in the phytohormone response in *A. thaliana* were hypothetical proteins. Thus, it was difficult to determine whether the pathways 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 (Cooke et al. 2001; Bajguz and Czerpak 1998; Stirk et al. 2002). In addition, brassinosteroids were chosen because their role in algae have not been fully examined.

4.2 Growth Rates

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 are generated through the standard deviation of the growth rates calculated. Larger changes in growth rate may occasionally occur in smaller concentrations of the phytohormone, such as in the auxin trials (See Fig. 1). The potential reasons for this occurrence will be further explored in the Discussion.

Dry algal mass was also measured and averaged across trials. The number of trials conducted for each concentration of phytohormone was 8. The growth rates and final algal masses were plotted together to compare the effect of the phytohormone with the growth rate.

4.2.1.1 Auxin

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 $52.9 \pm 5.0\%$ 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 auxin slightly increased the final mass of the algae at 10^{-9} and 10^{-12} M concentrations; 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.

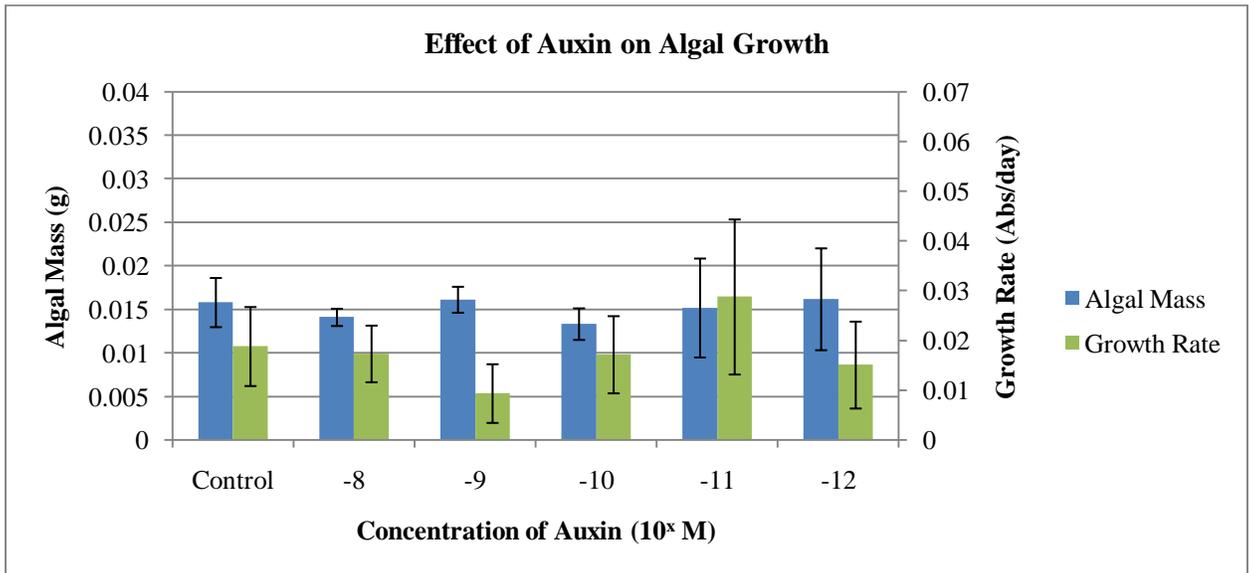


Figure 17: Graphical representation of the mean effects of differing concentrations of auxin (indole-3-acetic acid) on *C. reinhardtii* growth.

4.2.1.2 Brassinolide

The application of various concentrations of brassinolide had similar results: an increase at certain concentrations of the phytohormones, but large errors. There was a smaller increase in growth rate in comparison to auxin, but at the 10^{-12} M level of

brassinolide, the growth rate increased by approximately $62.7 \pm 33.0\%$. Application of brassinolide caused a slight decrease in algal mass at all concentrations, with larger decreases occurring with the more dilute concentrations of phytohormone.

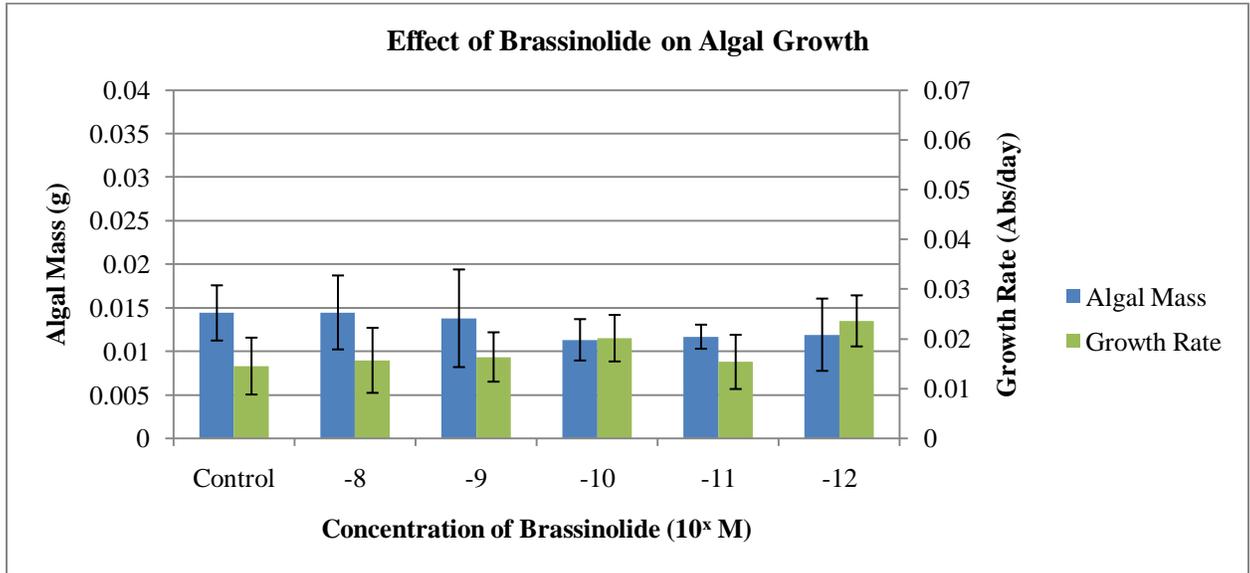


Figure 18: Graphical representation of the mean effect of brassinolide on the growth rate of *C. reinhardtii*.

4.2.1.3 Zeatin

The application of zeatin had an adverse effect on algal growth at high concentrations. Zeatin caused a slight decrease in algal mass at all concentrations, with the decrease peaking close to the concentrations of 10^{-9} M and 10^{-10} M. This was mirrored in the growth rate, with decreases in growth rate ranging from 16% to 48%.

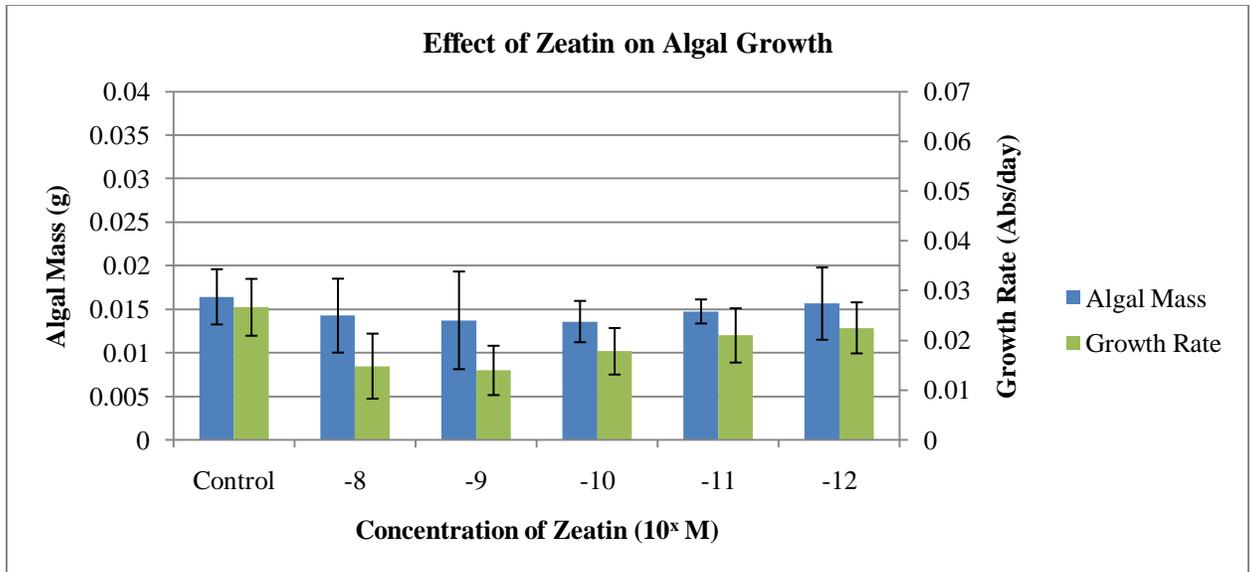


Figure 19: Graphical representation of the mean effect of trans-zeatin-riboside on the growth rate of *C. reinhardtii*.

4.2.1.4 Trans-zeatin-riboside

The application of trans-zeatin-riboside seemed to have a positive effect on the growth rate of the algae at higher concentrations. The highest growth rate was a 31.1 ± 21.5% 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 occurring at concentrations of 10⁻⁹ and 10⁻¹¹ M.

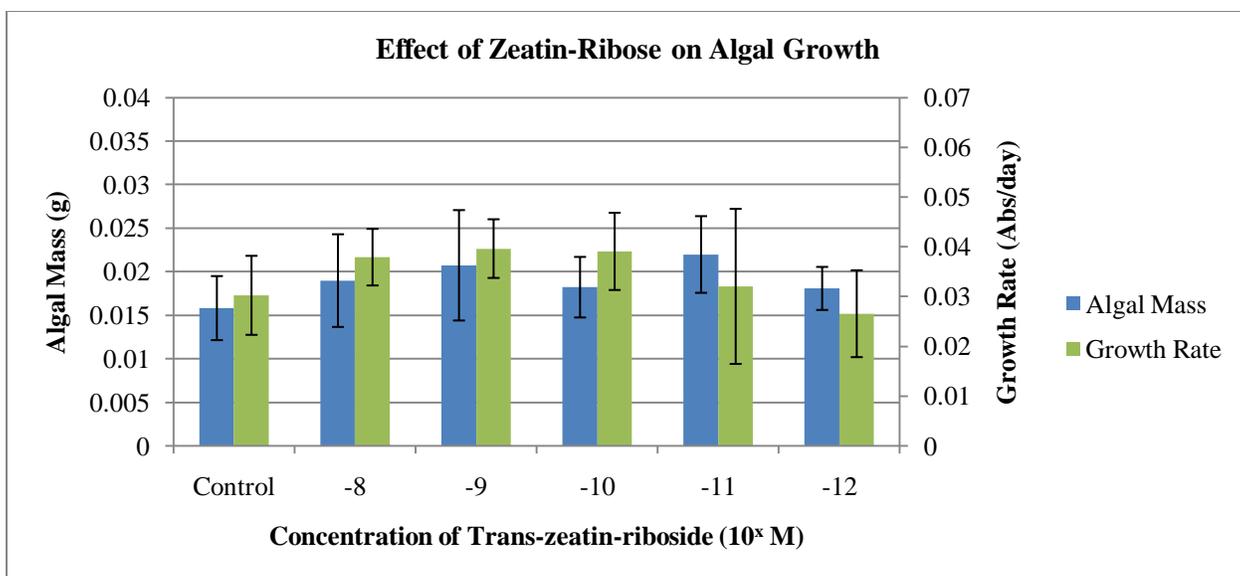


Figure 20: Graphical representation of the mean effect of various concentrations of trans-zeatin-ribose on the growth rate of *C. reinhardtii*.

4.2.1.5 Auxin & Zeatin

The application of both auxin and zeatin did not appear to affect the growth rate significantly except for at zeatin concentrations of 10⁻¹² M. Treatment of algae with auxin-zeatin combination caused a slight increase in algal mass at all concentrations except for 10⁻¹¹ M, with the noticeable increases occurring at concentrations of 10⁻¹⁰ and 10⁻¹² M.

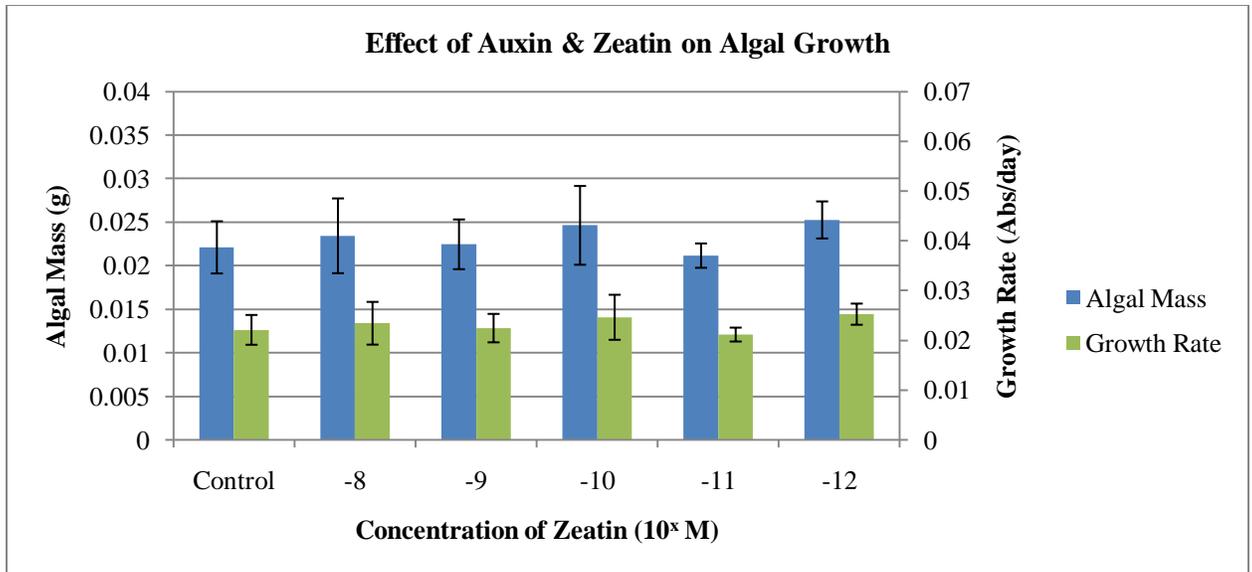


Figure 21: Graphical representation of the mean effect of both auxin and zeatin at [auxin] 10^{-11} M and varying concentrations of zeatin on the growth rate and dry algal mass of *C. reinhardtii*

4.2.1.6 Auxin & Trans-zeatin-riboside

The application of both auxin and trans-zeatin-riboside seemed to have a varying effect on the growth rates of the algae. The highest growth rate was seen in the 10^{-12} M concentration of auxin and trans-zeatin-riboside, but it was only a slight increase in growth rate from the control groups. Application of auxin and trans-zeatin-riboside decreased algal mass at all concentrations except for 10^{-11} , at which concentration the mass was increased slightly.

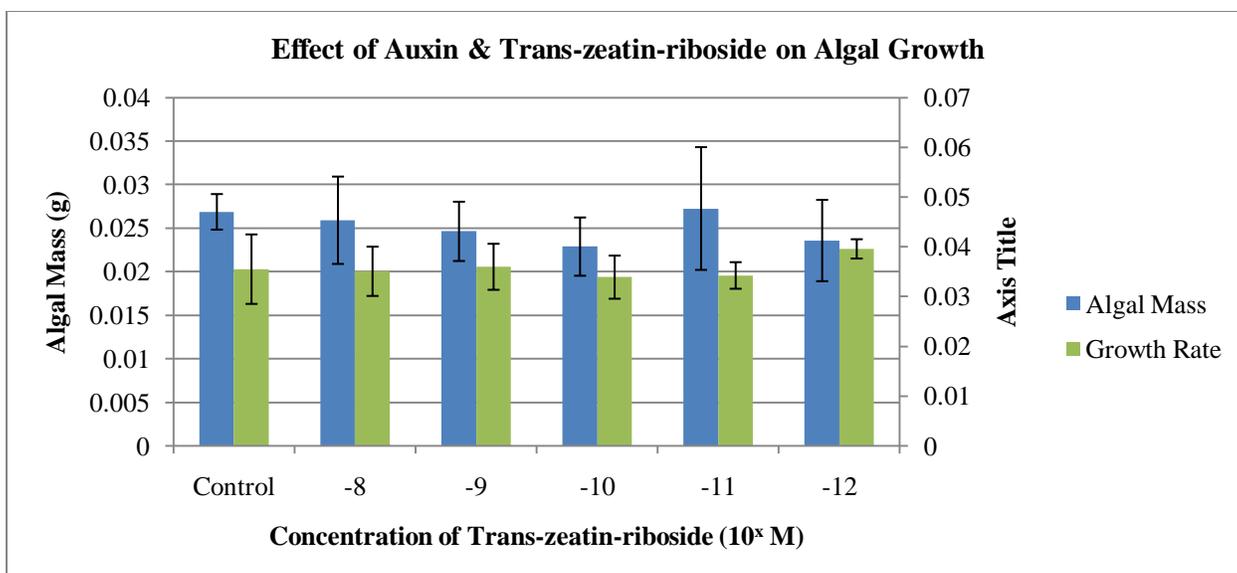


Figure 22: Graphical representation of the mean effects of various concentrations of trans-zeatin-riboside on the growth rate of *C. reinhardtii*. The concentration of auxin is 10^{-11} M.

4.2.1.7 Overall

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 7. The data shows that it is likely, but not certain, that auxin by itself has the most positive effect on growth rate of the algae, while auxin and zeatin together pose a close contender. Brassinosteroids also seem to have a positive effect on the growth of the algae. Meanwhile, the other three options (zeatin, trans-zeatin-riboside, and auxin with trans-zeatin-riboside) seem to have very little effect on the algal growth—either slightly positive or slightly negative. The growth rate and final algal mass seem to be related when they both change—the phytohormones either cause increases in both growth rate and final algal mass or decreases in both.

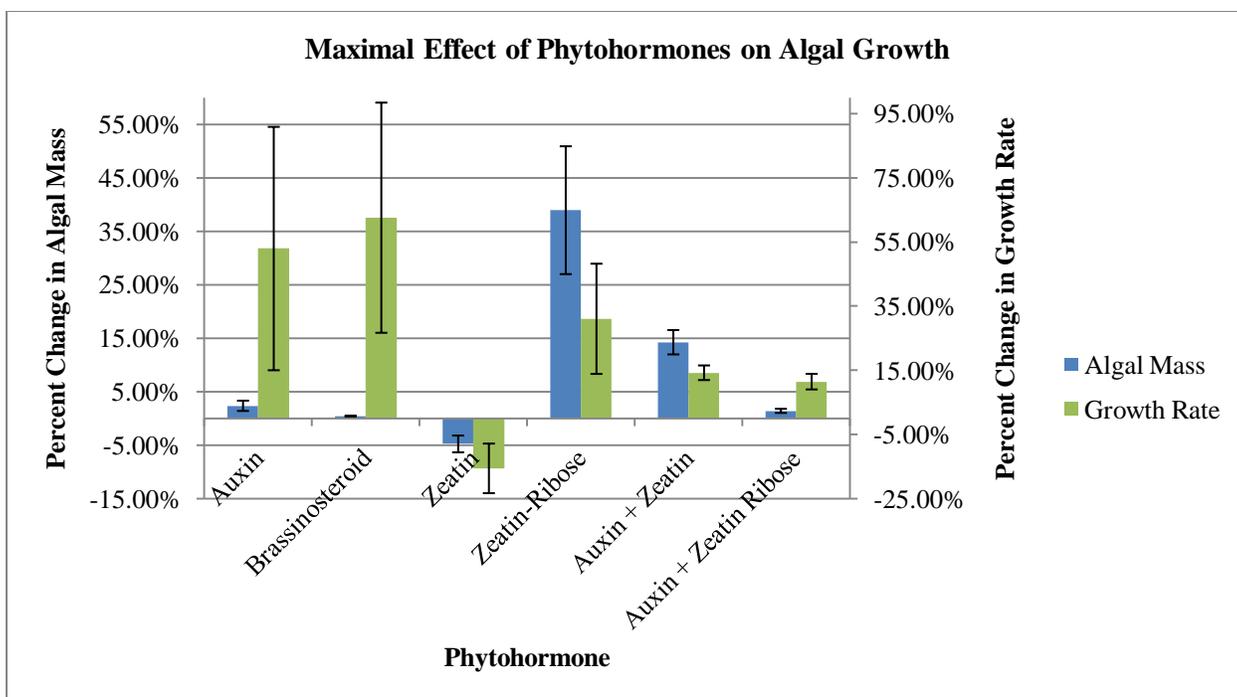


Figure 23: A graphical representation of the six most extreme changes due to phytohormone treatments on the change in growth rate of the algae. The addition of auxin and brassinolide produced the greatest increases. The concentrations for auxin, brassinolide, zeatin, and trans-zeatin-riboside were 10^{-11} , 10^{-10} , 10^{-9} and 10^{-9} M.

4.2.2 Statistical Analysis

In order to determine the validity of our results, a one-factor analysis of variance (ANOVA) was executed. ANOVA is a statistical tool that looks at several groups of data, and compares the variation within each of the groups to variation among the groups themselves. Based on these variances, ANOVA 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 to each other and a control. In other

words, each ANOVA calculation looked at a single metric (growth rate, final mass, final dry weight, lipid mass, lipid specific weight) of a single hormone, with 6 groups (1 group of controls and 5 groups, each of a single phytohormone at a single concentration). The confidence factor used was 95%.

A Matlab m-file was written to test the various concentrations of phytohormones against their respective controls, 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 [CITATION: MATHWORKS]. 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.

ANOVA tests were performed for each phytohormone based on five metrics: growth rate (absorbance/day), final mass of algae (g), final dry mass of algae (g), total mass of lipids (g), and lipid mass as a percentage of dry mass (g/g). 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 .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 .05 or

less were found, the m-file would provide 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 brassinolide showed an improvement, with a 1% chance that the difference was a result of random chance. This result was encouraging, because despite the sources of large error in the results, the ANOVA test supported the results we had obtained. The fact that none of the other groups statistically showed that they improved growth rate is discouraging, but because of the magnitude of error discussed above, effects from phytohormones cannot be conclusively ruled out.

Table 20: Growth rate and mass data statistics

Phytohormone	Metric	P value	Statistically Significant Groups
Auxin	Growth Rate (abs/day)	0.0064	11 >> 9
Auxin	Mass of Growth (g)	0.84	
Auxin	Mass of Extracted Algae (g)	0.79	
Brassinosteroid	Growth Rate (abs/day)	0.0122	12 >> 11, Control
Brassinosteroid	Mass of Growth (g)	0.41	
Brassinosteroid	Mass of Extracted Algae (g)	0.999	
Zeatin	Growth Rate (abs/day)	0.0168	Control >> 8, 9
Zeatin	Mass of Growth (g)	0.64	
Zeatin	Mass of Extracted Algae (g)	0.93	
Trans-Zeatin-Riboside	Growth Rate (abs/day)	0.484	
Trans-Zeatin-Riboside	Mass of Growth (g)	0.17	
Trans-Zeatin-Riboside	Mass of Extracted Algae (g)	0.48	
Auxin and Zeatin	Growth Rate (abs/day)	0.0214	10, 12 > 9
Auxin and Zeatin	Mass of Growth (g)	0.478	
Auxin and Zeatin	Mass of Extracted Algae (g)	0.606	
Auxin and Trans-Zeatin-Riboside	Growth Rate (abs/day)	0.558	
Auxin and Trans-Zeatin-Riboside	Mass of Growth (g)	0.7288	
Auxin and Trans-Zeatin-Riboside	Mass of Extracted Algae (g)	0.4027	

4.3 Lipid Analysis

4.3.1 Lipid Dry Weights

The dry weight of the lipids after extraction was divided by the mass of the algae from which the lipids were extracted. This calculation resulted in the specific weight of lipid after the treatment. This information shows 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 weight is a good measure of a phytohormone's potential in increasing lipid yields.

4.3.1.1 Auxin

The application of auxin resulted in higher lipid yield at 10^{-10} M concentration, while causing decreases in specific weight for certain phytohormone concentrations. Taking into account the experimental uncertainty, however, makes it difficult to draw any conclusions—the yields are approximately the same.

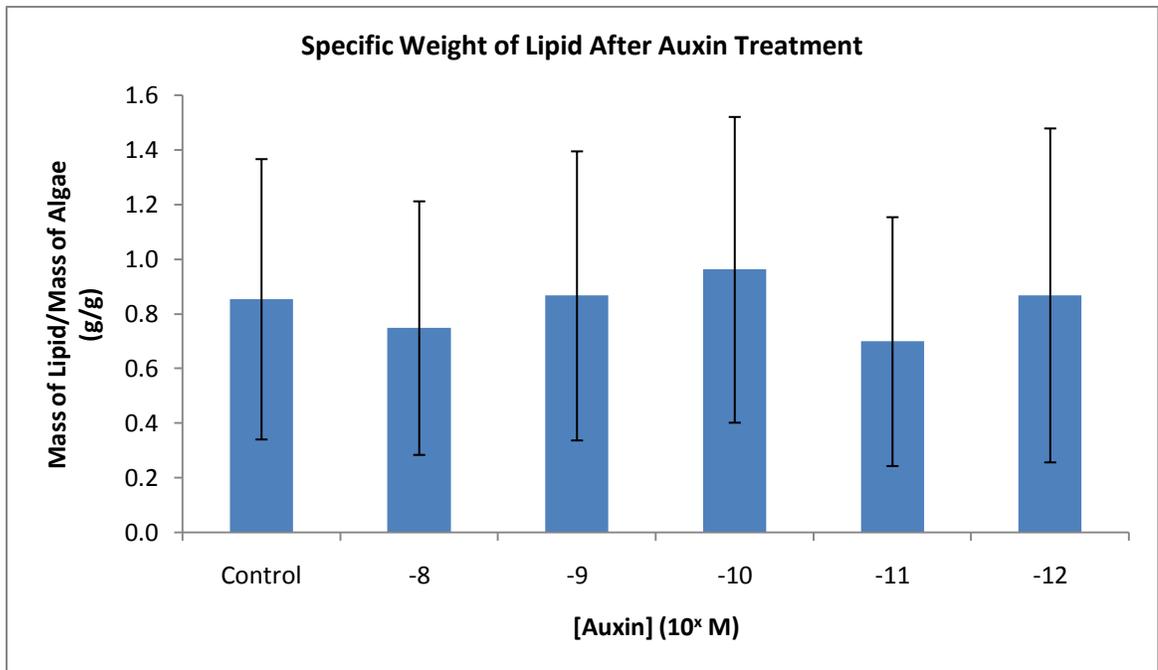


Figure 24: Graphical representation of change in lipid –algae mass ratio after application of auxin

4.3.1.2 Brassinolide

Application of brassinolide to algae resulted in a barely noticeable increased lipid yield at all concentrations except for 10^{-8} M.

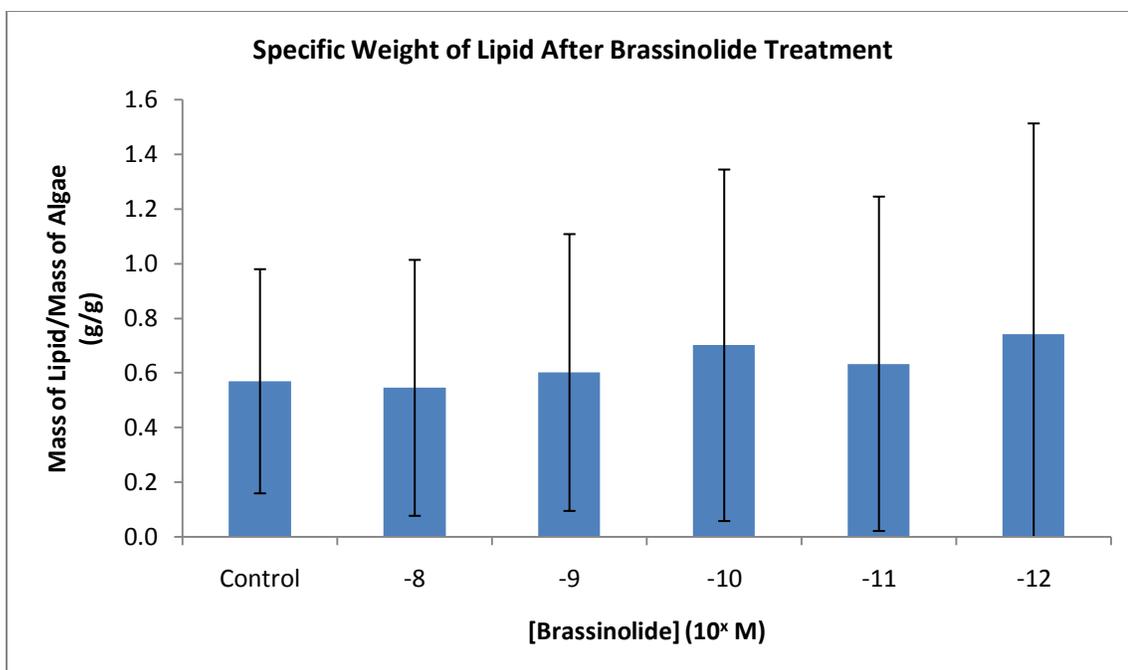


Figure 25: Graphical representation of change in lipid –algae mass ratio after application of brassinolide

4.3.1.3 Zeatin

Lipid yield resulting from application of zeatin was changed very little in all but 10⁻⁸ M concentration. At the 10⁻⁸ M concentrations, algae appeared to produce less lipid per gram of cell weight.

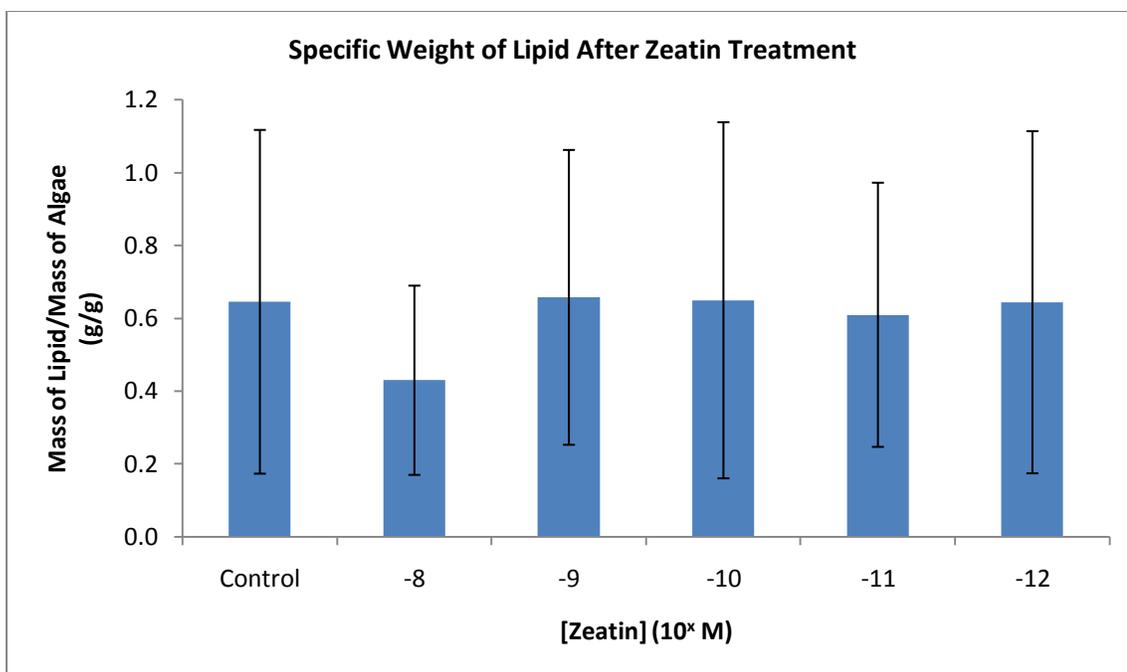


Figure 26: Graphical representation of change in lipid–algae mass ratio after application of zeatin.

4.3.1.4 Trans-zeatin-riboside

Application of trans-zeatin-riboside decreased lipid yield slightly at most concentrations, but decreased it noticeably at 10^{-8} M concentration. The 10^{-10} M concentration of zeatin-ribose allowed it to have the least detrimental effect on the

algae's specific weight of lipid.

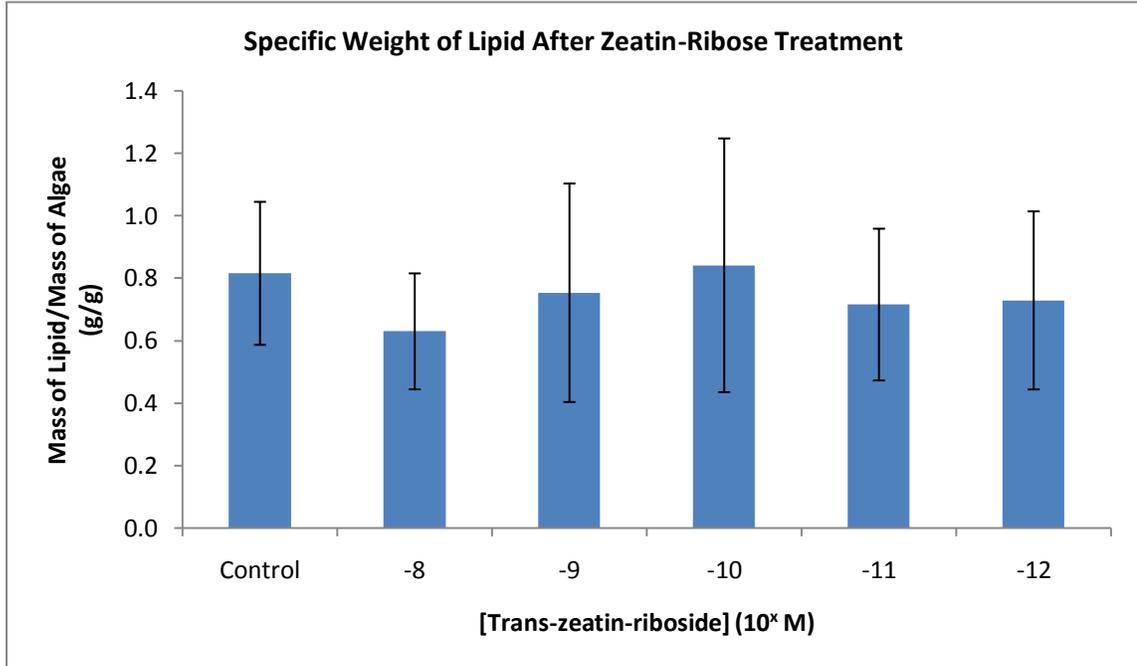


Figure 27: Graphical representation of change in lipid –algae mass ratio after application of trans-zeatin-ribose

4.3.1.5 Auxin & Zeatin

Application of auxin & zeatin substantially decreased lipid yield at all concentrations except for 10^{-9} M, at which lipid yield remained the same.

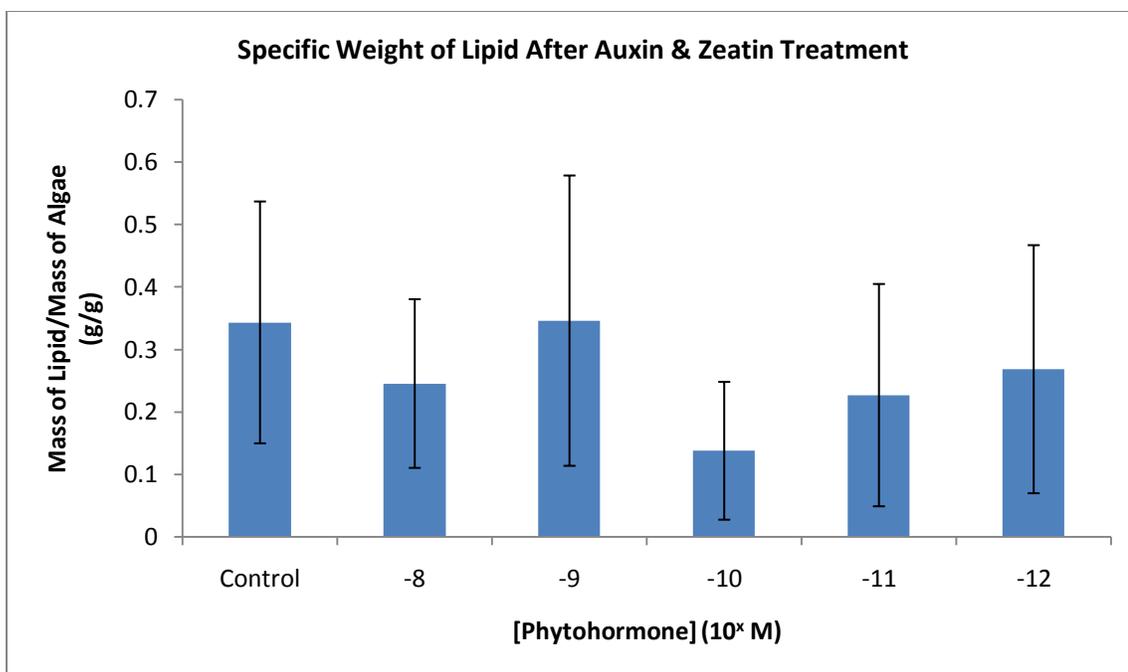


Figure 28: Graphical representation of change in lipid –algae mass ratio after application of auxin & zeatin

4.3.1.6 Auxin & Trans-zeatin Riboside

Application of auxin and zeatin ribose increased lipid yield noticeably at 10^{-9} and 10^{-10} M concentrations, but 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.

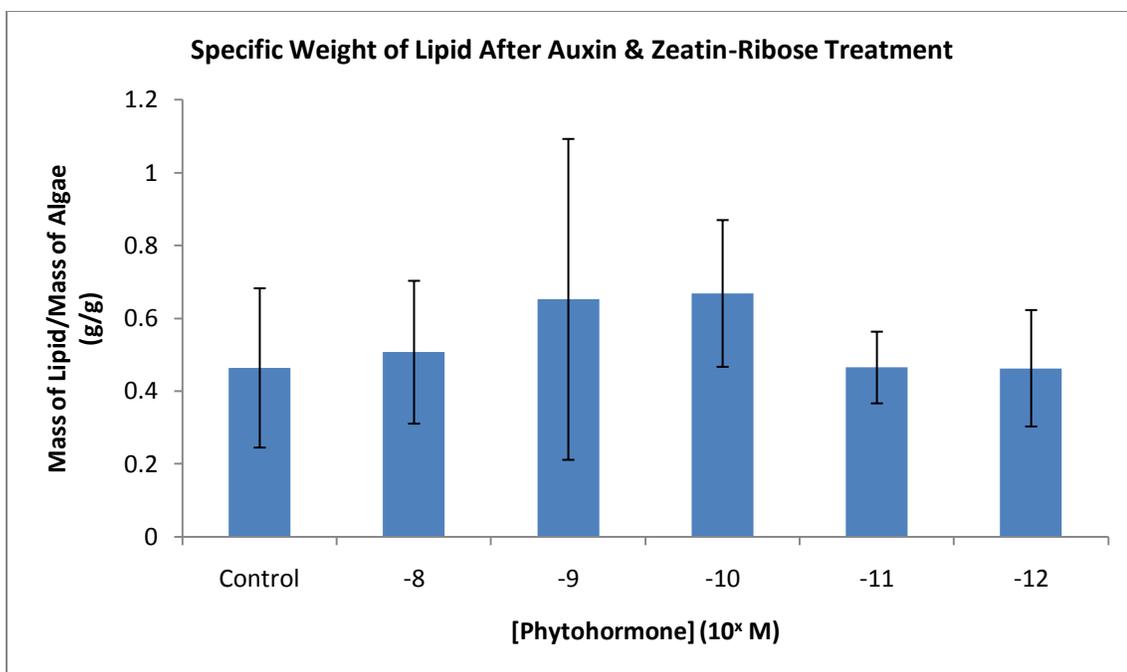


Figure 29 Graphical representation of change in lipid –algae mass ratio after application of auxin trans-zeatin-ribose

4.3.1.7 Overall

The phytohormone treatments had varying effects on algal lipid yield. Auxin, trans-zeatin riboside, and the auxin – trans-zeatin-ribose combination increased lipid

yield. Brassinolide, zeatin, and the auxin – zeatin combination decreased lipid yield.

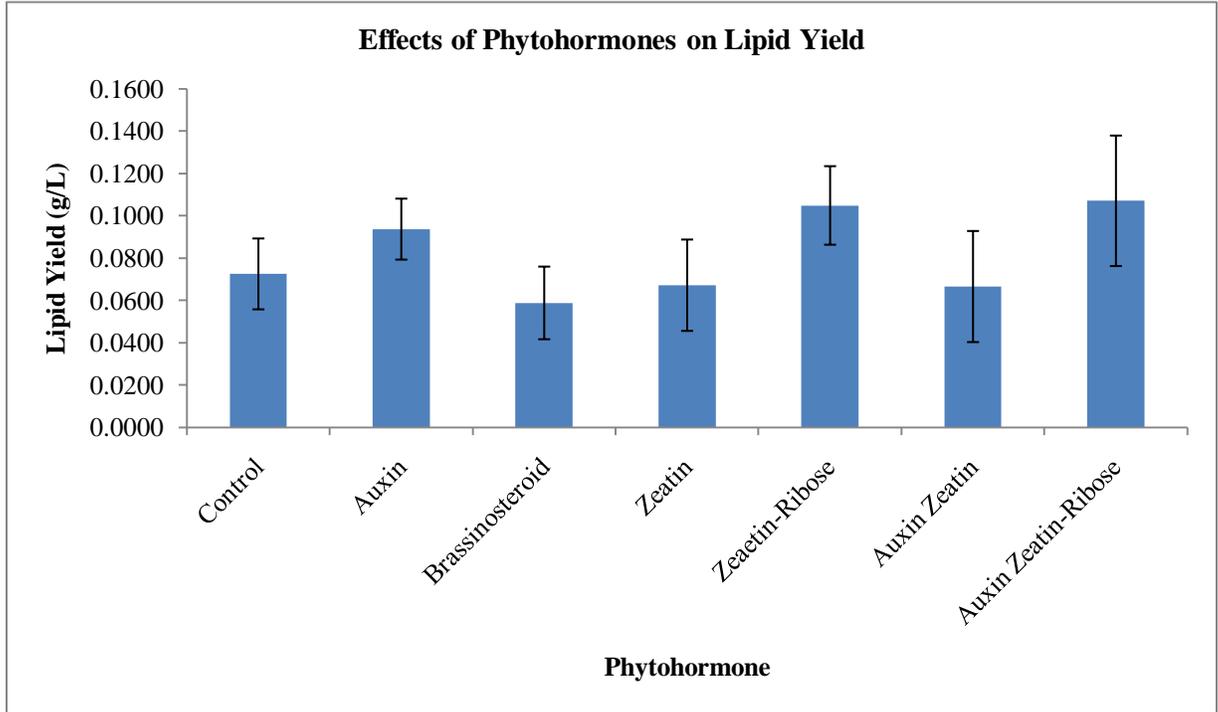


Figure 30: Effect of various phytohormones on algal lipid yield.

4.3.2 Lipid Concentration

The lipid concentration was calculated as in A.6. The lipid concentration was a measure of how able the algae was to generate lipid after the application of the phytohormone.

4.3.2.1 Auxin

The effect of auxin on the lipid concentration was varied, leading to the belief that there was no significant dose response. The 10^{-8} and 10^{-11} M concentrations both had a decreased lipid concentration. The 10^{-9} and 10^{-12} M concentrations, however, had a

slightly opposite effect. After taking into account the experimental uncertainty, there appears to be no significant change.

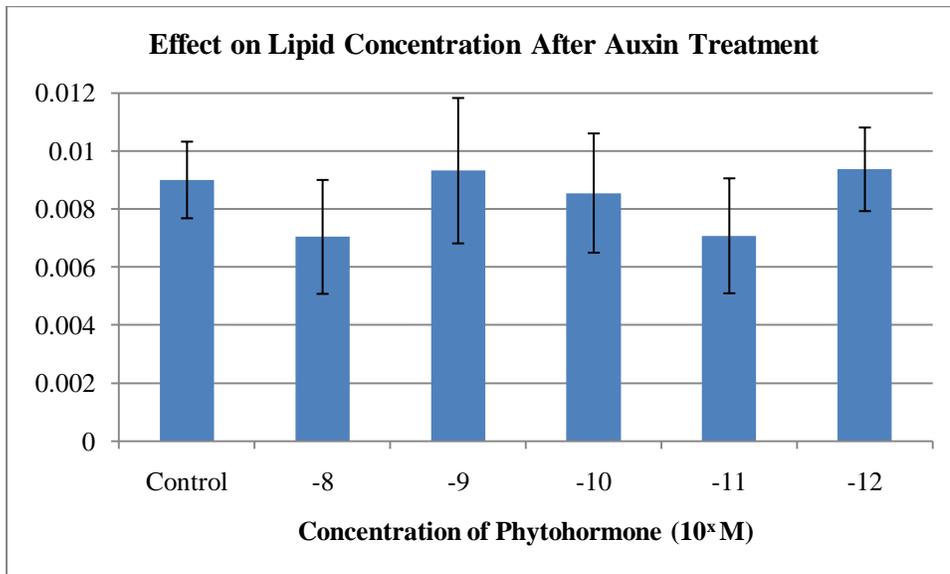


Figure 31: The effect of auxin on the lipid concentration of the algae after 10 days of growth with n=8.

4.3.2.2 Brassinolide

Brassinolide did not seem to have a significant effect on the overall lipid concentration at any concentration of the phytohormone. There was no significant change in any of the values and the experimental uncertainty renders the data essentially the same.

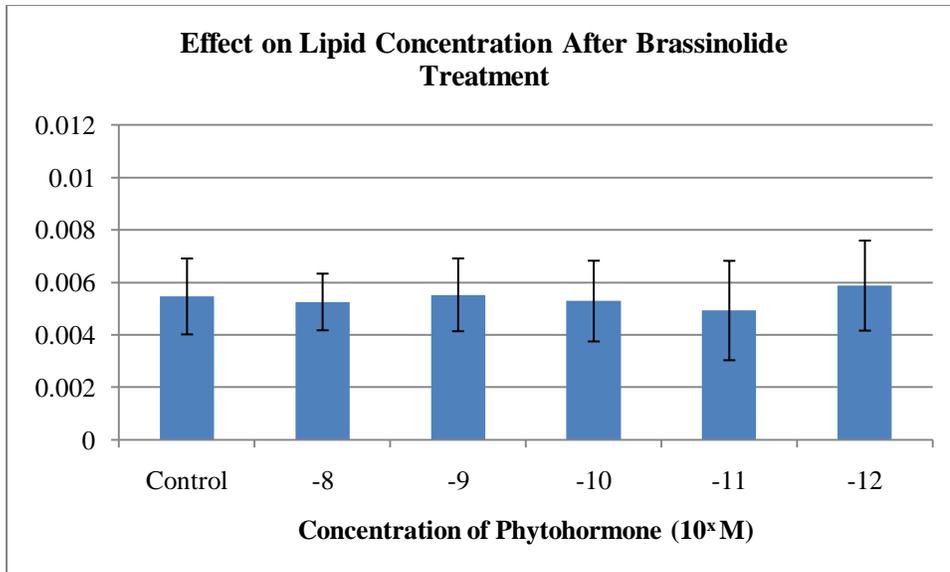


Figure 32: The effect of brassinolide on the lipid concentration of the algae after 10 days of growth with n=8.

4.3.2.3 Zeatin

Zeatin caused a noticeable decrease in the lipid concentration at the 10⁻⁸ M level, but did not appear to have as detrimental of an effect at the lesser concentrations. None of the trials with zeatin applied, however, were able to reach the control group in terms of lipid concentration.

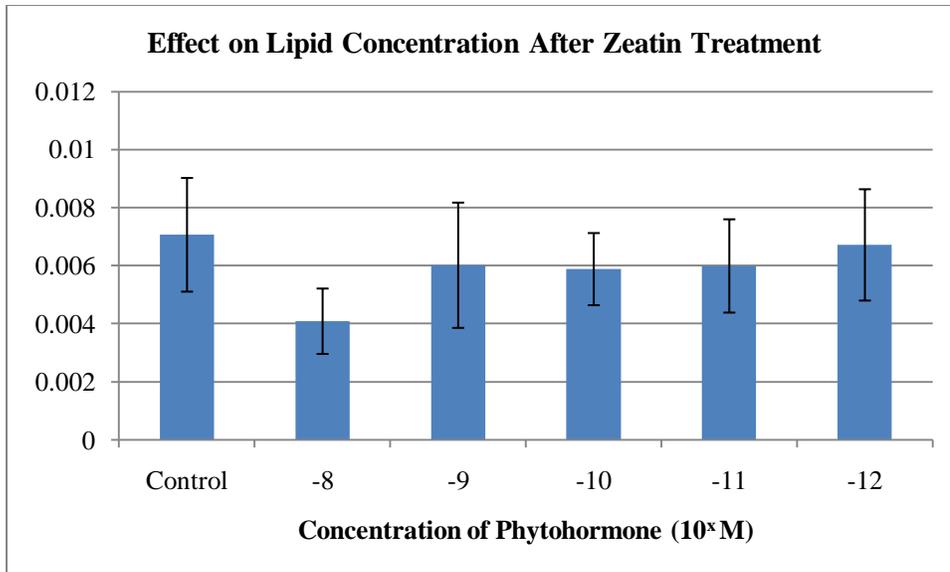


Figure 33: The effect of zeatin on the lipid concentration of the algae after 10 days of growth with n=8.

4.3.2.4 Trans-zeatin-riboside

The effect of trans-zeatin-riboside was positive for all trials except for 10⁻⁸ and 10⁻¹² M concentrations of the phytohormone. The lipid concentrations were approximately 20% greater for the other three concentrations: 10⁻⁹ M, 10⁻¹⁰ M, and 10⁻¹¹ M. The apparent benefit from the addition of trans-zeatin-riboside led to experimentation with the combination of auxin and the phytohormone.

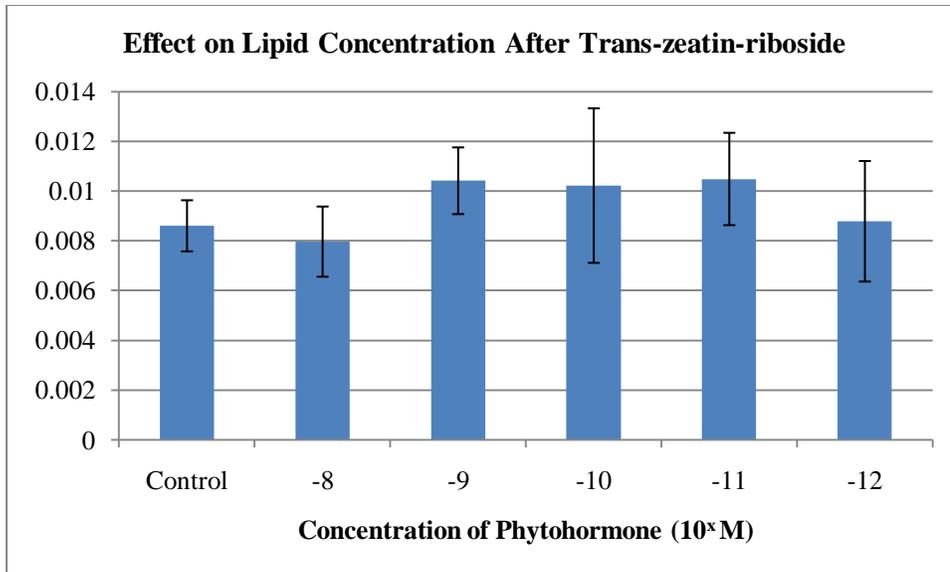


Figure 34: The effect of trans-zeatin-riboside on the lipid concentration of the algae after 10 days of growth with n=8.

4.3.2.5 Auxin & Zeatin

The concentration of auxin was held constant at 10^{-11} M. The combination of auxin and zeatin did not have a positive effect on the lipid concentration of the algae. There were no concentrations at which the lipid concentration was significantly higher; in fact, at 10^{-10} M zeatin, there was a $55.3 \pm 31.2\%$ decrease in lipid concentration.

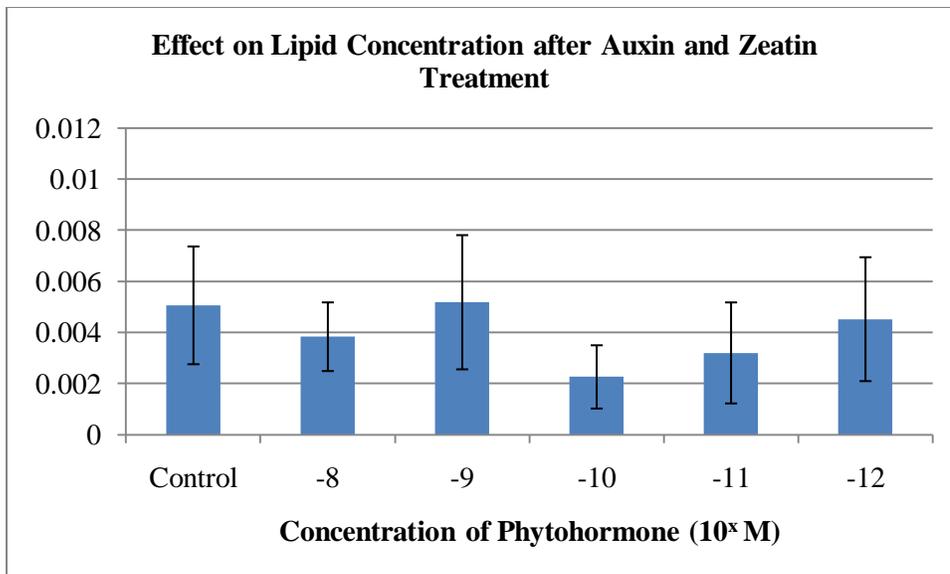


Figure 35: The effect of auxin at 10^{-11} M and varying concentrations of zeatin on the lipid concentration of the algae after 10 days of growth with n=8.

4.3.2.6 Auxin & Trans-zeatin-riboside

The effect of the combination of auxin and trans-zeatin-riboside seemed to have an increase in lipid concentration at most concentrations of the phytohormones. As in 4.3.2.5, the concentration of auxin was held constant at 10^{-11} M, while the concentration of trans-zeatin-riboside was varied. The 10^{-9} and 10^{-10} M concentrations of trans-zeatin-riboside had the largest increase in lipid concentration.

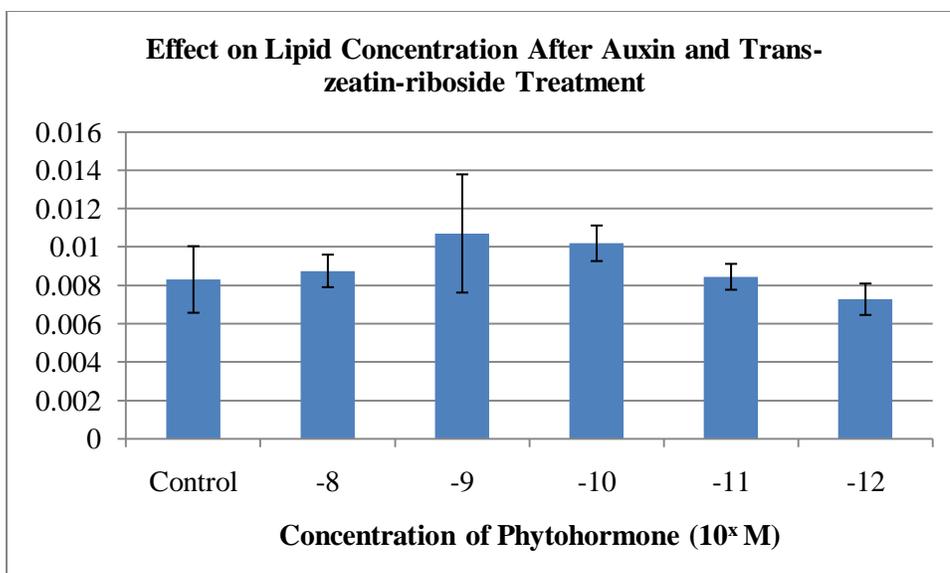


Figure 36: The effect of auxin at 10^{-11} M and trans-zeatin-riboside at varying concentrations on the lipid concentration of the algae after 10 days of growth with n=8.

4.3.2.7 Overall

The lipid concentration is one of the measures of algal lipid yield optimization that is important in considering the effectiveness of a phytohormone in increasing biofuels production. The phytohormones that showed the most promise in increasing lipid concentration was the combination of auxin and trans-zeatin-riboside at concentrations of 10^{-11} and 10^{-9} M respectively.

4.3.3 Statistical Significance

As with the growth rate trials, the lipid data was run through ANOVA to test for statistical significance. 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-riboside treatments were statistically different from each other (10^{-9} M had a higher lipid specific weight than both 10^{-8} and

10⁻¹²M), but were inconclusive when compared against the control. Table 8 shows the results below. The large error associated with our measurements are likely a result of the insufficient number of trials, biological variability, and human error. As a result lipid concentration tests could not be run with the combinations of multiple phytohormones in any meaningful way.

Table 21: Statistical analysis of lipid data

<i>Phytohormone</i>	<i>Metric</i>	<i>P value</i>	<i>Statistically Significant Groups</i>
Auxin	Mass of Extracted Lipids (g)	0.53	
Auxin	Lipid Mass per Total Mass (g/g)	0.9	
Brassinosteroid	Mass of Extracted Lipids (g)	0.73	
Brassinosteroid	Lipid Mass per Total Mass (g/g)	0.47	
Zeatin	Mass of Extracted Lipids (g)	0.28	
Zeatin	Lipid Mass per Total Mass (g/g)	0.79	
Trans-Zeatin-Riboside	Mass of Extracted Lipids (g)	0.13	
Trans-Zeatin-Riboside	Lipid Mass per Total Mass (g/g)	0.0125	9 >> 8, 12
Auxin and Zeatin	Mass of Extracted Lipids (g)	0.5577	
Auxin and Trans-Zeatin-Riboside	Mass of Extracted Lipids (g)	0.8457	

4.4 Liquid Chromatography – Mass Spectrometry

4.4.1 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 the total masses 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.

4.4.2 Control Results

Figures 31 and 32 show typical TIC and TAC chromatograms for the control group, respectively. Because of the gradient, the acetonitrile did not have too much effect on the TIC baseline until after about 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 being passed through during the flushing phase.

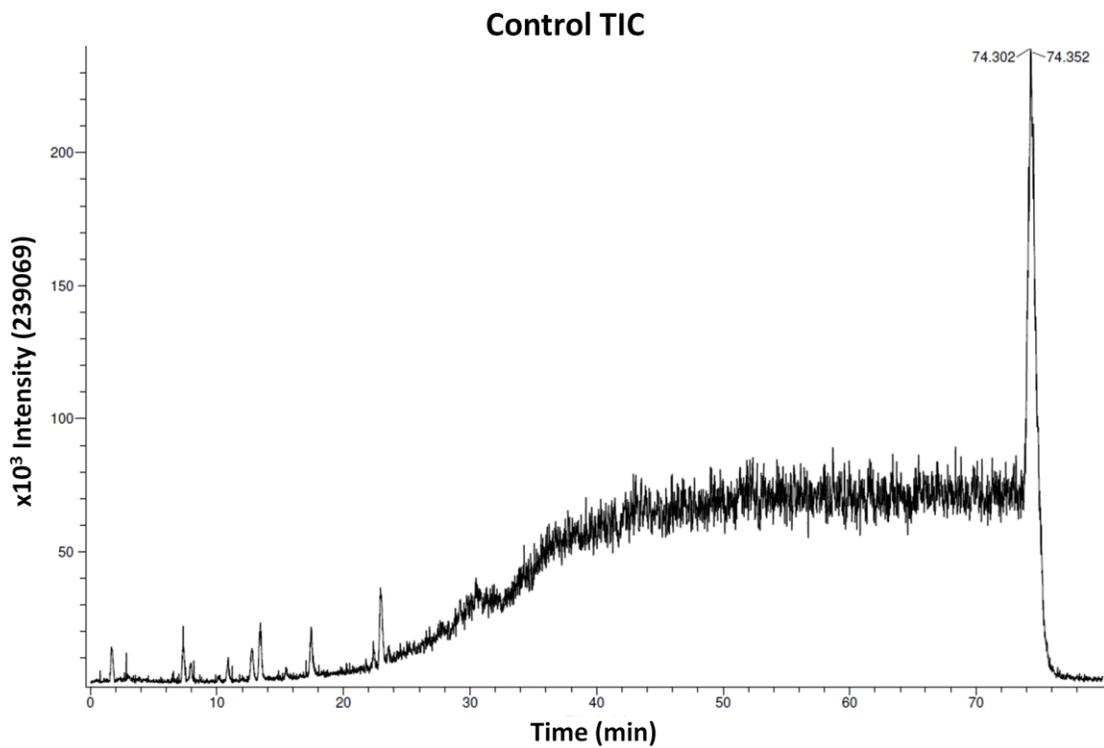


Figure 37: Total intensity chromatogram for a typical control sample. The noticeable increase in the baseline is due to the concentration of acetonitrile increasing.

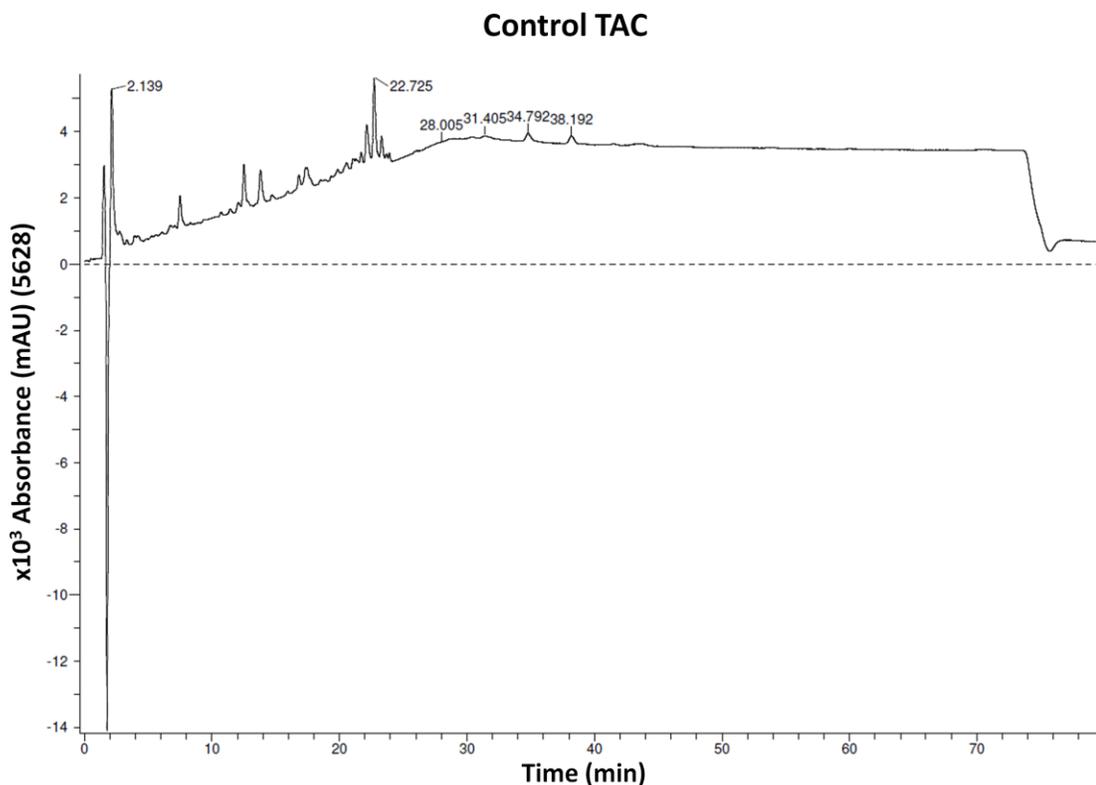


Figure 38: Total absorbance chromatogram for a typical control sample. Peaks can be seen past the 20 minute mark that cannot be seen in the TIC. It is possible that other peaks are being hidden by the solvent. However those peaks are relatively small, so their impact on the total lipid analysis is not too large.

From these two figures, we had to determine 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. 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. 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 33 and 34 below are magnifications of Figures 31 and 32. The peaks are marked by time stamps as shown in the figures.

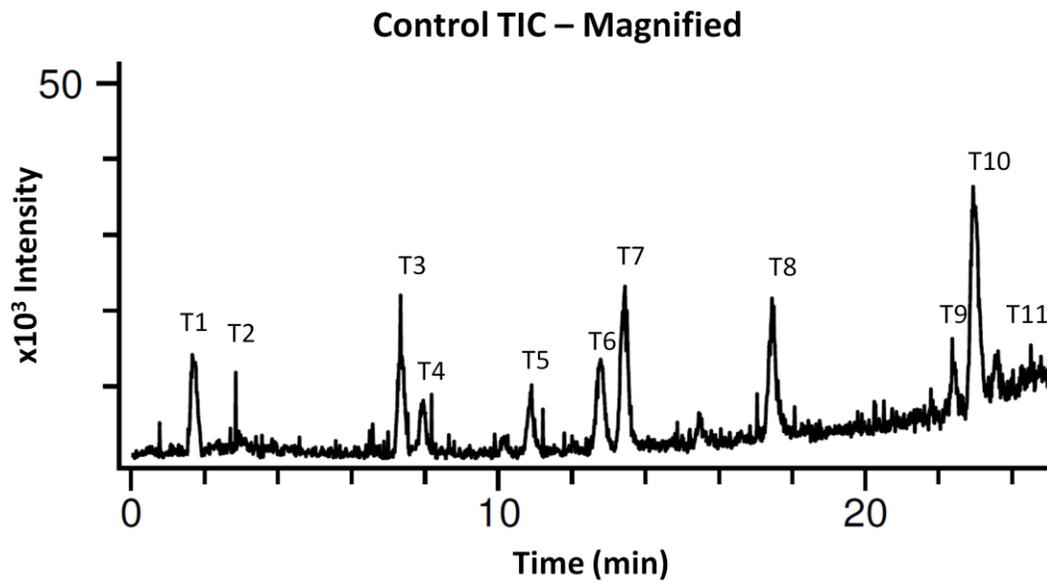


Figure 39: Magnified view of the Control TIC in Figure 31.

Control TAC – Magnified

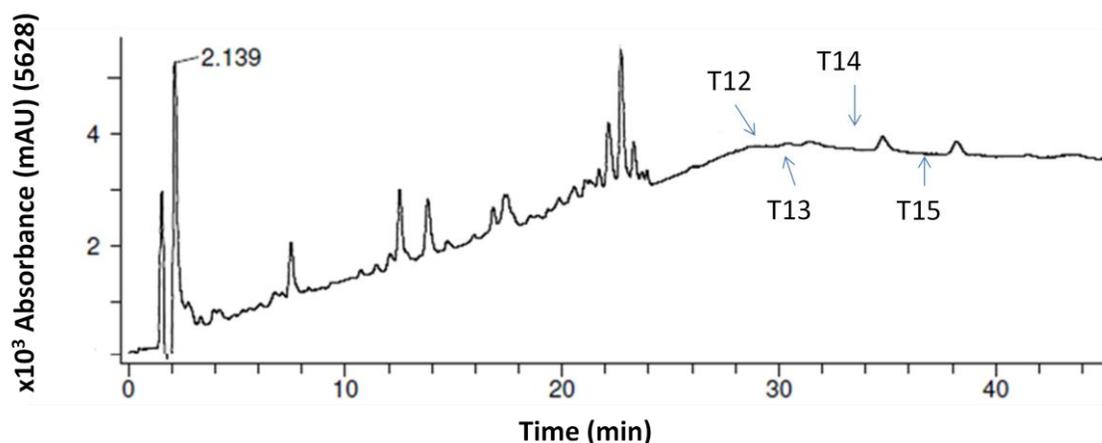


Figure 40: Magnified view of the Control TAC in Figure 32.

After marking the peaks in the chromatograms, each peak was analyzed to determine which lipids were present in the peak. Shown below in Figures 33 and 34 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 were identified by their m/z values found in literature or an online lipid database [cite 1]. The identity of the lipids at T3 can be found in Table 9. The identity of the lipids at T7 can be found in Table 10. All of the identified lipids found at each time point across all control samples were aggregated and displayed in Table 11. Note that the thick black lines in the mass chromatograms of Figure 24 and 25 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.

Mass Chromatogram of Control Timepoint 3

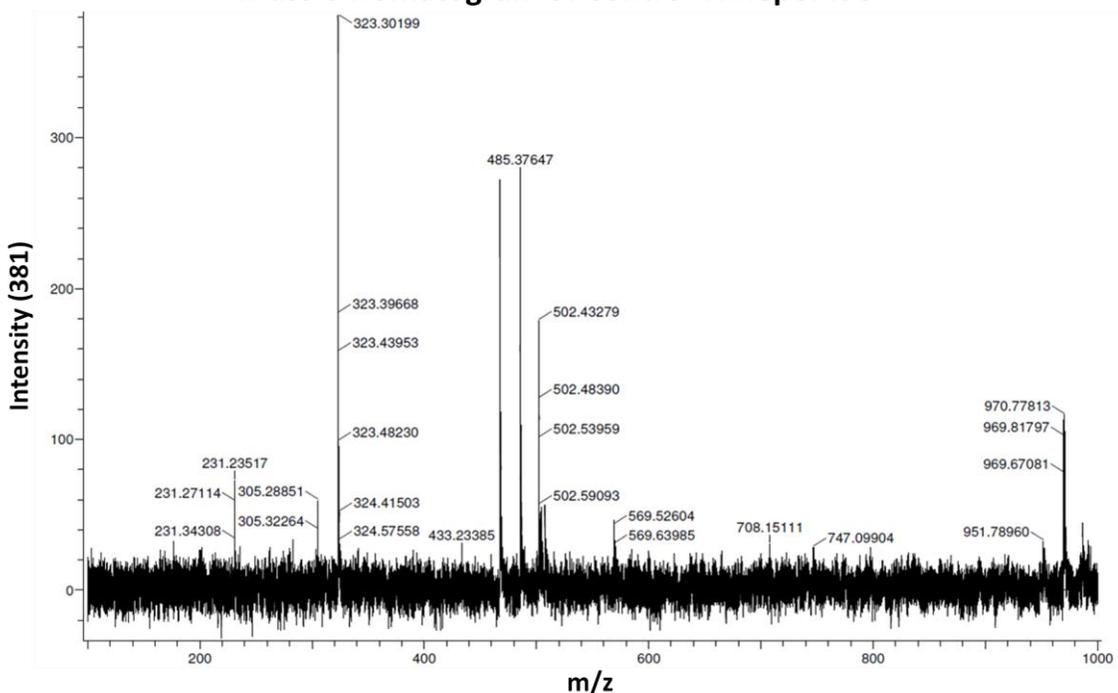


Figure 41: Mass chromatogram of timepoint 3 which corresponds to the peak at 7 minutes and 30 seconds in the TIC and TAC. The identities of the peaks are shown in Table 9.

Table 22: Compilation of m/1 peaks and corresponding lipids at T3. The classes of the lipids found are glycerophospholipids, diacylglycerols, and free fatty acids.

Lipids Found At T3	
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

Mass Chromatogram of Control Timepoint 7

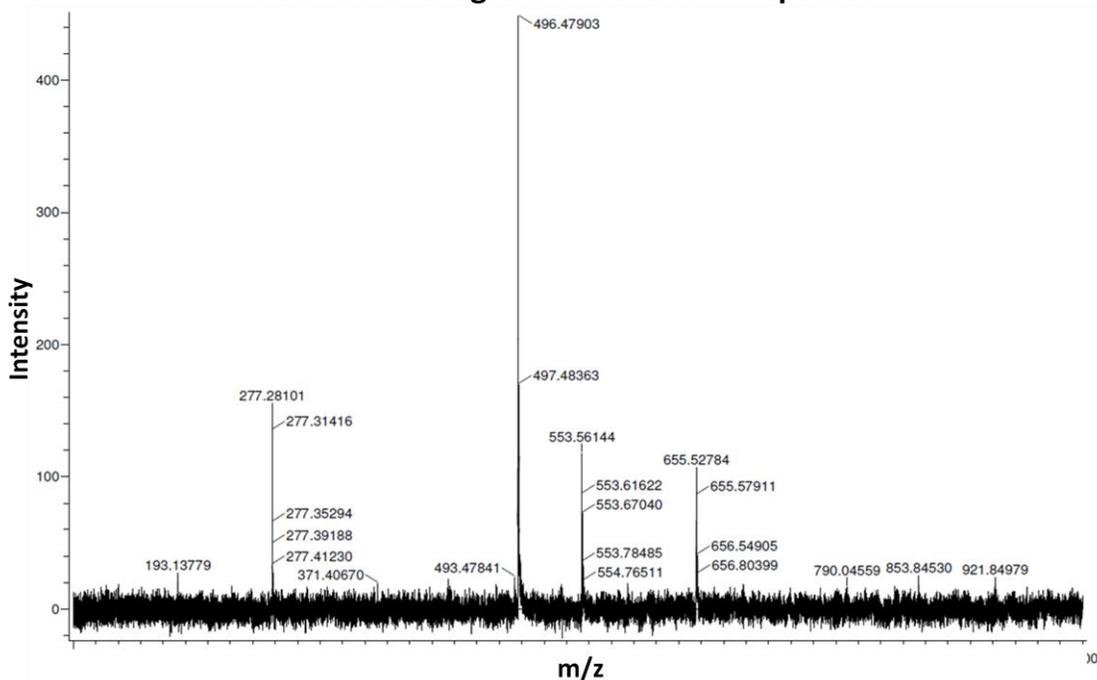


Figure 42: Mass chromatogram of timepoint 7 which corresponds to the peak at the TIC and TAC at 13 minutes and 30 seconds. The identities of the lipid were are shown in Table 22.

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

Lipids Found At T7	
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
105	2-Amino-3-hydroxypropanoic acid	Amino Fatty Acids
146	3-hydroxy-3-methyl-2-oxo-pentanoic acid	Free Fatty Acids

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
639	Diacylglycerol (17:0/20:0)	Diacylglycerides
639	Diacylglycerol (18:0/19:0)	Diacylglycerides
639	End of run flush	
639	Triacylglycerol(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

4.4.2.1 Phytohormone Application

The follow 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. It is important to note that only salient lipids are listed in the tables; lipids that are present in the controls can still be present in the phytohormone-treated samples. 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.

4.4.2.2 Auxin at 10^{-8} M

The first treatment was the Auxin treatment. Figure 26 shows the TIC for Auxin at a 10^{-8} M dose. This first plot looks very similar to the control as the same peaks are observed at the same times. Figure 27 shows the M/Z peaks which come from T10.

There are a number of important lipids that were observed for this treatment that are in different amounts than the controls. Table 12 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).

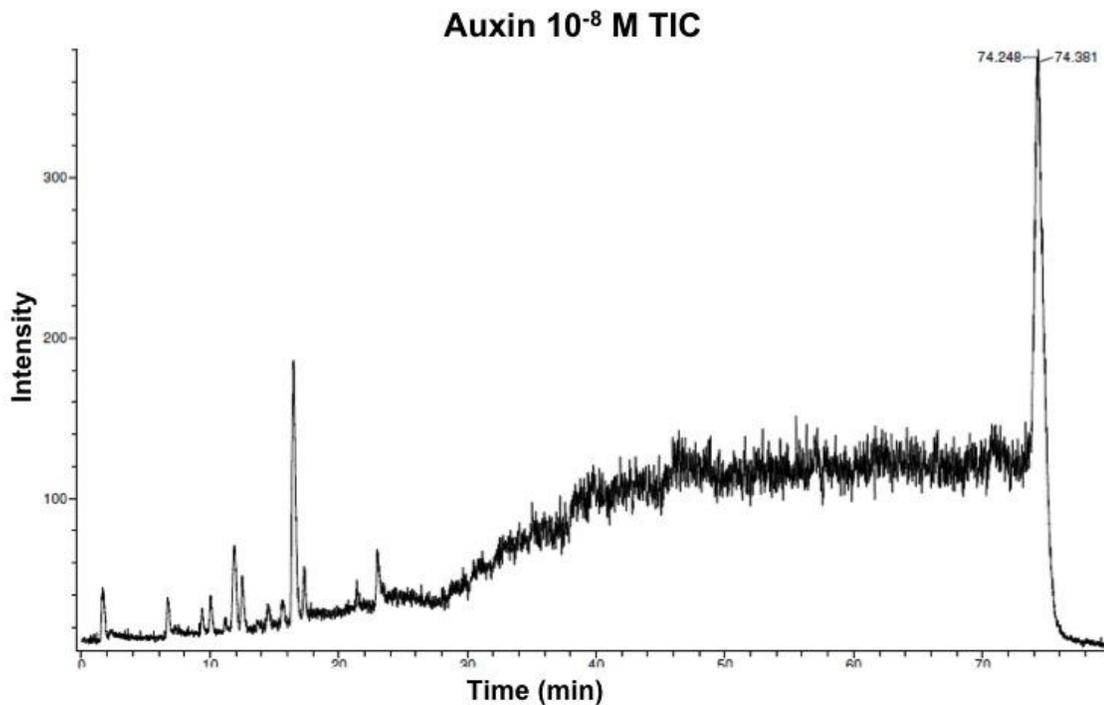


Figure 43: TIC of auxin at 10⁻⁸ M. Notice the increases at the peak around T10 (16 minutes). The mass chromatogram of the peaks is found in Figure 38.

Mass Chromatogram of Auxin (10^{-8} M) Timepoint 10

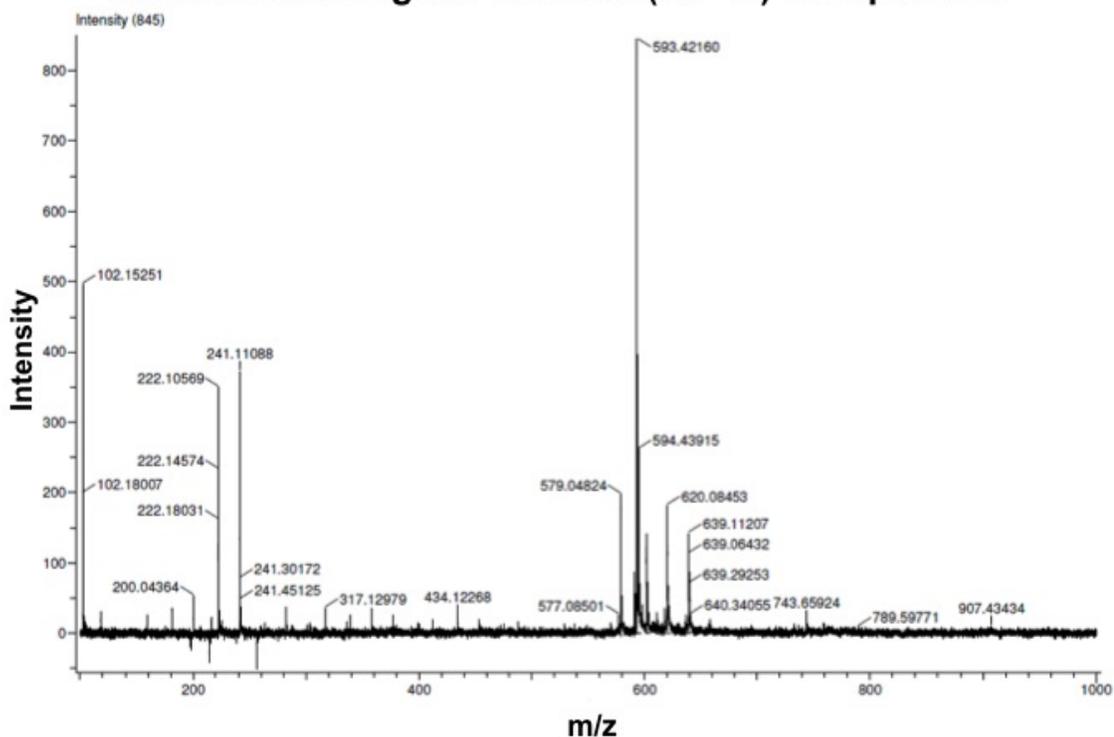


Figure 44: Mass chromatogram of T10 of auxin at 10^{-8} M. The identities of the lipids are shown in Table 25.

Table 25: Compilation of the production of lipids that deviated from control s for auxin 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	□

4.4.2.3 Auxin at 10^{-11} M

The next phytohormone treatment that showed a noticeable change in the lipid production was auxin at 10^{-11} M. 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 of Auxin (10⁻¹¹ M) Timepoint 14

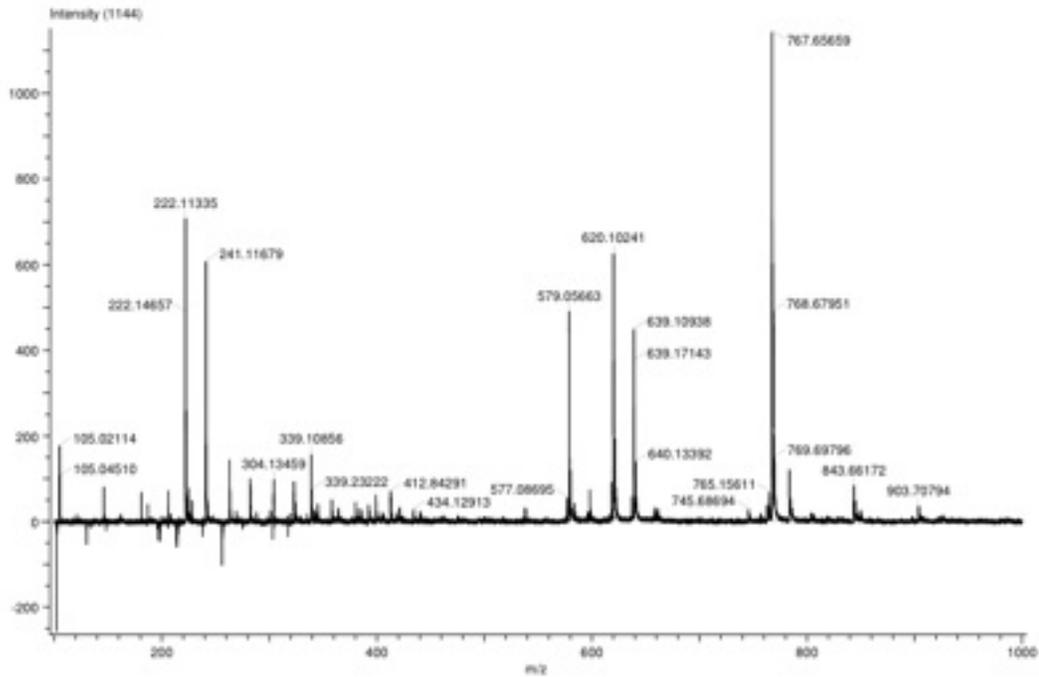


Figure 45: Mass chromatogram of timepoint 14 for the treatment of auxin at 10⁻¹¹ M. The identities of the lipids are shown in table 26.

Table 26: Compilation of the identified lipids produced from a treatment of auxin 10^{-11} M that deviated from the controls.

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

4.4.2.4 Brassinolide at 10^{-9} M

The second treatment was brassinosteroid at 10^{-9} M. Increased levels of Dodecatetraenedioic acid, N-(3-oxo-octanoyl)-homoserine lactone, Elmircic 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 brassinosteroid treatment but not the control. These lipids are from the free fatty acids and glycerophosolipids categories.

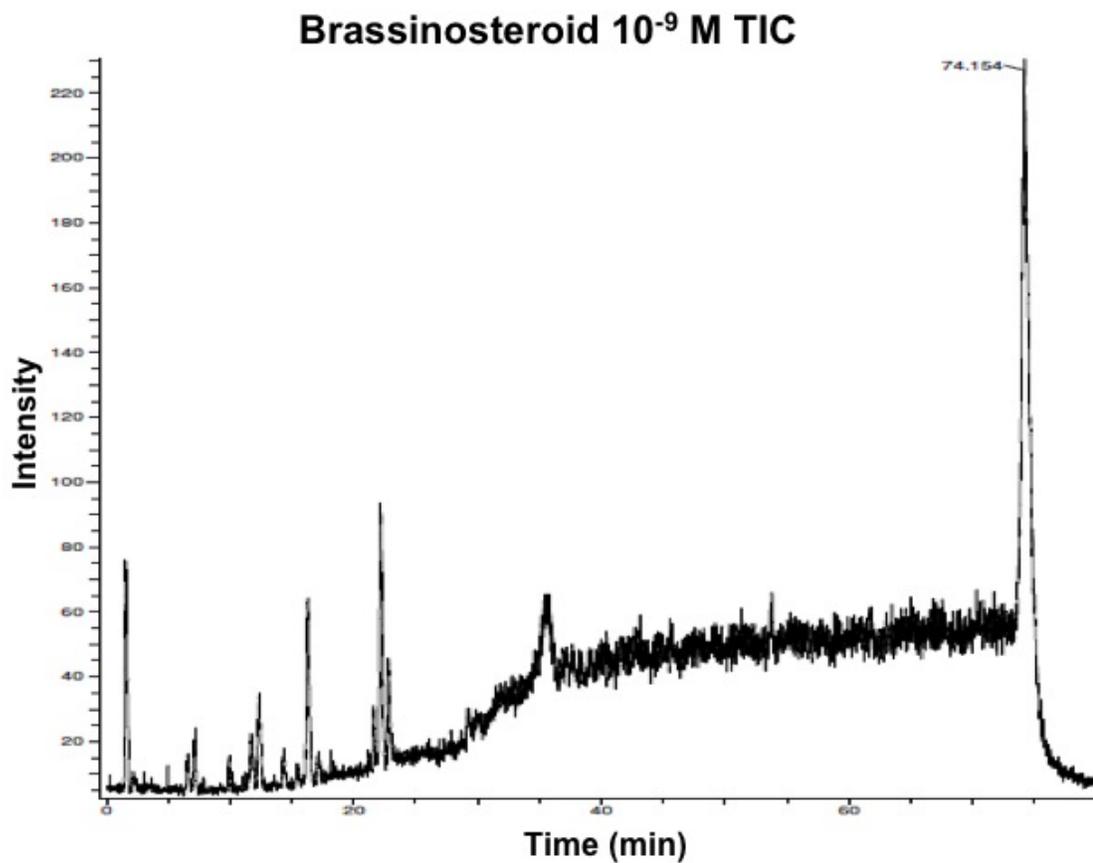


Figure 46: TIC of brassinosteroid at 10^{-9} M. Noticeable changes in lipids are found at T13. The mass chromatograms of T13 is shown in Figure 41.

Mass Chromatogram of Brassinosteroid (10^{-9} M) Timepoint 13

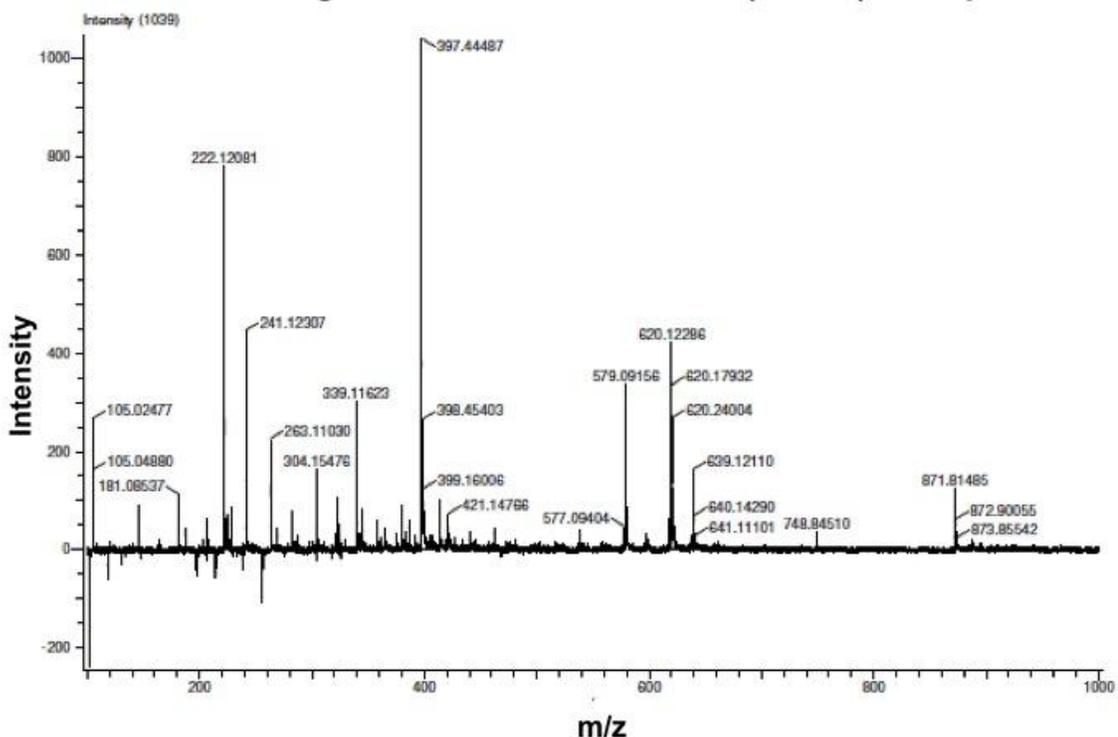


Figure 47: Mass chromatogram of T13 after a treatment of brassinosteroid 10^{-9} M. The identity of the peaks were found in Table 27.

Table 27: Compilation of identified lipids from a treatment of brassinosteroid 10^{-8} that deviated from the control.

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

4.4.2.5 Zeatin at 10^{-8} M

The next treatment was zeatin at 10^{-8} M. Peaks of interest for zeatin treatments were found at the m/z values of 609, 743, and 816. 609 correlates to diacylglycerol within the larger class of diacylglycerides. This molecule was found at increased levels as compared to the control. 743 corresponds to monogalactosyl-monoacylglycerol a member of the glycerolipids family. This molecule was also found at increased levels as compared to the control. The final m/z value of 816 corresponds to glycerophosphocholine which is a member of the glycerophospholipid family and was also found at an increased level.

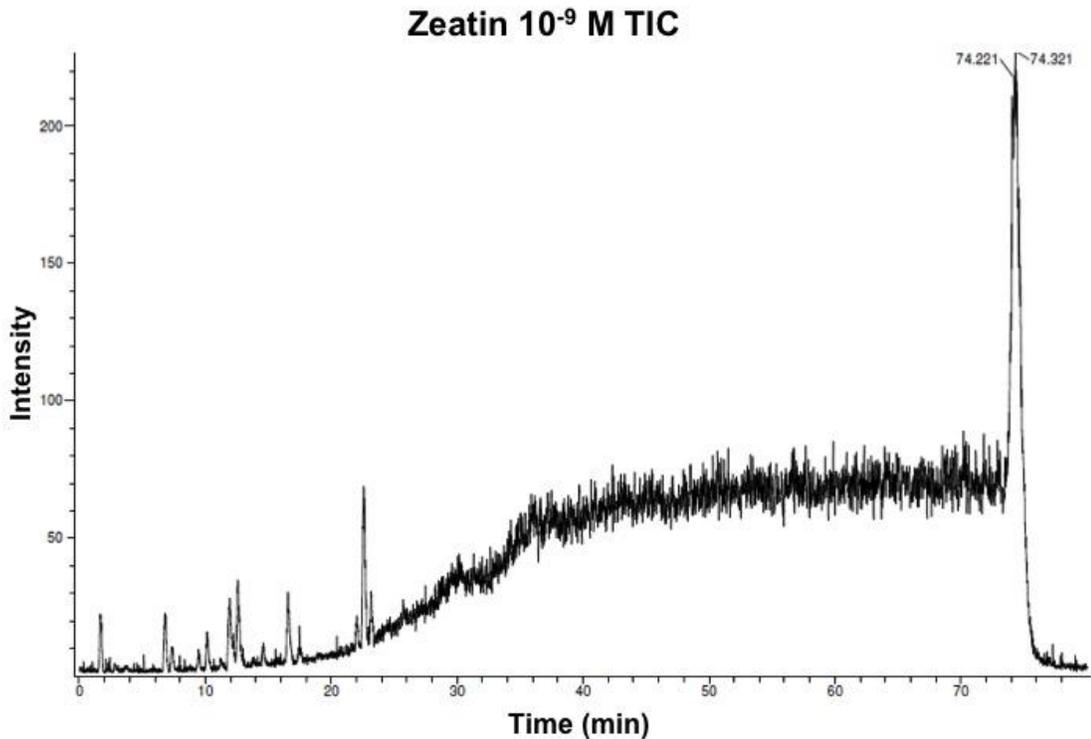


Figure 48: TIC of lipids after treatment of zeatin at 10^{-9} M. The lipids at T9 showed a difference in the lipids found in the control. The mass chromatogram of T9 is found in Table 43.

Mass Chromatogram of Zeatin (10^{-9} M) Timepoint 9

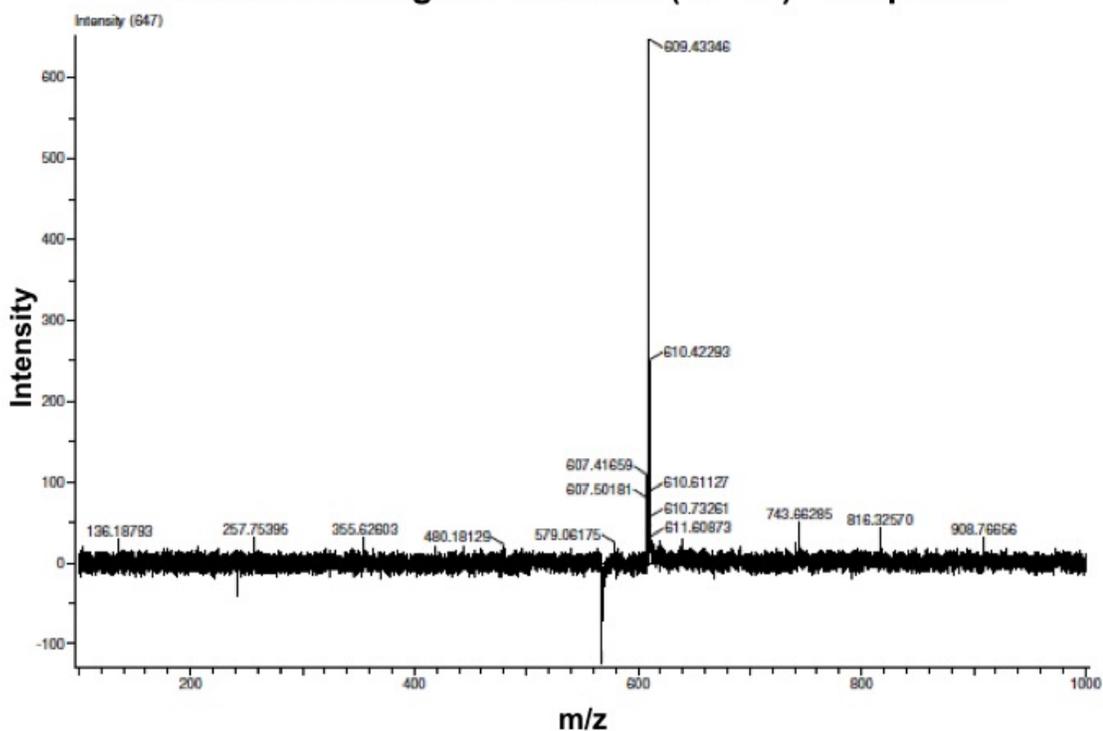


Figure 49: Mass chromatogram of the lipids found at T9. The identities of the lipids are found in Table 28.

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

m/z	Molecule Assignment	Class	Change
609	Diacylglycerol (16:1)	Diacylglycerides	<input type="checkbox"/>
743	Monogalactosyl-monoacylglycerol (MGMG 32:6)	Glycerolipids	<input type="checkbox"/>
816	Glycerophosphocholine (18:1/20:0)	Glycerophospholipids	<input type="checkbox"/>

4.4.2.6 Zeatin-ribose at 10^{-11} M

The final treatment was zeatin-ribose at 10^{-11} M. 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. Trilauroyl-glycerol was present, but it could not be quantified.

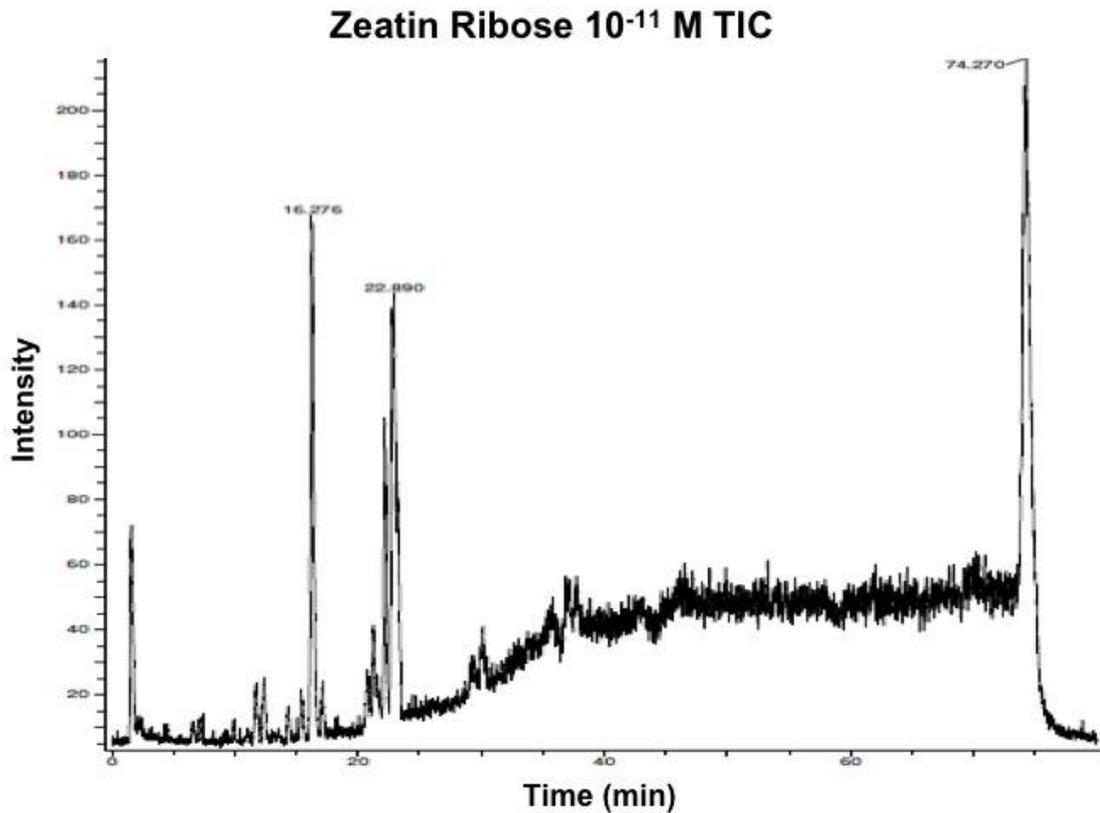


Figure 50: TIC of lipids after a treatment of zeatin ribose at 10^{-11} M. The mass chromatograms of T8 and a new peak at 28.2 minutes are found in Table 45 and 46.

Mass Chromatogram of Zeatin Ribose (10^{-11} M) Timepoint 8

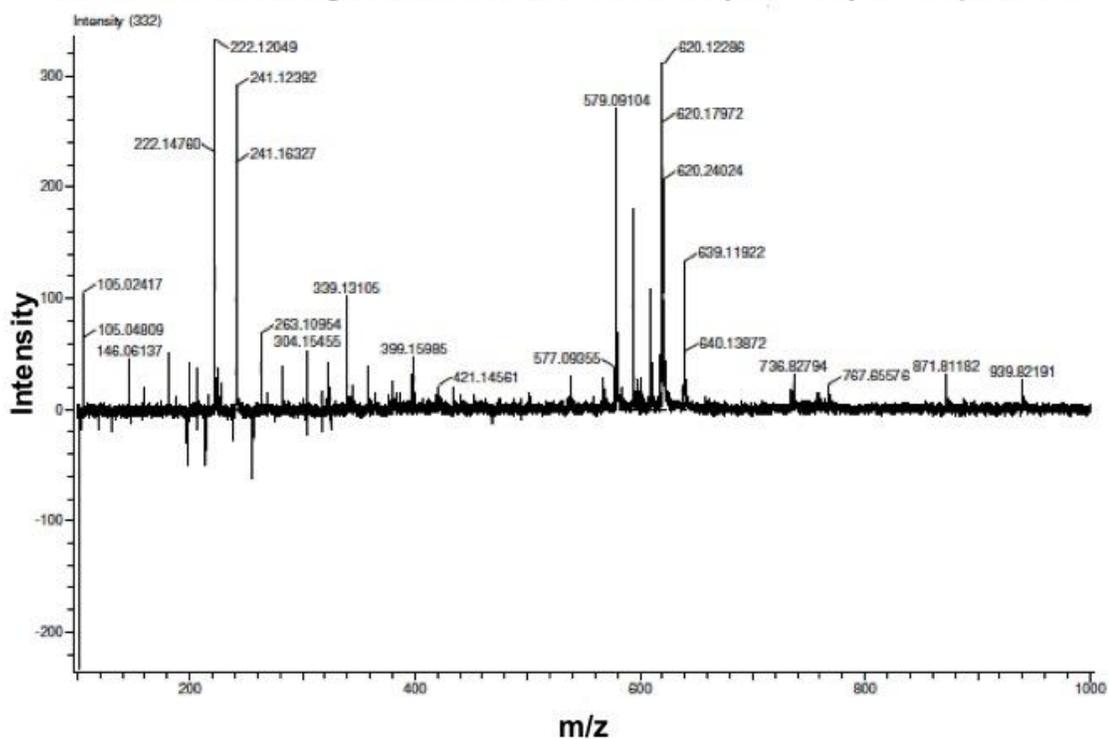


Figure 51: Mass chromatogram of the lipids at T8 after a treatment of zeatin-ribose at 10^{-11} M. The corresponding lipids are found in Table 29.

New Lipids found with Zeatin Ribose (10^{-11} M) at 28.2 Minutes

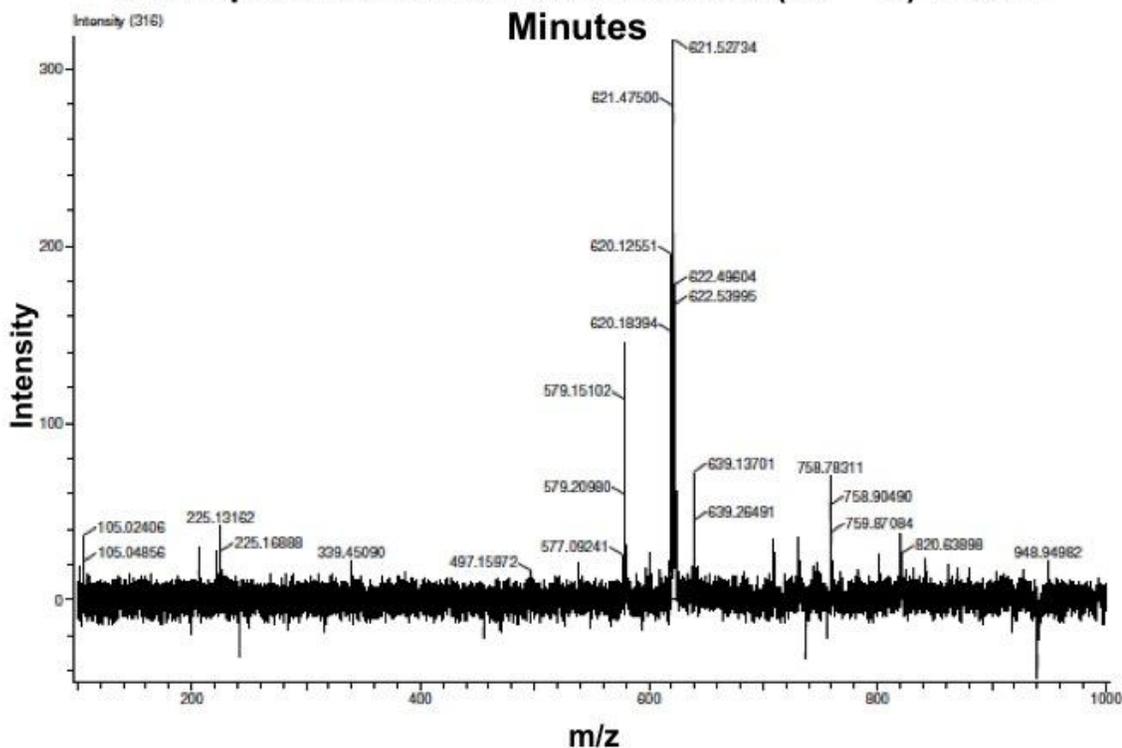


Figure S2: Mass chromatogram of the lipids found at the new peak on the TIC at 28.2 minutes. This peak appeared after a treatment of zeatin ribose at 10^{-11} M. The identities of the lipids are listed in Table S9.

Table S9: Compilation of lipids produced after a treatment of zeatin ribose at 10^{-11} M that deviated from the controls.

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 [GP06]	□
639	Trilauroyl-glycerol	Triacylglycerol	?
736	PS(16:0/16:0)[U]	Diacylglycerophosphoserines [GP0301]	□
247	C5 isoprenoids	Isoprenoid	□
377	dimethylallyl-diphosphate	Isoprenoid	□

579	Phthioceranic acid	Branched fatty acids	<input type="checkbox"/>
620	1,2-dipentadecanoyl-sn-glycero-3-phosphate	Diacylglycerophosphates	<input type="checkbox"/>
758	GlcCer(d18:0/20:0)	Neutral glycosphingolipids [SP05] Simple Glc series [SP0501]	<input type="checkbox"/>

4.5 Confocal Microscopy

After cells were dyed with Nile Red, there were two photomultiplier tubes (PMTs) that were activated to detect two ranges of wavelengths. 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. In the below images in Fig. 25, 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 spectra includes the wavelengths that the first PMT was detecting, and thus red dots appear in Fig. 25a.

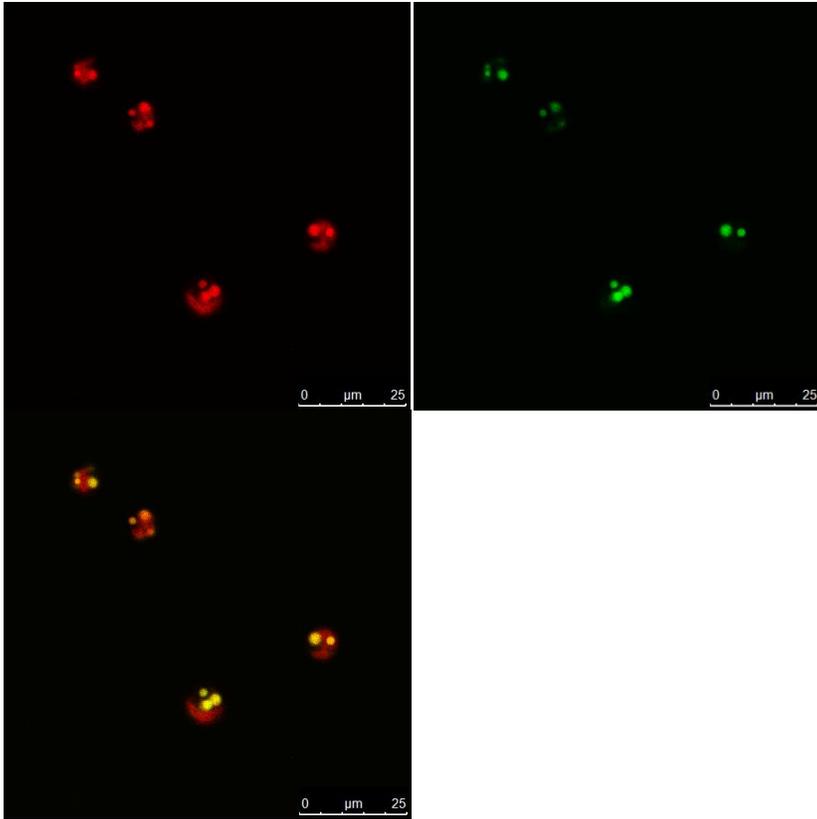


Figure 53: Algae fluorescing naturally (left). Lipid droplets dyed by Nile Red fluorescing (center). Overlay of both images (right).

All figures shown below will have two example images.

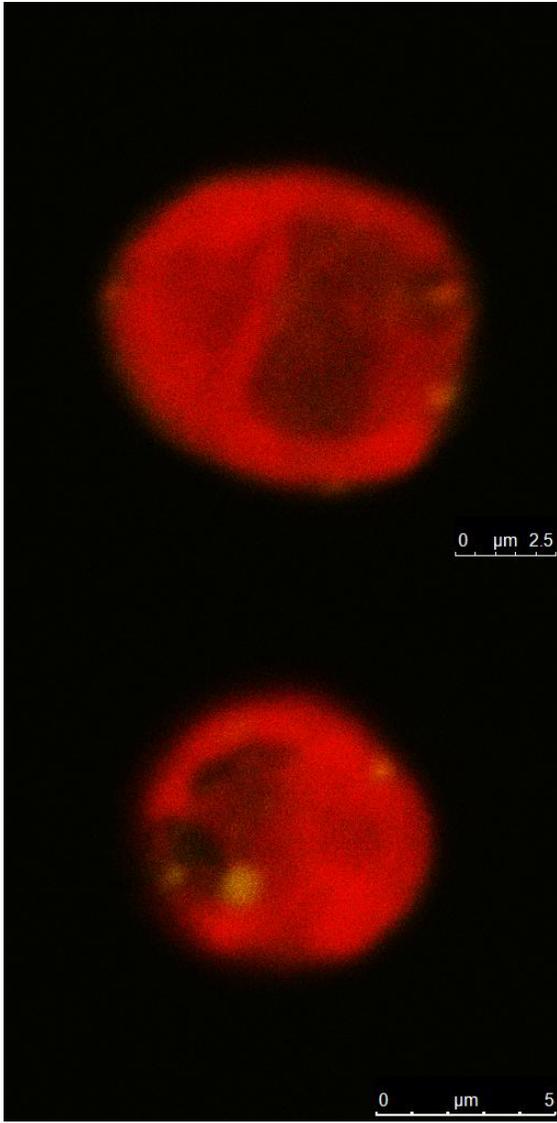


Figure 54 Control cells dyed with Nile Red. Notice the small number of lipid bodies.

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

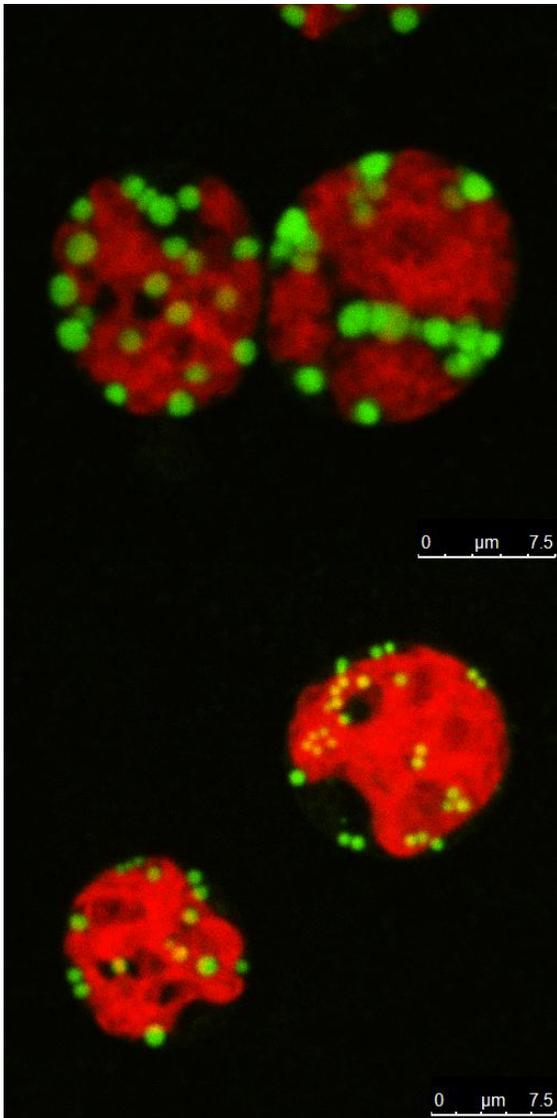


Figure 55: Cells with auxin applied to them. Notice the large number of lipid droplets and the small size of each of them.

The cells seemed to grow of highly variable size and this is apparent in some of the following images.

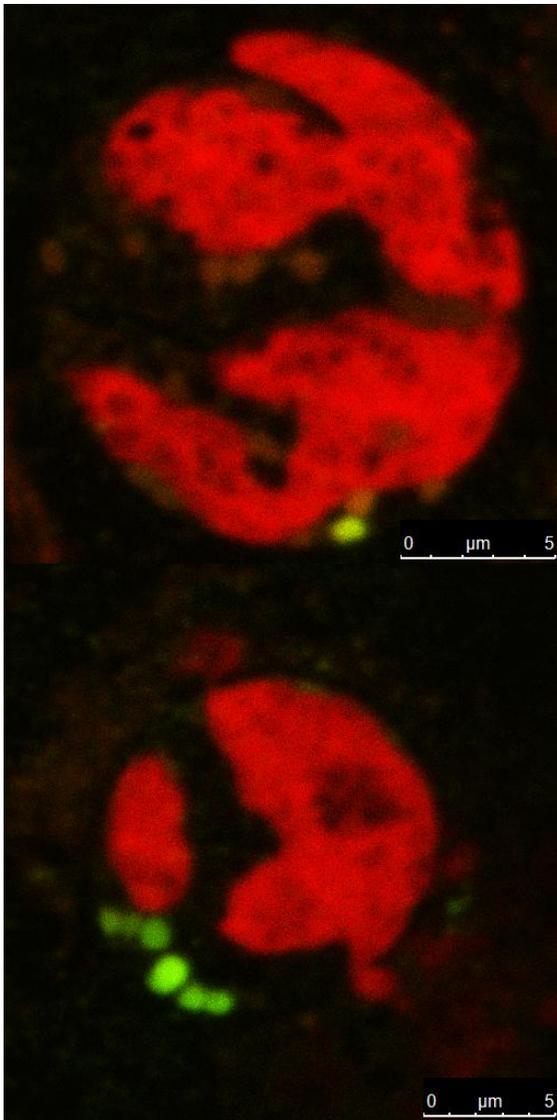


Figure 56: Cells with brassinolide applied to them. Note the larger reticulum in the cells.

The addition of brassinolide resulted in cell morphology where there appeared to be large gaps in the auto fluorescence of the cell, while still causing slightly more lipid droplets stained by Nile Red. The reason for the increased reticulum size is unknown. Upon application of zeatin, however, there are more lipid droplets than control groups. The lipid droplet sizes relative to cell size are larger compared to other groups.

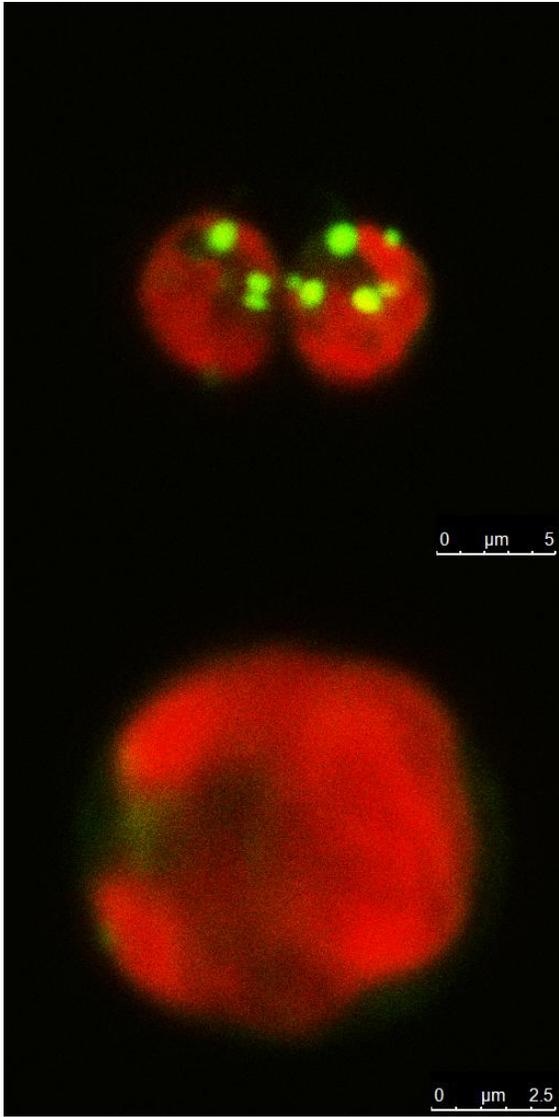


Figure 57: Cells with zeatin applied to them. Notice that there are medium sized lipid droplets, but not very many of them.

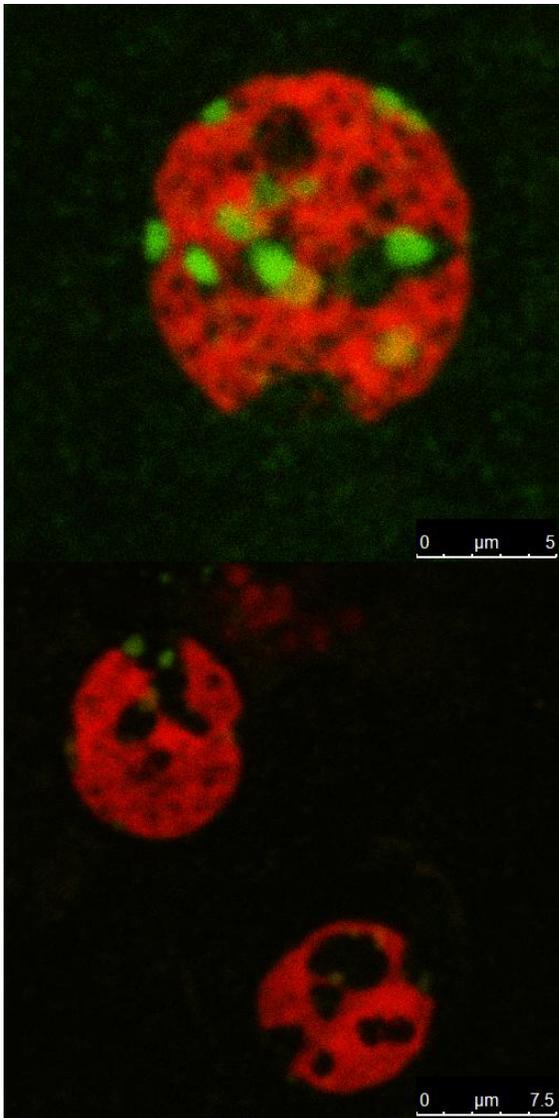


Figure 58: Cells with trans-zeatin-riboside applied to them. Notice that there are larger lipid bodies, but also larger reticulum. There are also more lipid droplets than in zeatin, but still fewer than auxin.

Trans-zeatin-riboside caused an increase in the number and size of lipid bodies relative to cell size, but had a similar effect as brassinolide on the autofluorescence of the cell. It appeared that the gaps were not interconnected as in Fig. 27, but instead separated into many parts.

5. Discussion

5.1 Overview

Our primary objective throughout our research has been to find a way to improve the production of algal biofuels, specifically the efficiency of lipid production, to a more economically practical level given our limited resources. In order to accomplish this, we focused on the areas to which we could add new information. From our review of the literature, we determined that there are two possible routes to increasing lipid production and decreasing costs. The first is to modify the algal metabolism to cause the algae to grow faster, or to produce more lipids. The second is to develop methods of algal processing that are cheaper, faster, and overall more efficient 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 retrieving the algae, 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 in a modern industrial setting. Additionally, we do not have the resources to create a similar set-up on a small scale, nor would it be effective for our purposes. However, if we can find ways to improve qualities of the algae, this can be incorporated into the current techniques. This led to our decision to modify the algae through the use of phytohormones.

The motivation for the exogenous application of phytohormones to modify the algae came from knowledge gleaned from various scientific research papers on the subject. We determined that by experimenting with nutrients, 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 nutrients. 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. This possible connection was analyzed through bioinformatics, namely the comparison of *C. reinhardtii* and *A. thaliana*. While there were few phytohormone receptors or pathways that matched explicitly, there was considerable evidence that suggested a connection. Thus, we decided to apply auxins, brassinosteroids, and cytokinins to *C. reinhardtii* to see if there was any effect. The two primary areas where we expected to see changes were in the growth rates and lipid yields of *C. reinhardtii*. We monitored the growth rates through the use of spectroscopic data collected and the dry weights of the algae. In order to analyze lipid yield changes, LC-MS and confocal microscopy were used in addition to measuring the dry weights of the lipids. Our experimentation and analysis determined that all the phytohormones had some effect on the growth rate, lipid yield, and cell morphology of *C. reinhardtii*, with a mixture of auxin and trans-zeatin ribose yielding the most promising results.

5.2 Bioinformatics

To determine whether particular phytohormones would have an effect in *C. reinhardtii*, the genome of *C. reinhardtii* 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 determined and annotated, many of the results included hypothetical proteins, which were disregarded in the analysis.

5.2.1 Abscisic Acid

Previous research on the signal transduction pathway for abscisic 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; Riano-Pachon et al. 2008). 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. Only hypothetical proteins displayed matches with the proteins in *A. thaliana*. Due to this, abscisic acid was not selected for further investigation.

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 CO₂, 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. 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 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 (Jacobs 1951; Jacobs et al. 1985; Cooke et al. 2001). 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.

5.2.3 Brassinosteroids

The signal transduction pathway for brassinosteroids is better known 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, which is encoded by the *bin2* gene (Li and Nam 2002). When brassinosteroids bind on the membrane, BIN2 is inactivated by an unknown

mechanism, which allows hypophosphorylated nuclear proteins such as Brassinazole resistant (BZR1) to suppress transcription (He et al. 2002). 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, including ZMP1 and VPS34, has functions related to locomotion and are not associated with the phytohormone response, indicating that the proteins only share conserved domains. In addition, since BZR1 and BES1 are not proteins critical to the brassinosteroid response, the strong matches were not entirely significant. Proteins more closely associated with the brassinosteroid response pathway, such as BIN2 and BRI1, did not give any strong matches. Despite the lack of matches to justify the use of brassinosteroids in the experiments, they were chosen to serve as a possible negative control. In addition, the response of *C. reinhardtii* to brassinosteroids can be more fully investigated since minimal research has been conducted regarding the relationship between brassinosteroids and *C. reinhardtii*.

5.2.4 Cytokinins

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; 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 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. Trans-zeatin and trans-zeatin-riboside were selected on the basis of their prevalence in the regulation of cell division for *A. thaliana*.

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. Most of the weak matches consisted of hypothetical proteins, indicating that ethylene was not a good candidate for further research.

5.2.6 Giberellins

Previous research has not elucidated the signal transduction pathway for gibberellins. 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). However, the GID1 protein did not yield any matches. In addition, other proteins that have been proposed to be involved in the pathway did not give any

noteworthy matches. Thus, it was concluded that gibberellins were not likely to initiate a response in *C. reinhardtii*.

5.2.7 Summary

The bioinformatics analysis of the *C. reinhardtii* genome did not give strong evidence that phytohormone signal transduction occurs in the algae in a similar fashion to *A. thaliana*. Considering that *A. thaliana* and *C. reinhardtii* are 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. Virtually none of the components found in the *A. thaliana* pathways were found in *C. reinhardtii*; thus it is possible that the phytohormone signal transduction pathways in *C. reinhardtii*, if they exist, may involve different proteins. Many of the proteins that were strong matches were hypothetical proteins; since the *C. reinhardtii* genome has not been fully analyzed, it is possible that there are homologous proteins that are involved in phytohormone signal transduction. Thus, for a bioinformatics analysis of *C. reinhardtii* to be successful, future research must focus on identifying and characterizing proteins that have yet to be annotated 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. 2001). In addition, substances similar to cytokinins and brassinosteroids are produced by *C. reinhardtii* under certain conditions (Bajguz and Czerpak 1998; Stirk et al. 2002). In spite 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 trans-zeatin. The effects of increased lipid yield were more difficult to ascertain due to the error in our lipid dry mass data. However, confocal microscopy and LC-MS were used to verify our suspicions on the effects of phytohormones on lipid yields. Generally auxin and brassinosteroids increased growth with little effects on the lipid yield while cytokinins either increased or decreased the growth rate while having a greater effect on increasing lipid yield. A mix of phytohormones had the best effect by increasing both growth rates and lipid yields.

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 weights of the algae, and lipid yields. The morphology of the cells and the identities of the lipids found in typical *C. reinhardtii* cells were determined. 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 ± 0.7 mg algae/L/day grown in a linear growth phase. Algal growth is documented to occur in sigmoidal growth phase. While establishing our growth conditions, we inspected all variable to determine the root cause of the linear growth. However, we were unable to determine the cause within our growth conditions. All samples consistently exhibit a linear growth phase. The suspected causes of this linear growth rate are scrutinized in our analysis of our methodology below. Regardless, 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 ($\text{g}_{\text{lipid}}/\text{g}_{\text{algae}}$) of the algae was found to be $60.1 \pm 13.6\%$ for control. This value represents the percentage of lipid by mass of the algae. A higher number indicates increase production of lipid content per algal cell. Although this is higher than the literature value of 20%, this was consistent throughout all of the controls (Sialve 2009). In addition to the specific lipid weight, we calculate the lipid concentration ($\text{g}_{\text{lipid}}/\text{L}_{\text{volume of solution}}$). For the control the mean lipid concentration was 0.075 ± 0.016 g/L. This value takes into account the growth rate and the specific weights of the lipids of the algae. Provides a more accurate representation of the effects of a phytohormone treatment. The confocal microscopy imaging of the controls showed

very few lipid bodies. This is consistent with the study conducted by 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 purpose of this was 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 24. Among these we found triacylglycerides (TAG), free fatty acids (FFA), glycerophospholipids (GP) classes of lipids. 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 a result we present them,

however, we do not discuss their roles in the cell. The MGMG and SQMG are thylakoid membrane lipids expected in organisms containing chloroplasts. However, these two lipids have previously 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 chain 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 be unique to 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 diacylglyceryl-*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 Auxin

Auxins, as a class of phytohormones, affect the activity of the cell at the transcriptional level. They accomplish the control of transcription through auxin response factors (ARFs), which dimerize and bind to DNA. In higher plants, auxins play a role in root initiation and elongation in shoot cells and inhibit lateral bud

formation. For these reasons, we analyzed the effects of the exogenous introduction of auxins.

Though auxins are naturally present in algae, the concentrations at which they are found are minute (less than 10^{-12} M) (Cooke, 2001). 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 operation 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 $53\pm 5\%$ relative to control. Conversely, the minimal growth rate was found at a concentration of 10^{-9} M (Fig. 17). The higher concentrations of auxin may reflect the inhibitory effects of auxins found in higher plants, which provides a possible rationale for the decline in growth rates. It is noteworthy to observe that 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, an additional pathway may become activated due to higher ligand binding threshold, which

improves the growth rate. In this manner, auxin may participate in multiple signaling pathways based on its concentration.

The greatest algal dry weights of the algae exposed to auxin occurred at concentrations of 10^{-9} M and 10^{-12} M, similar to the concentration of auxin which best increased the algal growth rates. At these concentration, there was a 2% increase in mass over the control samples; however, this is not a significantly different change. There was also lower variability among the dry weights of the samples exposed to various phytohormone concentrations when compared to the changes in the algal 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). The standard deviation for this result is large enough render the effect practically insignificant. Through the addition of auxin, lipid concentration ($g_{\text{lipid}}/L_{\text{volume of culture}}$) increase negligibly for 10^{-12} M auxin (Fig 31). Notably at concentration with the fastest growth rate, 10^{-11} M, a decrease of 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.

Despite the variations found within our data, our 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. 49) compared to very few in control (Fig. 48). 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-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. Further work can be conducted to illustrate how auxin affects these pathways.

5.3.3 Brassinosteroid

As with auxins, brassinosteroids are another class of phytohormones that are naturally found in several species of green algae. According to our literature review, algae produce brassinosteroids in the vicinity of 10^{-13} M, whose result is roughly reflected in our control group (Cooke, 2001). In higher plants, these hormones are involved in the growth of pollen, seeds and young plant tissue. It has been concluded that the level of cell division caused by these hormones correlates directly with their concentrations. Therefore, we predicted that brassinolide, our brassinosteroid of choice due to its natural presence in *C. reinhardtii*, would also have similar effects in algae.

According to our results, brassinolide best increased the growth rate of algae at a concentration of 10^{-12} M, with an increase of $62 \pm 33\%$ (Fig. 18). The growth rates decreased at higher concentrations of brassinolide, however, none of the treated samples' growth rates fell below the growth rate of the control. This result indicates

that brassinolide increases the growth of algae under all tested concentrations. Brassinolide activates pathways with similar functions as auxin to increase growth rates. The greatest increase in growth rate was seen at 10^{-12} M. Increasing the dose of brassinolide reduced growth rates closer to the level of the controls. We predict that brassinolide 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 weights of the samples showed a different trend. When exposed to lower concentrations of brassinolide, the dry algal weights of the samples were lower than the dry weights of the control groups. At higher concentrations, however, the dry weights approached the level of the control groups. The largest decrease in dry algal mass was 21%, over controls, and observed at a concentration of 10^{-12} M. This result can be attributed to the fact that when *C. reinhardtii* rapidly divides, the cell volume of each dividing cell decreases significantly. 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 weights of algae, brassinosteroids do not prove to be an effective treatment.

Brassinosteroids, like auxins, yielded an increase in specific lipid weight. We found the optimal brassinolide concentration to be 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 brassinolides . 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 weights and dry algal weight, 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 control and the five brassinolide concentrations were found for lipid concentrations (Fig. 32).

A qualitative evaluation of cells exposed to brassinolide 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 resulting void stretches across the entirety of the cell, it is most likely a type of reticulum.

Samples treated with Brassinosteroid 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. Of these concentrations, 10^{-9} M showed notable changes in features of lipids produced as 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 necessary 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

Trans-Zeatin is classified as a cytokinin. Cytokinins play roles in chloroplast development and preventing overgrowth by imposing limits on higher plant growth. Unlike the brassinosteroids and auxins previously mentioned, these hormones are not naturally found in algae. In plants cytokinins can increase growth rates through encouraging cell division (Tarakhovskaya et al. 2007; Riou-Khamlichi et al. 1999). Since algae are related to plants, we predicted that cytokinins would most influence cell division in our trials.

According to our data, however, 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^{-8} M with a 48% 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 that only the inhibitory effect of trans-zeatin was observed which is in opposition to our hypothesis.

Trans-zeatin had an interesting effect on algae morphology. Under trans-zeatin treatment *C. reinhardtii*, 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.

Despite this decrease in growth rate, the lipid yield from this treatment showed some positive signs.

The dry algal weights for all treatments showed little differences in mass. The greatest decrease in mass as compared to the control 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. Even with lower growth rates, the dry masses were all relatively similar to one another and the control. It appears that trans-zeatin causes cells to increase in individual weight but causes a decrease in overall cell division.

We found that at a concentration of 10^{-9} M trans-zeatin yields the highest specific lipid mass increase. However, since the increase in production was as little as 2%, it is likely that trans-zeatin did not have a positive effect on lipid yields. At higher concentrations (e.g., 10^{-8} M trans-zeatin), 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 concentration spectrum show negligible changes relative to control. Most notably a decrease of 43% in lipid concentration was found at 10^{-8} M. 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.

When viewed with the confocal microscope, trans-zeatin treated cells showed a small amount (<10) of rather large lipid bodies (Fig. 51). 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.

To analyze lipids types, LC-MS was performed on samples treated with trans-zeatin at concentration in the range of 10^{-8} M to 10^{-12} M. At all concentrations lipids identified in the control samples were also characterized. Of these concentrations, notable changes relative to controls were observed in lipid features for trans-zeatin-treated samples at 10^{-8} M. The identified lipids are presented in Table 28. Increases in Monogalactosyl-monoacylglycerol, DG, and glycerophosphocholine were observed; however, no novel lipids were identified. This particular concentration of trans-zeatin leads to a suppression of the growth rate relative to control.

Additionally, excessive clumping was observed in the sample flasks for this phytohormone. These two features of the growth period of this sample 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 a division event of 2^n . 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 corroborate this conjecture.

5.3.5 Trans-zeatin-riboside

Trans-zeatin-riboside is another cytokinin that our team decided to experiment with. As previously mentioned, cytokinins possess the ability to increase growth rates in higher plants but also have an inhibitory characteristic as well. For example it is known that cytokinins play roles in limiting plant development in order to prevent overgrowth (Werner et al. 2001). We believed that this cytokinin would decrease the growth rate of our algae similar to trans-zeatin.

Our results show that growth rate actually increased at concentrations of 10^{-12} to 10^{-9} M of trans-zeatin-riboside, with 10^{-9} M being the optimal concentration for increasing growth (Fig. 20). At this concentration there was a $31 \pm 21\%$ increase in growth compared to the control. The application of 10^{-8} M slightly showed a slight decrease compared to the optimal growth rate. This is most likely due to the fact that such a large amount of cytokinin would activate other pathways responsible for directing nutrients to various pathways.

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 would result 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 algal dry weights. 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 algal dry mass was 38% found in the 10^{-11} M trials (Fig. 20). Once again, this illustrates the tradeoff between growth rate and final masses. The cell sizes increase when division does not occur, thereby low growth shows larger algal cell mass.

In regards to the specific lipid weight, the most effective concentration of trans-zeatin-riboside was at 10^{-10} M, at which we observed an increase of 3% (Fig. 27). However, at a concentration of 10^{-8} M, we did note a decrease in specific lipid mass by about 23%. Due to the variation in our tested concentrations, we believe that trans-zeatin-riboside does not directly affect the production of lipids in algae. Similar to trans-zeatin, trans-zeatin-riboside mainly affected the growth rate of the algae. The lipid concentration increases for 10^{-9} M, 10^{-10} M, and 10^{-11} M trans-zeatin-riboside by 20% relative to control (Fig. 34). A notable here is that 10^{-9} M also displays the highest growth rate. Since a decrease in specific lipid mass at 10^{-9} M was not observed, the increase in growth rate contributed primarily to the increase in lipid concentration. This is optimal for biodiesel production because the lipid production is not compromised by the change in growth rate. This means the production of the final product benefits from both growth rate and total lipid content.

When viewed under the confocal microscope, a trans-zeatin-riboside treated algal cell shows a large number (<20) of large lipid bodies (Fig. 52). An interesting

morphological characteristic of this cell is that there appears to be many small black voids within the cell. The circular shapes of these cells were present in all of the photos taken for trans-zeatin ribose treated samples. These circular objects were too large to be proteins; therefore, it is likely to be the formation of many small vesicles. Determining the use of these vesicles could reveal 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 in concentration of 10^{-11} M. These findings are 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 rate seen in trans-zeatin, trans-zeatin-riboside increases growth rate relative to controls. Thus, we suspect the cell does not divert all of its lipid resources to storage, as suggested by the decrease in specific lipid weights of trans-zeatin ribose treated cells. These lines of evidence point to regulation of alternate pathways by trans-zeatin-riboside. As a result lipid type features 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 characterization is consistent with cellular metabolic processes.

5.3.6 Auxin & Trans-zeatin

A combination of auxin 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 auxin 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 auxin and 10^{-10} M of trans-zeatin and a treatment of 10^{-11} M of auxin and 10^{-12} M of trans-zeatin. Both of these combination treatments increased the growth rates by 17% and 19%, respectively (Fig. 21). There were no significant changes to dry algal mass relative to control (Fig. 21)

It is interesting to note that treatments of only trans-zeatin at 10^{-10} M and 10^{-12} M both showed no increase in growth rate. Also, treatments of trans-zeatin and auxin treatments separately both only show minor increases in dry algal weight. However, when *C. reinhardtii* is exposed to both auxin and trans-zeatin, it shows a greater increase in growth rate and dry algal masses. The presence of two optimal concentrations for the mixed phytohormone treatment reveals that auxin and trans-zeatin work by synergistically 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 weights of algae (Fig. 28). The only exception to this observation was found at a concentration auxin at 10^{-11} M and trans-zeatin at 10^{-9} M, at which point there was a 0.1% increase – a negligible change. The auxin-only trials did not exhibit a clear trend on the effects of auxin on specific lipid weights in algae and trans-zeatin-only trials did not show a clear decrease in specific lipid weights. The decrease in specific lipid weights may then be attributed to cross effects on metabolic pathways. Also, the decrease in specific lipid weights 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 auxin and 10^{-10} M trans-zeatin showed a $55\pm 31\%$ (Fig. 35)

LC-MS and confocal microscopy experiments were not run on the mixed treatment algae due to budget constraints. However, it would corroborate the findings of specific lipid yields and lipid concentrations. Additionally, noting changes in cellular morphology features would allow visualization of the specific metabolism of *C. reinhardtii*. We expect to see differences the quantity of lipid bodies, however, the types of lipids may not change. On the other hand, the lipid types may alter, while lipid bodies do not change in quantity.

5.3.7 Auxin & Trans-zeatin Riboside

The combination of auxin and trans-zeatin-riboside also had a synergistic effect on the growth rates and lipid yields of *C. reinhardtii*. Like the previous mixed phytohormone treatment, a mixture of auxin and trans-zeatin-riboside resulted in

effects that differed from the individual treatments of auxin and trans-zeatin-riboside. Individually, auxin and trans-zeatin-riboside both increased growth rates by $53\pm 5\%$ and $31\pm 21\%$ respectively. Together, auxin and trans-zeatin-riboside only increased growth rates by 11% at a concentration of 10^{-11} M auxin and 10^{-12} M trans-zeatin-riboside (Fig. 22). It is difficult to ascertain 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 cytokinins. The mixed treatment yielded the greatest increase in dry algal mass of 1.5% when treated at a concentration of 10^{-11} M auxin and 10^{-11} M trans-zeatin-riboside. This increase was negligible when considering the errors in our measurements.

The combination of auxin and trans-zeatin-riboside phytohormones showed a significant increase in the production of lipids. At a concentration of 10^{-11} M auxin and 10^{-10} M trans-zeatin-riboside, we observed a 44% increase in specific lipid weights (Fig. 29). The lipid production levels tapered off at the other tested concentration levels of trans-zeatin-riboside with auxin constant at 10^{-11} M. It is important to observe that none of the levels indicated a decrease in lipid production. Of particular interest is that individually, auxin and trans-zeatin-riboside did not increase lipid production, but rather increased growth rates. However, the

combination of phytohormones significantly increased specific lipid weights while only slightly increasing the growth rate of the algae.

In the end, the auxin and trans-zeatin ribose 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 33% relative to control (Fig. 36). That particular treatment yielded 0.107 ± 0.039 g lipid /mL of culture. The control culture only yielded 0.083 g lipid/mL culture. This is an increase of 0.014 g lipid /mL of culture relative to control. This increased concentration came from not only the increase in growth rates relative to other controls (i.e. auxin, brassinosteroid, etc.), but also lipid concentrations relative to other controls (i.e. auxin, brassinosteroid, etc.). Although there were higher increases in growth rates and higher increases in lipid concentrations from other phytohormone treatments, this particular treatment yielded a good balance in both growth and lipid yield, because generally large increases in growth rates will stunt lipid production and large increases in lipid production will stunt growth rates.

Unfortunately, due to budget constraints, we were unable to perform confocal microscopy and LC-MS analysis. 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 noteworthy.

5.4 Methodology Analysis

5.4.1 Growth Rates

In assessing the full validity of these findings, the limitations of methodology under our particular conditions 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 an exponential growth pattern. Our algal system, however, maintained a linear rate. We suspect that certain growth variables 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 CO₂ supplied from the air. Though we used a shaker table to aerate the algal flasks, the possibly reduced volume of CO₂ available may not have allowed the algae to reach their maximum growth potential. Pumping higher concentrations of CO₂ into the growth flasks could eliminate this growth pattern; however, this was beyond our means. Another possible reason for the observed linear growth was that our inoculation procedure made it difficult for our cultures to reach exponential growth rates. We introduced such small amounts of algae that the growth rates determined by spectroscopy might not have measured the true content of the algae in the algal growth system. Along with CO₂ levels, humidity and the minute human errors associated with having fourteen separate scientists working on this project may have contributed to the observed linear growth pattern.

This growth pattern was initially a salient feature of our algae. In order to recreate an exponential growth pattern as documented in the work of others, we explored various conditions, including light levels, temperature, and alternative inoculation procedures. The linear growth pattern, however, remained a constant feature for the algae. 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. Additionally, 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 weights of the algal samples. The algae were centrifuged and the excess supernatant was discarded. After lyophilization the masses were taken. 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 weights trend with spectrophotometric measurements of growth. However, there were isolated examples of significant deviation from these trends for selected trials. 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. As a result, we could not obtain optimum amounts of lipids in general. This limiting factor made it difficult to precisely quantify lipid yields 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. 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 weights against each sample's algal mass. This reduced the degree of variance observed in final lipid accumulation. Although the inefficiency of our extraction apparatus reduced our lipid dry weights, 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 minor 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. Additionally, 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 absorbency of UV light between 190 -400 nm for Acetonitrile, a solvent ramp was produced in the absorbency 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. The peaks of greater intensity than the solvent background were 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. Although we were able to resolve lipids in addition to those derived from absorbance measurements, 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 our comparisons

between control and phytohormone-treated samples are due to relative comparison of intensity, as the calculation of exact ratios carries a great degree of uncertainty. Further, the software paired with our LC-MS does not calculate integrated peak areas. This adds to the lack of exact quantification lipid amounts after separation. In analyzing the mass spectrum, we found additional ambiguity in ascertaining the exact structures of the lipids. Based on our matches in the Nature Lipidomics Lipid Map database, AOCS Lipid Library, and other sources, many of the m/z peaks returned several possible isomers. 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 ± 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 would allow resolution of these discrepancies.

5.5 Economic Analysis

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 CO₂ and heat (<http://www.worldwatch.org/node/5391>). Taking a base cost of \$33 per gallon, this means that for algal biofuel to be produced at a cost similar to that of crude oil,

the cost must be decreased by a factor of 10. 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. With a government subsidy, the cost of algal biodiesel could be significantly reduced. The U.S. government 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 cleaner energy. On the other hand, oil production is currently one of the most heavily subsidized industries. 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. 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. This possibility should not be ignored when considering the economic viability of algal biofuel in the future.

However, there are many costs associated with producing biodiesel. There is the cost of the media, the phytohormones, and the materials and energy used for growth and extraction.

Table 1- Costs of extracting lipids from algae under our experimental scale

Input	Cost per Sample of 150 mL (\$)
Media + Phytohormone	0.02
Extraction Solvents	1.17
Thimble	3.16
Centrifugation	0.02
Lyophilization	0.36
Electricity - Lighting	0.21
Total Cost	4.94

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. Considering that companies can currently produce biodiesel at \$33 per gallon we know that the costs can be driven down significantly from the experimental costs. Also, the \$33 per gallon can be considered a baseline and did not take into consideration the improvements seen in our experiment. In the case of auxin plus zeatin-ribose, the weight percentage of lipids was determined to be 68%, which is approximately two times greater than the 35% seen in the controls of the auxin plus zeatin-ribose samples. If this gain could be replicated in large-scale

production, the final cost of biodiesel could be cut significantly. Aiming for a target price of \$4/gal and using the lipid mass percentage of 68%, it would be necessary to grow algae to a density of 130 g/L. This is much higher than the approximately 10 g/L density achievable by *C. reinhardtii*, but the lipid weight percentage increase is a positive first step and if it can be generalized, then the \$4/gal target price is within range for other types of algae such as *C. prothothecoides* which have been shown to grow in densities up to 45 g/L.

(<http://www.springerlink.com/content/13446v1p63707q73/>)

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 ± 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

Our most notable result from treatment of all phytohormones tested was with the combination of auxin at 10^{-11} M and tran-zeatin-riboside 10^{-9} M. This combination of phytohormones at these concentrations yielded an increase growth rate 11% over controls with a corresponding algal mass change, specific lipid mass increased 51%, the lipid concentration increased 33%. Although the increases in growth rate and lipid concentration were not the highest, the most important aspect of these results is the lipid concentration. This includes a concurrent calculation of growth rate and total lipid content of the algal cells. When compared to all controls, the relative change of 33% in lipid concentration was significantly greater. The closest alternative in this assessment is trans-zeatin, individually, at 10^{-9} M. If we discount the error in these calculations due uncontrollable forces at the experimental scale, the combination of auxin at 10^{-11} M and trans-zeatin-riboside at 10^{-9} M provides the biggest improvement towards production of biodiesel via lipids of *C. reinhardtii*.

In order translate these findings for practical application, further research in several facets of this work need to be pursued. Firstly, the phytohormone signal transduction pathways in *C. reinhardtii* must be established to better understand the role of phytohormones in the species and algae in general. Next, the method of identification and quantification of lipid classes must be perfected upon 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. Next,

the process must be scaled up for mass production; costs must be reduced and the process must be streamlined through the use of better instrumentation and growth methodologies. As a last step to further reduce costs and improve efficiency of the pipeline, future ventures should look to exploring genetic engineering to induce specific algal metabolic pathways. Doing so would eliminate exogenous delivery of phytohormones thereby reducing a major cost component of this pipeline.

Based on finding, we believe metabolic engineering involving phytohormones and their pathways could potentially lead to significant reduction in production costs of biodiesel. Our experiments do not address the changes that the algae would incur at industrial scale level. However, even small contribution in improving growth rates and lipid production in the algae can be magnified at the industrial scale. Various changes in addition to our experiment conditions could further lend to secondary small benefits to this production pipeline.

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 biofuels are derived. However, due to time and budget constraints and the intensive research already being conducted in this area, our research focused on a novel way of increasing the economic viability of biofuels from algae by treating it 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.

7. Appendices

A.1 Data from Growth Trials

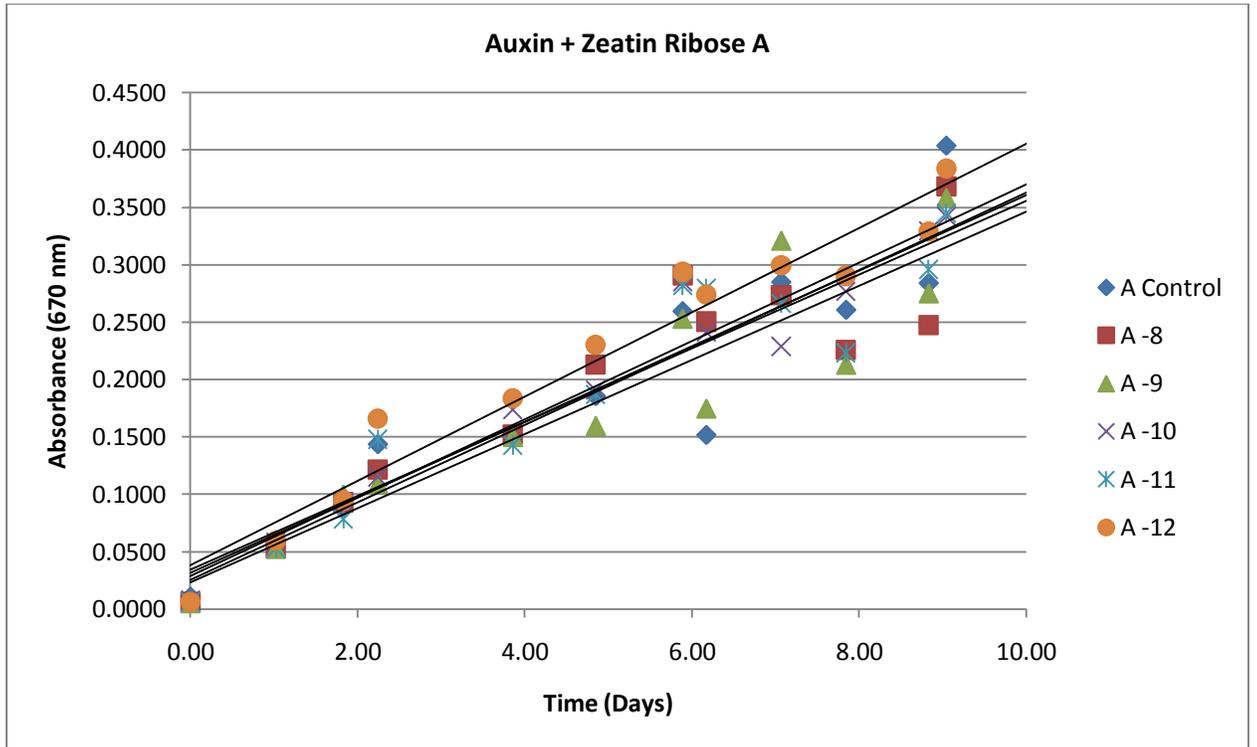


Figure 59: An example of the growth data for one concentration of auxin for one trial over the span of ten days

A.2 Linear Regression

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
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 Weight of Lipid

The specific weight 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.

No appendices are included in this draft.

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 good 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; smaller value indicates a closer match

Gas Chromatography - Mass Spectrophotometry (GC-MS): A technique that combines gas-liquid chromatography to separate substances and mass spectrometry to identify compounds.

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.

Homologous: Similar in function or characteristics

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 another alcohol; 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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