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From Pond to Pump: Microalgae as a Feedstock for Biodiesel

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EXECUTIVE SUMMARY

The global reliance on fossil fuels is an unsustainable practice that has led to the depletion of finite resources and the accumulation of greenhouse gases in the atmosphere, leading to global climate change (Demirbas 2010). Transportation accounts for about a third of carbon dioxide (CO_2) emissions in the United States. Demand for oil continues to increase and resources are becoming more uncertain. Increased production of renewable fuels such as biofuels can help ease the dependence on fossil fuels. Although interest and production in biofuels has been increasing in the past decade, current feedstocks do not meet efficiency needs and some may even lead to environmental damage and increased greenhouse gas emissions (National Research Council 2010). Microalgae are arguably the only source of renewable biodiesel that is capable of meeting the global demand for transport fuels due to their high productivity and ability to store large amounts of lipids (Demirbas 2010).

Algal biofuels have strong advantages over first-generation feedstock such as corn, palm oil, and soybeans. Algae culturing systems can be located on land that is unsuitable for agriculture, can be coupled with flue gas CO_2 mitigation, wastewater treatment, and the high-value byproducts from algae biofuel production ease production costs (Li et al. 2008). The potential for algae as biodiesel is great, as algae are highly productive, and the oil content can exceed 80 percent by weight compared to 5 percent for the best agricultural oil crops; however, microalga-based systems still have poor volumetric efficiencies, which make them costly compared with petroleum fuels (Amaro et al. 2011). Research and innovation into algae biofuels, specifically biodiesel, could help the industry grow and become a major provider of liquid fuel.

Most of the algae known to produce large amounts of lipids (more than 20 percent of their biomass) are of the Divisions Cryptophyta, Chorophyta, and Chromophyta (Darzins et al. 2010). *Dunaliella tertiolecta* is a green alga species that has been studied for biofuel production. *Dunaliella tertiolecta* is relatively easy to cultivate and has a high growth rate and lipid content (Tang et al. 2011). In this study, a photobioreactor was designed and constructed for the cultivation of microalgae in a laboratory, with attention to feasible scale-up possibilities for industrial productions. *Dunaliella tertiolecta* was cultivated in

the bioreactor and experiments were performed to study the algal growth rate, lipid production, and potential harvesting techniques.

The first objective of this experiment was to determine the effects of carbon dioxide on the growth rate of *Dunaliella tertiolecta*. The CO_2-enhanced (~1-2% CO_2) cultures had a growth rate of 2 doublings per day compared to the ambient air (~0.04% CO_2) cultures with a growth rate of 1.4 doublings per day, indicating that algae farm integration with CO_2-emitting industrial plants could both increase biofuel production as well as mitigate CO_2 and harmful pollutant emissions.

Lipids are the cellular components that are extracted from microalgal cells for the conversion to biodiesel and new, inexpensive methods to increase lipid content are needed to make microalgae a viable feedstock. Both a salt shock (5%) and decane treatment (1%) showed an increase in lipid production during the stationary phase of growth compared to untreated cultures.

Finally, experiments were performed to compare the effectiveness of autoflocculation and Chitosan flocculation with samples of *Dunaliella tertiolecta*. Chitosan is a non-toxic flocculant made from grinding and processing the exoskeletons of crustaceans to acquire the polysaccharide chitin (Lavoie and de la Noue 1983). Chitosan flocculation at a concentration of 2 g/L was the most effective means of separating the algal cells from the medium, compared with autoflocculation and chitosan at a concentration of 1 g/L. Mixing helped dissolve the powder and effectively bind to the algal cells and over 99% of the cells coagulated on the bottom of the container after three days, making them more available to be harvested.

The growth, lipid induction, and flocculation experiments have implications for algae biofuel productions and scalability to commercial-size operations. Optimizing biomass production simultaneously with increasing lipid content will result in the highest net biodiesel yields (Packer et al. 2011). Finding effective, affordable, and environmentally-friendly harvesting methods is also an area of important algae biofuel research (Chen et al. 2011). Using innovation and integrating production systems with other industries can help the algal biofuel industry become a viable option for renewable liquid fuel production.

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BACKGROUND

Introduction

The use of fossil fuel energy is widely accepted as unsustainable due to the depletion of finite resources and the accumulation of greenhouse gases in the atmosphere, leading to global climate change (Demirbas 2010). By sector, transportation is the second largest source of CO_2 emissions in the United States; in 2006, transportation accounted for 28% of the total U.S. greenhouse gas emissions, closely following electricity generation at 33% (U.S. EPA 2011). The majority of emissions released by transportation is a result of burning petroleum, including gasoline, diesel, and jet fuel. Furthermore, the U.S. imported more than 63 percent of its oil in 2008, costing approximately $200 billion (Pimentel 2008). As demand for oil increases and resources become more scarce and uncertain, a diversity of renewable energy sources should be developed. Among the renewable energies, one of the most important sources in the near future is biofuel, which will play a large role in easing the dependence on fossil fuels (Demirbas 2010).

Biofuels—all fuels derived from recently-produced organic matter or biomass—have the potential to provide numerous economic, social, and environmental benefits including reduced greenhouse gas emissions, national fuel security, rural development, and cleaner air and water quality (Gupta and Demirabas 2010). Biofuels are favorable to other potential renewable fuels such as hydrogen in the regards that they are fully fungible with current infrastructure and can be utilized without investments in new cars, storage facilities, or other technologies (Neal 2012). Biofuel production and use has been on the rise in the past decade, yet current feedstocks (e.g. corn) are unsustainable and do not adequately meet efficiency needs (National Research Council 2010). Microalgae are arguably the only source of renewable biodiesel that is capable of meeting the global demand for transport fuels due to their high productivity and ability to store large amounts of lipids (Demirbas 2010). While large-scale algae production has been proven successful for other industries (e.g. algal nutraceuticals), economic and technological barriers still limit the expansion of the algae biofuel industry (Huesemann and Benemann 2009).

Most industrial research has been focused on outdoor cultivation of microalgae in geographic regions that are amenable to year-round, large-scale production (Countway

2011b). This approach has potentially limited the consideration of particular algal species as well as cultivation methods. More research, innovation, and technology is needed to make algae biofuels a viable and significant replacement of fossil fuel use.

This report is divided into three sections: (1) an overview of current biofuel production, including the potential for large-scale algae biodiesel operations, (2) a summary of algae cultivation methods and photobioreactor design, and (3) an experimental study on the response of the green alga *Dunaliella tertiolecta* to growth manipulation, lipid induction, and harvesting methods.

Biofuels

Introduction

Liquid biofuels include all liquid fuels that are derived from organic matter or biomass (Worldwatch Institute 2007). In this report, the term "biofuel" refers only to liquid biofuels, and does not include biofuels in the solid form such as wood or grasses. Compared to petroleum-based fuels, biofuels offer numerous benefits, including reduction of greenhouse gas emissions, rural development, regional reduction of poverty, national fuel security, and long-term sustainability (Gupta and Demirabas 2010). These advantages will likely lead to rapid growth of biofuels used as automotive fuel in the next decade. Chemically, biofuels are compositionally comparable to petroleum fuels, and although some properties vary slightly, biofuels may have better fuel properties for transport fuel than petroleum fuels do. The greatest difference between biofuels and petroleum fuels is the oxygen content; biofuels are 10-45 percent oxygen by weight, whereas petroleum fuels contain essentially no oxygen (Gupta and Demirabas 2010). Oxygen as a part of the fuel composition promotes more complete combustion so that less carbon monoxide, soot, and unburned hydrocarbons are emitted from the tailpipe (Looper 2007). Oxygen-bearing compounds are often added to gasoline in the U.S. because of this improved combustion. The two dominant liquid biofuels in the market contribute approximately two percent of total transport energy today (IEA 2011): (1) ethanol is used as a substitute for gasoline, and (2) biodiesel is used in place of petrodiesel (simply called diesel) (Gupta and Demirabas 2010). To make biofuels a smart alternative to petroleum, the best feedstock(s) must be identified that reduce negative economic, social, and environmental impacts. A feedstock refers to the raw material that

is used in an industrial process, in this case to be converted into fuel (Worldwatch Institute 2007). Depending on the desired fuel product, favorable biofuel feedstocks are high in sugar, starch, or oil content. Currently, the most common feedstocks are sugar cane and corn for ethanol production, and soybean and oil palm for biodiesel production (Marshall 2009).

First-generation biofuels are those that are currently produced at a commercial scale, and are used in the transportation system. First-generation biofuels are made from sugar, starch, or vegetable oil. The two most widely produced first-generation biofuels are bioethanol and biodiesel (Worldwatch Institute 2007). The global biofuel production increased from 16 billion liters in 2000 to over 100 billion liters in 2010 (Figure 1). The biofuel industry is in the process of transitioning to second-generation or advanced biofuels. Second generation biofuels are produced from non-food biomass, including wastes and dedicated feedstocks such as switchgrass, jatropha, and algae (Sims et al. 2010). Cellulosic ethanol, algae fuels, biohydrogen, biomethanol, and wood diesel are a few promising second-generation biofuels that are currently under development.

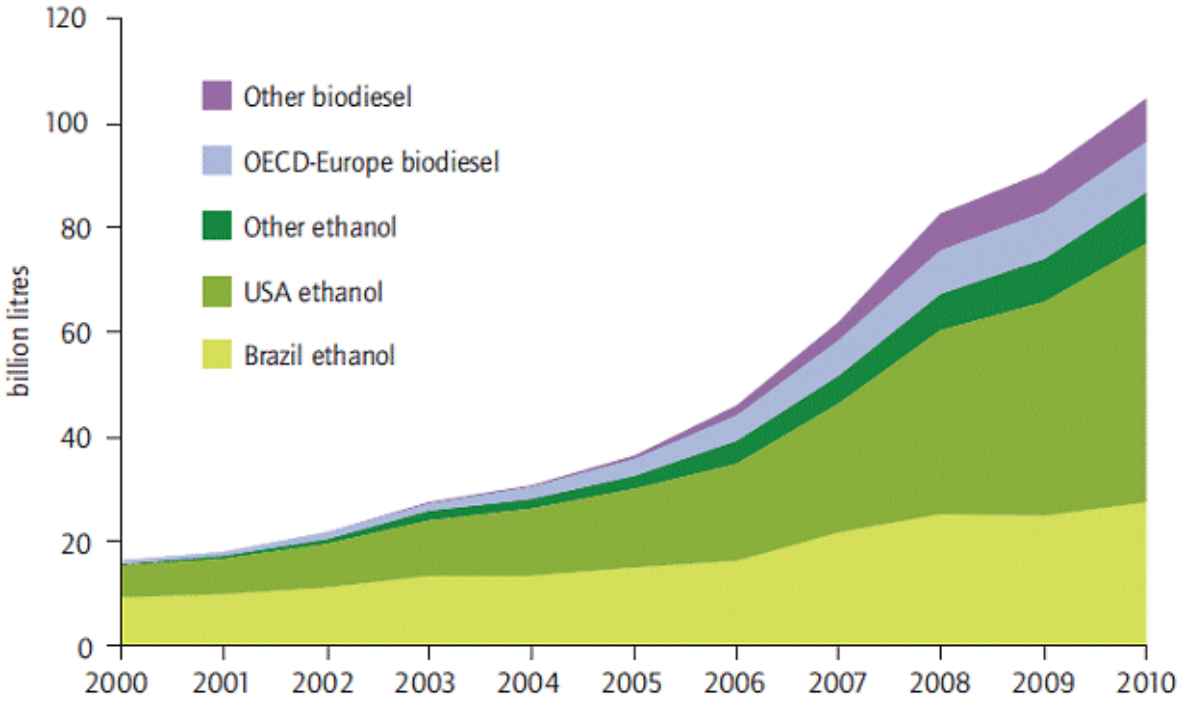

Figure 1. Global biofuel production increased from 16 billion liters in 2000 to over 100 billion liters in 2010 (International Energy Agency 2011)

Environmental, Social, and Economic Impacts

The biofuel industry has the potential to provide social and economic benefits across the globe. The concentration of petroleum resources in a few countries makes the global economy vulnerable to shocks and as stocks are being depleted, resources are becoming more scarce and uncertain (Childs and Bradley 2007). The decentralized production of biofuels, on the other hand, can reduce the risk of disruption and enhance national and global stability. Biofuels can be produced in almost every region of the world, eliminating the need for transporting fuel over long distances (Childs and Bradley 2007). Furthermore, the biofuel industry is also a source of jobs and economic development. In the United States, the ethanol industry is credited with directly providing about 200,000 jobs. In Brazil, half a million jobs are credited to the ethanol industry (Childs and Bradley 2007)

When considering biofuels from various feedstocks and production technologies, it is essential that a complete analysis is done to assess the direct and indirect impacts of the production process. A life-cycle analysis examines the environmental impact of a product or process from its beginning to the end of its relevant life (Worldwatch Institute 2007). Various life cycle analyses have been performed for first-generation biofuel crops with a wide range of results. Second-generation biofuels have a much more favorable environmental impact than first-generation biofuels do, yet life cycle analyses are still not widely available for second-generation biofuels. The majority of studies indicate that biofuels can reduce greenhouse gas emissions and can help alleviate dependence on petroleum fuels (Gupta and Demirabas 2010). However, environmental and social problems can also arise with biofuels during every stage of production. The major problems posed by expanding biofuel production, if not done carefully, are increased greenhouse gas emissions, increased land and water use, and threats to biodiversity and human health (National Research Council 2010).

One of the greatest motivations for developing the biofuel industry is concern for the environment and climate change. Combusting biofuels as opposed to fossil fuels could greatly improve urban air quality and reduce pollutants. The emissions from burning biofuels are most often cleaner than from petroleum fuels (Childs and Bradley 2007).

Blending biofuels with petroleum fuels has been proven to reduce sulfur, particulate, and carbon monoxide emissions from vehicles.

Biofuels offer a potential solution to the problem of large amounts of greenhouse gasses emitted by transportation. In theory, biofuels are carbon neutral because the carbon dioxide (CO_2) emitted during combustion is offset by the amount absorbed from the atmosphere to create the feedstock biomass (World Resources Institute 2006). While carbon is still emitted into the atmosphere upon combustion of biofuel, it is more favorable than burning fossil fuels, which are essentially carbon that has been stored in the earth for millions of years. However, this is a very simplified view of biofuels and does not account for energy input during all stages of production, nor does it address other environmental concerns posed by biofuel production.

In the development of second-generation biofuels, greater emphasis is put on reducing greenhouse gas emissions due to the debatable environmental impacts of first-generation biofuels. The U.S. Energy Independence and Security Act (EISA) of 2007 states that advanced biofuels must reduce lifecycle greenhouse gas emissions by at least 50 percent over baseline emissions (National Research Council 2010). A report by the National Research Council (2010) defines a sustainable biofuel system as one that is not only carbon neutral, but carbon negative; is nutrient and water conservative; provides biodiversity benefits; and has no negative impacts on human health. Biofuels that are carbon negative remove carbon from the atmosphere than they release through combustion, which can be achieved using a number of mechanisms for carbon sequestration such as recycling part of the biomass produced in the form of charcoal (Mathews 2008).

To determine if biofuels are a viable option for transportation fuel, the net energy output must be determined, which is often debated and difficult to measure because it involves processes throughout the life cycle of the fuel production. Measuring the energy input needed to produce the biofuel is crucial because the energy input is, in some cases, greater than the energy contained in the biofuels themselves (Sawin and Flavin 2006). To produce the crops from which biofuels are made, fossil energy is often used in the form of fertilizers, tractor fuel, processing energy, and transportation. Advances in technology, as well as the increasing use of bioenergy for feedstock processing, have reduced the

volume of fossil fuels used to produce biofuels from plant biomass (Sawin and Flavin 2006, Childs and Bradley 2007). Currently, all commercially produced biofuels purportedly have a positive fossil energy balance, calculated by the amount of energy contained in the produced biofuel per unit of fossil fuel input (Table 1) (Worldwatch Institute 2006).

Table 1. Fossil energy balances of selected fuel types based on data from Worldwatch Institute (2006). Calculations are based on the amount of energy contained in the listed fuel per unit of fossil fuel input. Ratios for cellulosic biofuels are theoretical.

Fuel (feedstock)	Fossil Energy Balance
Cellulosic ethanol	2–36
Biodiesel (palm oil)	~9
Ethanol (sugar cane)	~8
Biodiesel (waste vegetable oil)	5–6
Biodiesel (soybeans)	~3
Biodiesel (rapeseed, EU)	~2.5
Ethanol (wheat, sugar beets)	~2
Ethanol (corn)	~1.5
Diesel (crude oil)	0.8–0.9
Gasoline (crude oil)	0.8
Gasoline (tar sands)	~0.75

Before policies and subsidies are set in favor of increased biofuel production, it is critical to understand whether first-generation biofuels such as corn ethanol and rapeseed biodiesel deliver global environmental benefits. In the United States, protocols are currently being developed to measure the net greenhouse gas emissions from specific biofuel production systems. The U.S. Environmental Protection Agency (EPA) is creating a carbon accounting protocol for the Federal Renewable Fuel Standard, which raises questions about how to measure the total carbon impact of biofuel production (Marshall 2009). The issue of whether to include indirect land use changes, or secondary conversions, has not been resolved and complicates analysis. As biofuel production

expands throughout the world, it is essential to understand all direct and indirect impacts to guide policy.

Changes in land use, and the resulting effects on global greenhouse gas emissions, must also be factored into the environmental evaluation of biofuels. An increasing amount of land is being converted into agricultural land for biofuel crops such as corn and soybeans, which may result in a total net increase in carbon emissions. For example, the clearing of tropical forests in Southeast Asia for palm plantations, mainly for cooking oil, releases large amounts of carbon stored in the forests (Childs and Bradley 2007). Deforestation and forest degradation already account for up to 17% of global greenhouse gas emissions. Deforestation also results in a huge loss of biodiversity and other negative ecological effects. If the scale of first-generation biofuel production increases, large-scale redistribution of land will be necessary, and will push other agriculture or ranching uses into high-carbon forests and grasslands (Marshall 2009).

Another major concern with biofuel production is that it puts strain on existing agricultural systems and creates a "food vs. fuel" situation. The result is increasing food prices of important crops such as corn. Agricultural commodity markets have already been affected by the production of biofuels. For example, the price of sugar has gone up worldwide, partly attributed to the fact that 50 percent of the sugar cane crop in Brazil is dedicated to producing ethanol (Childs and Bradley 2007). In South Africa and the Scandinavian countries, staple food crops are banned for use as bioenergy because it directly competes with their use for food (Valentine et al. 2012).

The production of corn-based ethanol has been criticized for a number of negative environmental and social impacts. Corn is an annual crop that requires significant water inputs, fertilizers, and pesticides (National Research Council 2010). Compared to other crops, corn production has higher fertilizer requirement and creates nitrogen run-off, which negatively impacts water quality (Selman 2011). Mubako (2008) conducted research on water requirements of corn-derived ethanol production. The results show that corn ethanol is a very water-intensive product and the water to ethanol volumetric ratio of the major corn-growing U.S. states is 1174 to 1492. Furthermore, an investigation of agricultural and water quality impacts showed that, on average, 65.5 g nitrogen, 23.8 g phosphorus, and 1.03 g of pesticides are applied, and 4.8 kg of soil is eroded per liter of

ethanol produced (De La Torre Ugarte et al. 2010). Furthermore, corn ethanol has been associated with long-term degradation agricultural land due to large monocultures, tilling practices, and the lack of complementary crop residue use (De La Torre Ugarte et al. 2010).

Biofuel production is a complex system that needs to be evaluated on many levels to determine whether it is an environmentally, economically, and socially responsible option for transportation fuel. The current biofuel system is unsustainable, and second-and third-generation biofuels must meet higher standards for the expansion of the industry to be justified and responsible. Feedstocks and processes vary, and finding the most sustainable alternatives is key in guiding policy and commercialization.

Current Biofuel Production

Although biofuels have been used in the automotive industry since its inception, the global biofuel industry has grown dramatically in recent years. At the 1900 World's Fair, Rudolph Diesel was the first to run a diesel engine, powered by peanut oil (Pacific Biodiesel 2012). In 1941, Henry Ford constructed a car to be fueled by hemp ethanol (Lance 2009). Today, biofuels only provide about one percent of global liquid transport fuel (Worldwatch Institute 2006, Hammond et al. 2008). Global biofuel production tripled between 2000 and 2007, from 18 billion liters to about 60 billion liters, yet this only accounts for less than three percent of the global transportation fuel supply (Coyle 2007). The International Energy Agency (2011) reports that with the right policy framework, financial support, and scientific research, biofuels could provide 27% of total transport fuel by 2050. This projected use would reduce emissions of CO_2 by 2.1 gigatons. The upward trend in biofuel production is prominent within the U.S, as production expanded from 6 billion liters in 2000 to 34 billion liters in 2008. Within that time, the number of refineries also increased from 54 to 170 (Fulton 2011). Bioethanol, mainly from the U.S. and Brazil, constitutes the majority of biofuel production worldwide; strong state and federal mandates are in place and will continue to encourage industry growth. In 2010, the United States generated 57 percent of the total global output, or 49 billion liters of biofuel. Brazil produced 28 billion liters of biofuel, constituting 33 percent of the total global output (Worldwatch Institute 2011). While

bioethanol is largely produced in the U.S. and Brazil, the European Union and Southeast Asia are the major biodiesel producers.

Political and industrial support is necessary for a major transition away from petroleum transport fuels to occur. The rise in biofuel production can be partially attributed to new laws and mandates in a number of countries, including the United States, Brazil, Argentina, China, and Canada (Worldwatch Institute 2011). Adopted instruments that have been successful in increasing production include mandatory blending targets, tax exemptions, and subsidies (Sorda et al. 2010). Also, several prominent automakers such as Ford, General Motors, and Volkswagen have committed to manufacture more flexible-fuel ("flex-fuel") vehicles, which can run on various blends of ethanol and gasoline (Sawin and Flavin 2006). High oil prices have contributed to the growth and entrance of several large fuel companies into the industry. Flex-fuel vehicles are common in Brazil and many drivers have switched to sugarcane ethanol because it is cheaper than gasoline (Worldwatch Institute 2011). In the U.S., ethanol from corn is now at a competitive price with gasoline.

Biofuels in the United States

The United States government is committed to expanding the use of transportation biofuels and supports the growth of the industry through a number of programs and mandates. The U.S. Department of Energy seeks to increase research in the development and commercialization of economic biofuels produced from non-food sources as a near-term replacement for petroleum fuels (Silver 2010). Critical legislative actions include the Energy Policy Act of 2005 (EPACT), the Energy Independence and Security Act of 2007 (EISA), and the Farm Bills of 2002 and 2008 (National Research Council 2010). Historically, government support has been focused on ethanol production, with EPACT setting a goal of 28 billion liters produced by 2012, which was well exceeded (Sorda et al. 2010). Major financial support has been provided through the Volumetric Ethanol Exercise Tax Credit (VEETC), which provided companies with a tax incentive of $0.45 per gallon of pure ethanol that was blended with gasoline until the program's expiration in December 2011 (Worldwatch Institute 2011).

Regulations set by the U.S. Environmental Protection Agency (EPA) mainly focus on incorporating corn ethanol and cellulosic biofuels into petroleum transportation fuels, but

have recently been incorporating other advanced biofuels into production standards. The Renewable Fuel Standard (RFS), created under the Energy Policy Act of 2005, requires a minimum volume of renewable transport fuel be sold in the U.S. The goal of the program is to reduce greenhouse gas emissions, reduce reliance on imported petroleum, and expand the national renewable fuel sector. The original program required that 28 billion liters of renewable fuel be blended into gasoline by 2012. In 2007, the RFS program was expanded (RFS2) to require 515 billion liters of renewable fuel be blended into transportation fuel each year by 2022, and also included diesel mixtures in addition to gasoline. RFS2 also incorporated new categories of renewable fuel, and set specific volume requirements for each type: 57 billion liters per year will come from corn ethanol, 64 billion liters from cellulosic biofuels and biodiesel, and the remaining 15 billion liters from other advanced biofuels (U.S. EPA 2011). Producers are required to generate second-generation biofuels that reduce greenhouse gas emissions by at least 50 percent, and first-generation biofuels must reduce greenhouse gas emissions by at least 20 percent. Feedstock type and land use practices are also regulated. RFS2 went into effect in July 2010 (Sorda et al. 2010).

A World Resources Institute report titled *Plants at the Pump: Biofuels, Climate Change, and Sustainability* recommends that focus be put on guaranteeing environmental performance and improving technologies, rather than on increasing the scale of first-generation biofuel production (Childs and Bradley 2007). The U.S. Departments of Agriculture and Energy produced a joint study reporting 37 percent of U.S. transport fuel could be replaced with second-generation biofuels within the next 25 years, or 75 percent if vehicle fuel economy is doubled (Sawin and Flavin 2006). Policies are needed to advance the transition to the next generation of feedstocks that allow increased production at lower costs and that incorporate greater knowledge of the environmental impacts.

Biodiesel

Introduction

Biodiesel is one of the most commonly used biofuels and it is miscible with petrodiesel in any proportion. Biodiesel is an attractive alternative or extender fuel for conventional petroleum diesel fuel (petrodiesel) for combustion in compression-ignition

(diesel) engines (Moser 2009). The American Society for Testing and Materials (ASTM) defines biodiesel, designated "B100," as fuel composed of mono-alkyl esters of long-chain fatty acids (called fatty acid alkyl esters) derived from renewable vegetable oils or animal fats. This alternative or mixture offers a number of technical advantages over ulta-low sulfur diesel fuel (ULSD, <15 ppm S), which is most commonly used in automobiles. Feedstocks for biodiesel production vary in different parts of the world based on the availability of resources and conditions for growing feedstock crops. Potential biodiesel feedstocks can be divided into four major categories: (1) oilseeds, (2) animal fats, (3) algae, and (4) various low-value materials such as cooking oils, greases, and soap stocks.

Some of the technical advantages and disadvantages of biodiesel as compared to conventional petrodiesel are described in Table 2. Many of these problems with biodiesel can be mitigated through cold flow improver and antioxidant additives, blending with petrodiesel, and/or reducing storage time (Moser 2009). Diesel cold flow improver helps prevent fuel filter plugging in cold temperatures. Fuel additives are an indispensable tool in biodiesel production in that they improve ignition and combustion efficiency, stabilize fuel mixtures, protect the motor from wax deposition and abrasion, and reduce pollutant emissions (Ribeiro et al. 2007).

Table 2. Advantages and disadvantages of biodiesel compared to petrodiesel (Moser 2009)

Advantages	Disadvantages
Inherent lubricity	High feedstock cost
Low toxicity	Inferior storage and oxidative stability
Derivation from a renewable and domestic feedstock	Lower volumetric energy content
Superior flashpoint and biodegradability	Inferior low-temperature operability
Negligible sulfur content	Higher NOx exhaust emissions (in some cases)
Lower overall exhaust emissions	

Biodiesel Characteristics

A chemical process, called a transesterification reaction, is used to convert biomass into biodiesel fuel that is compatible with diesel engines. Combusting the raw material (viscosity generally 28-40 mm^2/s) in a diesel engine would lead to operational problems; transesterification of the oil is necessary to reduce the viscosity to a range close to that of conventional petrodiesel (generally 4-5 mm^2/s) (Knothe 2010). Vegetable oils and animal fats are ideal for conversion to biofuels because they contain triacylglycerols (TAGs), which consist of long-chain fatty acids bound to a glycerol backbone. During transesterification, the TAG reacts with a short-chain monohydric alcohol in the presence of a catalyst at elevated temperature. Methanol is the most commonly used alcohol because it is typically less expensive than other alcohols, but ethanol is used in some regions where it is less expensive, for example in Brazil. Generally, homogenous alkaline base catalysts such as sodium hydroxide, potassium hydroxide, or methoxide are used in commercial biodiesel production because they are faster and less expensive than acid catalysts. The products of transesterification are fatty acid alkyl esters (biodiesel) and glycerol. For every mole of TAG that undergoes transesterification, three moles of biodiesel and one mole of glycerol are produced. (Moser 2009)

Biodiesel standards (ASTM D6751 in the U.S.) are in place in many countries to ensure that high-quality biodiesel reaches the market. The quality of biodiesel is described by several properties, including low-temperature operability, oxidative and storage stability, kinematic viscosity, exhaust emissions, cetane number, and energy content. Biodiesel produced from different feedstocks have distinct fuel properties due to the unique chemical compositions of the raw materials. The fuel properties are determined by the fatty ester composition, as well as presence of contaminants and minor components. Contaminants are defined as unwanted or incomplete reaction products from the transesterification process, and may include free fatty acids (FFA), soaps, triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG), alcohol, catalyst, glycerol, metals, and water. Minor components are the naturally occurring parts such as tocopherols, phospholipids, steryl glucosides, chlorophyll, fat soluble vitamins, and hydrocarbons (Moser 2009). According to the American Society for Testing

Materials (ASTM), biodiesel from microalgae has similar properties to standard biodiesel and is more stable because of its higher flash point values (Demirbas and Demirbas 2010).

While biodiesel is predominantly used as transport fuel for automobile diesel engines, it has also become a considerable option for a number of additional applications. Other combustion-related applications of biodiesel include jet fuel as well as in diesel-fueled marine engines to reduce environmental impact. Fatty acid alkyl esters are a versatile material that can be used as a bio-based replacement for petroleum as heating oil, and in generators and turbines for the generation of electricity (Moser 2009). Biodiesel is often used to power underground mining equipment to mitigate harmful exhaust emissions. In a Mine Safety and Health Administration (MSHA) study of a Carmeuse limestone mine in Kentucky, a biodiesel blend B35 (35% biodiesel, 65% petrodiesel) resulted in a 50 percent reduction of diesel particulate matter inside the equipment cabs. As a result, the mine switched its entire operation to B99 despite the slightly higher costs (U.S. DOE 2009). Consequently, managers of ational parks and environmentally conscious locations may be especially interested in biodiesel for use in fuel-related applications (Moser 2009).

Current Biodiesel Production

World biodiesel production from vegetable oils and animal fats was 8.6 megatonnes (mt) in 2007, mostly from the European Union and the United States, but also increasingly from Argentina, Brazil, and Indonesia (International Grains Council 2008). In 2007, biodiesel production represented about seven percent of the total use of vegetable oil. The largest feedstock was rapeseed (canola) oil, with 4.5 mt of raw material used for biodiesel production. Soy oil and palm oil also constituted a large portion of the feedstocks: 22% (2.1 mt) and 11% (1.0 mt), respectively. The EU was the world's largest biodiesel producer, with an estimated 5.4 mt of output in 2007. The U.S. was the second largest producer, with 1.5 mt in 2007. Seventy-nine percent of the biodiesel production in the U.S. comes from soy oil. Other raw materials commonly used in the U.S. include animal fats and grease (80,000 tonnes), cottonseed oil, rapeseed oil, and corn oil (International Grains Council 2008). Current biodiesel feedstocks pose the same problems as the aforementioned first-generation biofuel feedstocks, such as low

energy yield, competition with food sources, and high CO_2 emissions. Research in new feedstocks, such as non-food crops, cellulosic plants, and algae will lead the future in biodiesel production.

Future of Biodiesel

Traditionally, oilseeds such as soybean, rapeseed, palm, corn, sunflower, cottonseed, peanut, and coconut oils have been the predominant feedstocks used worldwide. Genetic engineering and selective breeding have been used to increase yields of many biodiesel crops such as corn and soybeans. A major threat to the biodiesel industry is the high cost of feedstock acquisition, which can account for over 80 percent of the overall biodiesel production expenses (Moser 2009). The use of alternative feedstocks of varying type, quality, and cost could potentially solve this problem. Alternative feedstocks that have not yet been developed on a large scale include acid oils, used cooking oils and waste greases, non-food vegetable fats, and oils obtained from trees and microorganisms such as algae. Characteristics that make alternative oilseed feedstocks attractive for biodiesel production include adaptability to local growing conditions (rainfall, soil type, humidity, sunlight), regional availability, high oil content, favorable fatty acid composition, compatibility with existing infrastructure, low agricultural inputs (water, fertilizer, nutrients), definable growth season, potential markets for by-products, and the ability to grow on non-arable land. Feedstocks that meet a majority of these criteria will be the most promising sources of second-generation biodiesel (Moser 2009).

One of the most-promising biodiesel feedstock that meets many of the above criteria is microalgae; large amounts of lipid-rich algal biomass can be produced on land that is unsuitable for agriculture. Furthermore, the production of algal biodiesel can be coupled with flue gas CO_2 mitigation, wastewater treatment, and the production of high-value chemicals (Li et al. 2008). The potential for algae as biodiesel is great, as the oil content can exceed 80 percent by weight compared with 5 percent for the best agricultural oil crops; however, microalga-based systems still have poor volumetric efficiencies, which make them costly compared with petroleum fuels (Amaro et al. 2011). Research and innovation into algae biofuels, specifically biodiesel, could help the industry grow and become a major provider of liquid fuel.

Algae Biodiesel

Introduction to Algae

The term "algae" generally refers to aquatic eukaryotes that range from small, single-celled organisms to fairly complex multicellular forms. Microalgae, also called phytoplankton, are a diverse group of primitive organisms with a simple cellular structure and are ubiquitous throughout the world. Over 40,000 species of eukaryotic algae have been identified and many more are thought to exist (Darzins et al. 2010). A few thousand strains are kept in culture collections throughout the world and only a handful of species are cultivated at an industrial scale (Darzins et al. 2010). The Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA), located at the Bigelow Laboratory for Ocean Sciences in East Boothbay, Maine has over 2,700 species of marine, benthic, macrophytic, and freshwater phytoplankton and heterotrophic organisms in culture (NCMA 2011). Algae can live in saline or freshwater ecosystems; large-scale natural sources include bogs, marshes, swamps, salt marshes, salt lakes, as well as large blooms in the ocean (Darzins et al. 2010).

Microalgae are classified into at least 12 major divisions based on their pigmentation, life cycle, and cellular structure (Darzins et al. 2010). The three most abundant classes of microalgae are Bacillariophyceae (diatoms), Chlorophyceae (green algae), and Chrysophyceae (golden algae). Diatoms dominate the phytoplankton; these are the largest group of biomass producers on Earth, with over 100,000 species estimated to exist (Demirbas and Demirbas 2010). Green algae also comprise a diverse group of algae, consisting of approximately 350 genera and 2650 living species (UCMP 2011).

Most microalgae are photoautotrophic in that they use photosynthesis to convert the energy of sunlight into chemical energy, relying on sunlight, carbon dioxide, and water for survival (Countway 2011d). Bacteria of the Phylum Cyanobacteria are also considered algae and gain their energy through photosynthesis as well. The photosynthesis process is described by the following chemical reaction:

$$6CO_2 + 6H_20 + \text{light energy} \rightarrow C_6H_{12}O_6 \text{ (sugars)} + 6O_2$$

The sugars produced by photosynthesis are then converted into the cellular biomass including organic molecules such as lipids, carbohydrates, and proteins. Many species of microalgae are able to accumulate large amounts of glycerolipids, specifically

triglycerides (Darzins et al. 2010). Typically, lipids form in the outer membrane of the cells; however, some species produce significant amounts of storage lipids. Stress conditions, for example nutrient deprivation, can cause storage lipids to increase to up to 60 percent of the cell weight. Most of the algae known to produce large amounts of lipids (more than 20 percent of their biomass) are of the Divisions Cryptophyta, Chorophyta, and Chromophyta (Darzins et al. 2010).

Algae fix large amount of carbon dioxide (CO_2) and are an essential component of the global carbon cycle. Although algae only account for approximately 0.2 percent of global biomass generated by photosynthesis, they are responsible for about 50 percent of the total fixed organic carbon (Darzins et al. 2010). This is partly due to their high growth rates, at one to four cell doublings per day. Algae support the majority of life on Earth, forming the basis of the ocean food web and also responsible for contributing 40 to 50 percent of the oxygen in the atmosphere (Darzins et al. 2010).

Algae are more efficient at converting solar energy into biomass than most terrestrial plants, partly due to their large surface-area-to-volume ratio, allowing them to uptake large amounts of nutrients (Sheehan et al. 1998). Algae can convert up to five percent of the sun's energy into biomass, whereas corn and sugar cane only convert about one percent (Aswathanarayana 2010). This efficiency is largely driven by the fact that algae do not produce cellulose and lignin – both of which are required to hold plants against gravity, and which require significant energy input to produce (Neal 2012). While microalgae-derived fuel may not seem plausible to fill current demand, the difference in life cycles of primary producers on land and in the ocean must be kept in mind. Terrestrial vegetation, while often large in size, has low turnover and growth rates. Pelagic phytoplankton, although small, have high turnover rates and high growth rates. The annual production to biomass ratio of pelagic phytoplankton is between 100-300 whereas the ratio for terrestrial vegetation is 0.5-2.0 (Countway 2011d).

Essential nutrients for algal growth are carbon (C), nitrogen (N), phosphorus (P), and iron (Fe) (Chisti 2007). Nitrate is the primary source of nitrogen in the ocean, however, ammonium is the preferred source for many phytoplankton (Countway 2011d). Silicate is a major requirement for diatoms because they form silicon plates as a protective cell wall, but other phytoplankton do not require silica. In natural systems, major nutrients are not

evenly distributed in time or space and are not always available in a usable form (Countway 2011a). As a result, different species develop adaptations to a variety of resources and environmental conditions. Although individual cells vary considerably in nutrient requirements, the Redfield Ratio is a widely accepted "community average" of the elemental composition of marine organic matter. This C:N:P ratio, which was empirically developed from analyzing thousands of marine samples from different ocean regions, is 106:16:1(Redfield 1934). When nutrients are not limiting in natural or controlled environments, this ratio generally holds true for phytoplankton biomass.

Dunaliella- an algal biofuel contender

Dunaliella sp. is a green alga that is being explored for the purpose of producing biofuels. Algae of the genus *Dunaliella* belong to the Class Chlorophyceae*,* the Order Chlamydomonadles*,* and the Family Dunaliellaceae (Polle et al. 2009). The genus *Dunaliella,* represented by 27 euryhaline species, is one of the most studied groups of Chlorophyceae (Gonzales et al. 2009). They are unicellular green flagellates that lack a rigid cell wall, which is common to many other unicellular green algae. Twenty-three of the 27 species live in saline environments, and many are extremophiles, tolerating subzero temperatures and extreme salinities. *Dunaliella* are found all over the world, including the Dead Sea and salt lakes of the Antarctic. They can also thrive in habitats with a wide range of pH, light intensity and temperature. Certain species of *Dunaliella* are among the most intensively studied algae due to the accumulation of α- and ß -carotene that produce a red color when the cells are under stress. These pigments are utilized in the cell for osmoregulation and photo-protection (Countway 2011b). *Dunaliella* have been cultured since 1925 as a live feed organism in aquaculture and as a valuable source of ß-carotene (Subba Rao 2009).

Dunaliella can grow in a wide range of conditions, and the choice of medium depends on the utility of the cultures (Subba Rao 2009). *Dunaliella* species have been successfully grown autotrophically at temperatures ranging between 10˚C and 30˚C in various growth medium. Subba Rao (2009) determined that the maximum cell density varied between 0.3 x 10^6 ml^{-1} and 24 x 10^6 ml^{-1} between various *Dunaliella* species and so did the day of attaining their maximum density (4 to 35 days).

Dunaliella tertiolecta is a species that has been shown to have very high oil content, which has made it one of the common species used as a food source for larval fish and invertebrates in aquaculture (Countway 2011b). The green cells are about 6-9 μm in length, have an ellipsoidal shape and flagella that are about twice the length of the cell body (Guiry and Guiry 2012). This motile species has a high tolerance to salt, light, and temperature. *Dunaliella tertiolecta* is relatively easy to cultivate and has a high growth rate and lipid content (Tang et al. 2011). After investigating the effects of a number of environmental parameters on the growth rate and fatty acid methyl ester composition, Tang et al. (2011) conclude that *Dunaliella tertiolecta* is a good candidate for biofuel production.

Commercial Algae Production

Algae have been actively cultured by humans for centuries, mainly as a source of protein for food (Demirbas and Demirbas 2010). *Spirulina*, a blue-green alga hyped today as a "superfood," has been used as a food source since the 16th century and used by Aztecs and other Mesoamericans (Darzins et al. 2010). Large-scale cultivation of microalgae for a variety of practical uses was first taken up in Germany during World War II (Becker 1994). These studies provided early information on the growth, physiology, and biochemistry of algae. A 1953 book titled, *Algal culture: From Laboratory to Pilot Plant* by a group of scientists at the Carnegie Institution of Washington, focused on large-scale production of the green alga *Chlorella* for food, and addressed the issue of maintaining yields in large-scale outdoor cultures (Burlew 1953). Further research has shown that in addition to food, algal biomass can be used for other applications such as animal feed, biofertilizer, aquaculture feedstock, and can even act as a biological purifier for waste water. More recently, the production of a variety of compounds from microalgae, such as polysaccharides, lipids, proteins, carotenoids, pigments, vitamins, sterols, enzymes, antibiotics, pharmaceuticals, and several other fine chemicals has been investigated (Becker 1994). The various projected applications of algae are summarized in Table 3.

Table 3. Projected applications of commercially produced algae from data in (Becker 1994)

Category	Specific applications
Food	Protein supplement/fortification in diets for malnourished children and adults.
Feed	Protein/vitamin supplement in feeds for poultry, cattle, pigs, fish, and bivalves.
Health Food	Algal powder as ingredient and supplement in health food recipes and products.
Therapeutics	ß-Carotene as possible anti-skin-cancer treatment. Algal antibiotics as wound treatment, enzymatic hydrolyzates to promote skin metabolism. Prostaglandin stimulation by y-linoleic acid. Regulation of cholesterol synthesis. Isotopic compounds in medical research.
Pigments	ß-Carotene as food color and food supplement. Xanthophylls in chicken and fish feeds. Phycobilins as food color, in diagnostics, cosmetics and analytical reagents.
Source of chemicals	Glycerol used in foods, beverages, cosmetics, pharmaceuticals. Fatty acids, lipids, waxes, sterols, hydrocarbons, amino acids, enzymes, vitamins C and E. Polysaccharides as gums, visocifers and ion exchangers.
Fuel	Long-chain hydrocarbons and esterified lipids as combustible oil. Hydrogen, biogas, methane.
Hormones	Auxins, gibberlins and cytokines.
Others	Biofertilizer, soil conditioner. Waste treatment.

Only about 9,000 tonnes of algal biomass is produced worldwide each year (Darzins et al. 2010). Most commercial algae production is restricted to producing high-value health food or pigment, mostly in Asia and the United States (Becker 1994).Microalgal biomass is generally cultivated in open, CO_2-fertilized outdoor ponds and is limited to a few genera, including *Spirulin* sp., *Chlorella* sp., and *Dunaliella* sp. The majority of microalgal production in the U.S. comes from three companies: Earthrise Nutritionals, LLC. in California, Cyanotech Corporation in Hawaii, and Martek Biosciences Corporation in Maryland (Darzins et al. 2010). Earthrise Nutritionals introduced the cyanobacterium *Spirulina* to the natural foods market in 1979, and is now the world's

largest *Spirulina* farm (Earthrise Nutritional 2009). Cyanotech Corporation produces nutritional supplements and cosmetic products from microalgae grown in their 90-acre facility (Cyanotech 2011). Martek Co. make DHA Omega-3 supplements, which are a vegetarian alternative to the popular fish oil products (Martek 2012). These companies achieve yields of 50 tonnes of algal biomass per acre per year. Although these companies grow algae for purposes other than biofuel production, they demonstrate that large-scale production is feasible. If the lipid content of the harvested biomass could be made to reach 33%, more than 19,000 liters of biofuel per acre per year could be produced with the current biomass yields (Riesing 2009). However, this estimated yield does not account for losses during production and has not yet been accomplished at such a large scale.

Algae Energy

Using microalgae as fuel, whether burning the biomass directly or extracting cellular components for conversion, is essentially the same principle as using petroleum for energy. The energy of the sun is captured by plant biomass which is then converted into useable fuel. Petroleum is called a "fossil fuel" because it was formed by the geological compression and heating of the remains of algae and other small sea plants and animals that died millions of years ago and sank to the ocean floor (Gupta and Demirabas 2010). In the 1970s, efforts to find alternative energy increased and interest in microalgae as a source of renewable energy arose. However, there is still minimal industrial effort in the mass production of algae for biofuel conversion in spite of the economic potential. Microalgae have the potential to generate a variety of environmentally and economically sustainable biofuels, including biodiesel, hydrogen, methane, hydrocarbons, and bio-syngas – a liquid fuel produced by the Fischer-Tropsch process. Algae are possibly the most promising feedstock for biodiesel because they are among the most photosynthetically efficient organisms on Earth and store energy in the form of lipids and starch (Demirbas and Demirbas 2010). Demirbas (2010) estimates that a 1-hectare algae farm on wasteland can produce over 10 to 100 times as much oil as any other known fuel crop. Table 4 shows the oil yield of various biodiesel feedstocks, including algae. These projections are only estimates because algae biodiesel production of this scale has not been tested. Nevertheless, it is worthwhile to invest research into algae as a feedstock for

biofuel due to the high theoretical yields. Some scientists believe that algal biofuels alone can replace all fossil fuel consumption on earth (Francis 2010).

Table 4. Liters of oil per acre per year for various biofuel feedstocks (Stewart and Hessami 2005)

Biofuel feedstock	Liters of oil per acre per year
Corn	68
Soybeans	182
Safflower	314
Sunflower	386
Rapeseed	481
Oil Palm	2,404
Microalgae	19,000-56,800

There are several types of fuel that can be produced using microalgae as a feedstock. The algal biomass can be dried and directly combusted for power generation or other thermochemical conversions can be undertaken to generate synthetic gas and oils. The major disadvantages of direct combustion are high NO_x emissions and losses of nitrogen fertilizer (Huesemann and Benemann 2009). Thermochemical conversion processes, such as pyrolysis and gasification, have been successfully demonstrated in laboratory studies, but are difficult to scale up to commercial levels. Other fuel options from microalgae include ethanol from the fermentation of starch, and methane or other fuels produced by microbial action. However, the current yield of algal methane is only about half of the maximum achievable (Huesemann and Benemann 2009). More research will be needed to increase yields from this process to make it economically viable.

Currently, making biodiesel from extracted lipids is the most promising fuel application of microalgae. The lipids produced by many microalgae are suitable for conversion into liquid transportation fuel (Demirbas and Demirbas 2010); according to the American Society for Testing Materials (ASTM), biodiesel from microalgae has similar properties to standard biodiesel (Demirbas and Demirbas 2010).

The production of microalgal biodiesel requires large quantities of algal biomass and also high lipid content in the algal cells. Algal cellular compositions vary considerably,

and the fatty acid content of most green algae is between 10 and 30 percent of the dry cell weight (Colyer and Fogg 1955). Table 5, compiled by Demirbas (2010) shows the lipid content of various species recorded in selected studies. Under favorable growth conditions, the lipid content can increase to more than 60 percent of the dry cell weight (Sheehan et al. 1998). Studies have shown that lipid composition and content is influenced by a number of factors, including light, temperature, and nutrient availability (Demirbas and Demirbas 2010). As far back as the 1940s, nutrient deprivation was reported to significantly increase microalgal lipid storage (Demirbas and Demirbas 2010). In response to nutrient deficiency (nitrogen for green algae and silicon for diatoms) and other environmental stressors, many microalgae accumulate neutral lipids, in particular triacylglycerols (TAGs) (Huesemann and Benemann 2009).

Table 5. Oil content in selected microalgal species as reported in Demirbas (2010b)

Species	Oil content (% dry weight)
Botryococcus braunii	29-75
Chlorella spp.	29
Cyclotella DI-35	42
Dunaliella teriolecta	36-42
Hantzchia DI-160	66
Isochrysis spp.	7-33
Nannochloris	12 (6-63)
Nannochloropsis	46 (31-68)
Nitzchia TR-114	28-50
Phaedactylum tricornutum	31
Scenedesum TR-84	45

Current Algae Fuel Production

Currently, algae-derived fuels are not used or commercially available other than in very small-scale or experimental endeavors. A large market for algae-derived liquid fuels

does not exist; however, with further development, algal biofuels have the potential to become an environmentally friendly and economically viable alternative to fossil fuels. A report to the Bioenergy sector of the International Energy Agency (IEA task 39) determined that algal biomass is a feasible option for the production of liquid transportation fuels, and innovation will be needed to address technical inefficiencies at all stages of production to develop large-scale enterprises. Technological advances will most likely not be enough to make algae fuels economically competitive with conventional fossil fuels. A number of U.S. companies are logistically prepared to produce significant volumes of algae biodiesel, yet there are still barriers to realizing the potential yields. Government support through emissions trading schemes, carbon tax, legislation to reduce CO_2 emissions, or require biodiesel integration could provide incentive for transition (Darzins et al. 2010).

Few companies are currently producing energy from algae at a commercially viable scale, and these companies have designed innovative technologies and production systems to make this possible. Sapphire Energy in California has successfully produced "green crude" on a large scale, and aims to produce 4 million liters of biodiesel and jet fuel from algae per year starting in 2011 (Aswathanarayana 2010). Sapphire Energy, a leader in the industry, also has a 3,000-acre Integrated Algal Biorefinery in southern New Mexico. The world's largest commercial microalgae farm for biofuel production is set to reach full production in three years. The project, called the Malaysian Integrated Algae Valley, will grow freshwater microalgae on a 5,000-acre farm in the small township of Rompin, Malaysia. It will cost an estimated $383 million to undertake, and upon completion should produce 150,000 tonnes of biofuel per year. The fuel yield would average about 3,800 liters of biofuel per acre per year – lower than the projected estimates but higher than yields for any current biofuel crops. Algaetech International is providing the technology for the project (Saiful 2011).

Military Consumption

The military is the single largest consumer of petroleum in the U.S., accounting for nearly 80% of the U.S. government's energy consumption (Karbuz 2006). Increasingly, the military is investing more in clean and renewable energy sources. Aviation and ship fuel constitute the majority of fuel used by the military, liquid transport

biofuels will be essential. In an interview on February 8, 2012, Navy Secretary Ray Mabus stated that his primary goal for the U.S. Navy is that, by 2020, at least 50 percent of the naval energy consumption will be sourced from non-fossil fuels (Network 2012). All aircraft in the Navy and Marine Corp are certified for biofuels, and the surface fleet is in the process of certification. Mabus said that there is not only one technology that the military is looking to use, but rather a variety of sources. These fuels include second and third generation biofuels derived from algae, from *Camelina,* an inedible plant of the mustard family, and from other oil-rich crops.

In 2010, the U.S. Navy announced its plan to launch the "Great Green Fleet," a force of ships, planes, and submarines powered by renewable biofuels (Network 2012). The fleet will run on a 50-50 blend of advanced biofuels and traditional fuel. The first group of "Great Green Fleet" vessels is set to be tested in 2012, with hopes of becoming operational in 2016 (Goldenberg 2010). In December 2011, the U.S. Navy also announced a $12 million, 1,703,400 liter contract with Dynamic Fuels LLC – a joint venture of Tyson Foods Inc. and Solazyme – representing the federal government's largest purchase of advanced biofuels. 378,500 of the liters will be algae-derived fuel produced by Solazyme. The remainder will be from non-food-grade animal byproducts and waste cooking grease provided by Tyson Foods. The U.S. military requires that the biofuels they purchase do not compete with food sources, are produced domestically, and are drop-in replacements to traditional fuels (Snider 2011).

The adoption of renewable energy by the U.S. Navy is for the purpose of improving naval preparedness and fuel security, not to reduce carbon emissions. Mabus states that the U.S. is too dependent on energy procured from volatile places on earth, which makes it susceptible to supply and price shocks. For example, the current political unrest in Libya caused the price of oil to increase by $40 a barrel, which resulted in nearly an additional billion dollars spent on fuel by the U.S. Navy (Network 2012). Regardless of the motivation, the naval involvement will have a large impact on the algae industry and could help bring attention to algae-derived fuels for commercial use. As Mabus notes, the Navy has been a driver in energy use changes throughout American history. In the 1850s, naval ships were converted from sail to coal-powered, and transitioned again in the early 20th century to oil. In the 1950s, the Navy was the first to use nuclear power for

transportation. The Navy is once again changing the source of transportation energy in the U.S. (Network 2012).

Aviation Fuel

A promising industry with growing interest in algae biofuels is commercial aviation. On average, a Continental Airlines flight burns 68 liters per person to fly a passenger jet 1,000 miles (CFM International 2009). As a whole, the commercial aviation industry burns nearly 908 million liters of petroleum-derived 'Jet A' fuel per year (Biello 2009). Major U.S. and international airlines have shown interest in converting to renewable energy sources, due to both economic and environmental sustainability concerns. The industry group Air Transport Association hopes to source 10 percent of all aviation fuel from sustainable plant sources by 2017 (Biello 2009). This would ease the volatility of fuel prices and cut greenhouse gas emissions from aviation.

Continental Airlines, the world's fifth largest airline, conducted a test commercial flight powered by biofuel in 2008 on a two-engine Boeing 737-800. This represented the first demonstration flight by a commercial carrier in North America using biofuel, and the first demonstration in the world using fuel partially derived from algae. The flight operated with a blend of 50 percent biologically-derived fuel and 50 percent traditional jet fuel in one of the engines (Biello 2009). The alternative jet fuel, called synthetic paraffinated kerosenes, was a blend mostly derived from the *Jatropha* plant, but also contained 2.5 percent algae-derived fuel. More than 2,000 liters of algae oil were provided by Sapphire Energy for the blend. The fuel is a "drop-in" fuel, meaning that no modifications to the aircraft or engine were necessary to use the fuel, and the fuel met and even exceeded standards for jet fuel, denoted "aviation turbine fuel," set by ASTM (CMF International 2009). The jet successfully completed a two-hour test flight out of Houston and experienced no complications (Biello 2009). The second engine operated on 100 percent traditional jet fuel to allow for performance comparison between the two fuels (CFM International 2009, Continental Airlines 2009b). The results of the flight showed that the bio-based fuel performed as well as or even better than the traditional jet fuel; the biofuel blend displayed a 1.1 percent increase in fuel efficiency over traditional fuel. Based on overall life cycle analysis, Continental Airlines estimated that use of this blend could reduce greenhouse gas emissions by 60 percent to 80 percent compared to

traditional jet fuel use. The alternative fuel was also shown to not freeze at high-altitude temperatures, is lighter than traditional fuel, and did not negatively affect the engine or aircraft in any way (Continental Airlines 2009a).

During November 2011, Continental Airlines began using cleaner fuel blends for a number of domestic passenger flights. On November 7, Flight 1403, a Boeing 737-800, successfully completed the first commercial flight from Houston to Chicago on an algae-based biofuel, making it the first U.S. airline to fly passengers using a blend of advanced biofuel and petroleum jet fuel (Continental Airlines 2011). The "Eco Skies test flight" used a blend containing 60 percent conventional jet fuel and 40 percent algae-derived fuel produced by the company Solazyme (Plautz 2011). The same day, United Continental Holdings, the international 'parent' company of Continental Airlines, announced its agreement with Solazyme to purchase 76 million liters of algae fuel per year, beginning as soon as 2014. Solazyme was the first company in the world to produce a jet fuel derived 100 percent from algae biomass, called Solajet™, which is intended for both commercial and military applications. The biofuel meets the ASTM standards for aviation fuels, defined as hydro-processed esters and fatty acids (HEFA). Fuel safety and operational characteristics of HEFA are identical to conventional jet fuel, but the emissions are cleaner. No modification to the aircraft, engine, or operation is necessary with these drop-in fuels (Continental Airlines 2011).

ALGAL CULTIVATION

There are three stages in the production of algal biofuels: (1) the cultivation of algae, (2) the harvesting of algae, and (3) the extraction of oil and conversion to fuel. In each of these steps, there are currently many methods in use and being investigated. More research is needed to find the most efficient and economically sound technologies at each stage of production. Laboratory studies help determine the optimal conditions for algae production, whereas large-scale industrial productions test their viability and explore economical ways to produce substantial amounts of biofuel. For this study, a bioreactor was designed for the cultivation of microalgae in a laboratory, with attention to feasible scale up possibilities for industrial productions. *Dunaliella tertiolecta* was then cultivated in the closed photobioreactor and experiments were performed to study the algal growth rate, lipid production, and potential harvesting techniques.

Production Systems

There are currently two main algal culturing systems: photobioreactors and open ponds (Demirbas and Demirbas 2010). A photobioreactor is a closed-tank system, often a tubular or flat plate design, in which the algae are cultivated. Open-pond systems are outdoor shallow ponds that circulate the algae. Algal cultures can consist of a single or several strains that are best for producing the desired product. In both systems, water, nutrients, and CO_2 are provided in a controlled way, while oxygen produced by microbial respiration is allowed to escape. Natural sunlight can be utilized as an energy source, but laboratory photobioreactors generally use fluorescent lamps as a consistent light source. Efficient, large-scale productions feed fresh culture medium to the system at a continuous rate, as the same quantity of microalgal broth is withdrawn to keep growth rates high, nutrient levels constant, and harvesting continuous. The closed photobioreactor system and the open pond system both offer distinct advantages and disadvantages for culturing algae for laboratory and commercial purposes.

Open-pond systems are the simplest system and are cheaper to build and operate than closed bioreactors. Ponds are usually designed in a raceway configuration, with a paddlewheel to circulate and mix the algae and nutrients (Demirbas and Demirbas 2010). They are generally made from concrete or simply dug into the earth and lined with plastic. Issues of land use cost, water availability, climate conditions, and contamination

become problematic. Only a few algal species can successfully grow in outdoor pond systems.

Closed bioreactors are highly expensive compared to open ponds, however, they offer greater biological and technical control, and are important for producing fine chemical products (Demirbas and Demirbas 2010). They are the preferred method for scientific research because of the fine control. A single alga species can be grown in photobioreactors without competition or possible contamination, which is often a problem in open pond systems. Closed systems require less land than open ponds and can be very productive due to the control and optimization of environmental conditions. Demirbas (2010) states that high oil microalgae species have the potential to yield 19,000-56,800 liters of algal oil per year in an optimal-condition photobioreactor (over 200 times the yield from the best-performing plant/vegetable oils). New, innovative designs for photobioreactors are being developed to help maximize production and minimize costs. Currently tubular photobioreactors are considered best for culturing algal biomass on a scale sufficient for biofuel production. In these systems, carbon dioxide is injected to facilitate the exchange of CO_2 and oxygen, and also to create turbulence and prevent algae from remaining in the light-limited center zone of the reactor. These tubular reactors have high efficiency and biomass concentration, short harvest time (2 to 4 weeks), and a high surface-to-volume ratio (Demirbas and Demirbas 2010). Table 6 summarizes the comparative advantages and disadvantages of photobioreactor cultivation opposed to open pond systems.

Table 6. Advantages and disadvantages of photobioreactor cultivation (Francis 2010)

Advantages	Disadvantages
High Biomass Productivity and cell density	High capital cost associated with construction costs, circulation pumps, and nutrient-loading systems
Less contamination, water use, and CO_2 losses	Absence of evaporative cooling, which can lead to very high temperatures
Better light utilization and mixing	Accumulation of high concentration of photosynthetically generated O_2 leading to photo-oxidative damage
Controlled culture conditions	Absence of evaporative cooling, which can lead to very high temperatures

Photobioreactor Design

A closed photobioreactor system was designed and built for this study. The reactor was comprised of six 76.2 cm vertical acrylic tubes, with a 19 cm inside diameter and 6.35 mm thick walls. Acrylic was chosen because it is completely transparent, UV stabilized, resistant to corrosion, and does not leach chemicals into the water. Each tank had a volume of approximately 21.7 liters, which allowed for 20 liters of water with sufficient headspace for gas exchange with the water surface. Each tube was covered with a transparent acrylic cover, which was removable to allow for harvesting. Two aquarium pumps attached to air stones created gas circulation within each tube. A carbon dioxide tank was also attached to the aeration system and could be controlled to provide additional carbon dioxide to selected tubes. Agitation from the bubbling circulated the water and ensured that the cells did not settle on the bottom. Mixing is necessary for many reasons: it prevents cells from settling, avoids temperature gradients along the reactor, distributes nutrients, helps remove photosynthetically-created oxygen, and ensures that all cells are exposed to the light zone (Wijffels et al. 2009). The rate of the airflow could be controlled individually for each tube, or all could be set at the same rate. There was a second air valve in the cover of each tube to allow oxygen produced by the algae, as well as excess CO_2 to escape from the system. An accumulation of oxygen in the growth medium can lead to cell damage. The bioreactor was contained in a large environmental chamber that controls temperature, light, and humidity. The intention of the photobioreactor design was for the conditions in all six tubes to be identical before environmental manipulation experiments were performed and the conditions in selected tubes altered. Artificial light was provided from above the bioreactor and from both sides. A diagram of the photobioreactor that was specifically designed and built for this study is shown in Figure 2.

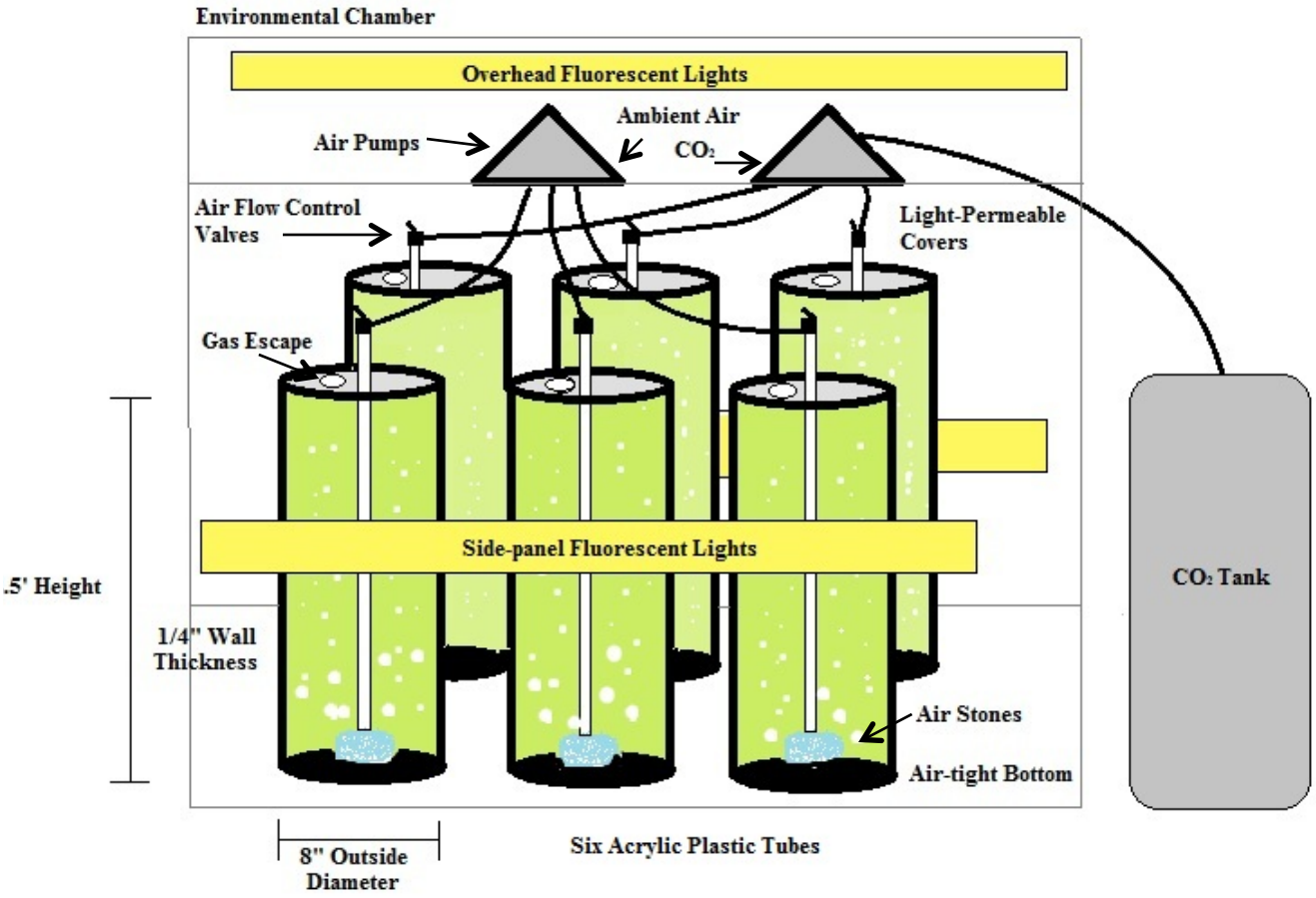

Figure 2. Diagram of photobioreactor designed and built for the culturing of *Dunaliella tertiolecta.* Six acrylic tubes, each 76.2 cm x 19.64 cm (6.35 mm-thick walls), hold 20 liters of water for a total of 120 liters. Aeration system supplies bubbling of controlled ambient air and carbon dioxide.

Culturing and Scaling Up

In the laboratory, growth conditions of algal cultures can be adjusted with a high degree of resolution and resources such as trace metals, vitamins, and antibiotics that are not necessarily viable for large-scale productions, can be utilized effectively to optimize growth. Lab-based algal culturing methods have been developed for a variety of downstream purposes. The following protocol is standard for laboratory algal cultures but has implications for commercial culturing ventures.

Enriched natural or artificial media can be used for the successful cultivation of algae. Micronutrients, trace metals, and vitamins are added to deionized water for freshwater species, and to either natural seawater or artificial seawater for marine and saline species. Artificial seawater is often the preferred medium for marine species because the nutrient levels in natural seawater vary in space and time. Culture media can be purchased or made to contain essential nutrients, vitamins, and trace metals. The medium is usually

dispensed into tubes, flasks and carboys and autoclaved for 15-45 min at 120˚C and 20 psi or sterilized at 80˚C for 1-2 hours to rid the media of any bacteria or cells that may affect the culture (Subba Rao 2009).

Starter cultures are generally maintained in 20-50 ml screw cap polystyrene sterile culture tubes or flasks and are then scaled up to Erlenmeyer flasks (125, 250, 500, or 1000 mL) (Subba Rao 2009). The flasks can be kept under constant fluorescent lights and movement by a shaker table to keep the algal cells in suspension. Cotton stoppers are used in each flask to prevent contamination while allowing for some gas exchange. For scaling up to larger volumes, tanks are washed with hot water, soaked in 10% bleach, and then thoroughly rinsed again with water. For commercial cultivation, it is important to minimize growth time and maximize production. By using exponentially growing culture as an inoculum, the lag phase can be reduced and higher density cultures yielded (Subba Rao 2009). The ratio of inoculum to fresh medium is 50:50 for volumes larger than 250 ml. Backup cultures are saved to prevent or eliminate crashes of cultures.

Assuming that there are no physical or nutritional constraints acting on the algae, the final yield of cells depends on the cell division rate and can be calculated with the formula $F=A*2^n$ after n doublings (A = Initial cell number, D = number of days, r = number of divisions per day, F = final cell number) (Subba Rao 2009). Cell densities based on theoretical counts are shown in Table 7.

Table 7. Theoretical algal cell densities based on division rates and inoculum size (Subba Rao 2009)

Divisions per day	**Cell numbers (x1000ml^{-1})**						
	Day 0	Day 1	Day 1	Day 2	Day 3	Day 4	Day 5
0	10	10	10	10	10	10	10
0.5	10	14	20	28	40	57	80
1.0	10	20	40	80	160	320	640
1.5	10	28	80	226	640	1,810	5,120
2.0	10	40	60	640	2,560	10,240	40,960
2.5	10	57	20	1,810	10,240	57,926	327,680

Growth Conditions

Environmental factors impact the growth rate of the algae; including light, temperature, CO_2, salinity, pH, phosphorus, nitrogen, and the presence of certain metals and vitamins. These factors can affect the growth rate of the algae as well as the cellular components and composition.

This section will focus on the cultivation of *Dunaliella tertiolecta,* a well-studied algal species for biofuel production, and will describe the bioreactor conditions in this study.

Light

Light is essential for algal growth because it provides energy that is harnessed by the cells and converted into chemical energy during photosynthesis. Photosynthesis rates increase with increasing light intensity up to a P_{max} value, at which point photo-inhibition begins (Countway 2011d). Photosynthetically active radiation (PAR) is the range of wavelengths (400 to 700 nm, also known as visible light) that photosynthetic organisms are able to use. The main pigment in phytoplankton is chlorophyll *a*, however other accessory pigments enable the cells to absorb light at different wavelengths of PAR (Countway 2011d). Because different algal species naturally live at different depths in the ocean, they are adapted to different light intensities. Light is measured as photons with an irradiance meter and is typically reported as μEinsteins/m^2/sec (1 Einstein = 1 mole of photons) (Countway 2011c). Light intensity is 4,000 μE/m^2/s for noon sun at the equator (for all wavelengths), and light intensity is 2,000 μE/m^2/s for the PAR maximum at the sea surface (Countway 2011c). A study by Tang et al. (2011) on the response of *Dunaliella tertiolecta* growth at different light intensities and exposures revealed that the species grew best at 350 μE/m^2/s with 24 hours of constant light. Trials using red LEDs (627 nm), white LEDs (420-750 nm), and fluorescent lights (400-800 nm) showed no difference in growth rate (Tang et al. 2011).

Commercial algae productions generally utilize sunlight as the light source for their cultures because is it free, and depending on location, is virtually unlimited. For experimental studies, bioreactors with controlled light fixtures allow for optimization by controlling the intensity and photoperiod duration.

The photobioreactor built for this study was designed to provide all six growth chambers with roughly equal light intensity. However, due to the alignment of the lights on the side and above the reactors, the two central growth chambers received some shading from the other tubes. The white reflection from the walls of the environmental chamber also created a light gradient within the space. Within the individual bioreactor tubes, the central column of water was shaded by the surrounding algal cells and less light could penetrate as density increases. The air stone on the bottom created constant turbulence, thus ensuring that all of the cells have equal access to the light zones, and it is important that bubbling began at the base of the chamber so that cells did not settle on the bottom.

Temperature

Temperature can have direct effects on the photosynthetic capacity, respiration rate, and growth rate of phytoplankton, and optimal temperatures vary for different algal species (Robarts and Zohary 1987). Many algal species are limited at low temperatures (below 15˚C) and are most productive at temperatures of 20˚C or greater. *Dunaliella tertiolecta* is a temperate algal species that can thrive at a wide range of temperatures. In the marine algae collection at the Provasoli-Guillard National Center for Marine Algae and Microbiota *Dunaliella tertiolecta* cultures are kept between 11˚C and 16˚C (NCMA 2011), but this species is often cultivated between 20˚C and 25˚C in the laboratory (Tang et al. 2011).

Salinity

The world's oceans have a mean salinity of about 3.5% by mass, or 35 ppt (mostly dissolved Na^+ and Cl^-) (Munn 2011). Salts slowly enter the ocean through weathering, which makes the overall salinity of the ocean highly conservative (Countway 2011c). However, salinity varies with depth (saline water is more dense than freshwater) and also with location (coastal, open ocean, latitude). In hypersaline environments salinity can be as high as 40 percent (Allred and Baxter 2012). These bodies of water tend to be landlocked lakes with high evaporation and are home to rich communities of "halophiles," or salt-lovers (Allred and Baxter 2012). Parts of the Great Salt Lake in Utah are saturated with dissolved salts (>30%). Although prokaryotes tend to dominate in extreme conditions, some eukaryotes can also thrive. Due to the wide range of salinities

in natural marine systems, different species of algae prefer distinct salinities. *Dunaliella tertiolecta,* for example, is able to tolerate a wide variety of salt concentrations. A study of five different nutrient concentration and salinity conditions found that strains of *Dunaliella tertiolecta* grew best when salinity was between 25% and 35% and nutrient concentrations were high (Fabregas et al. 1986). Strains of this species have been isolated from hypersaline lakes such as Lake Urmia in Iran, which has a salinity of greater than 30% (Fazeli et al. 2006, Pengra 2012).

Natural seawater can be used for large scale production of marine algal species; however, nutrient concentrations and the presence of other cells are more difficult to control when using natural seawater. A cheap solution, especially for laboratory studies, is a commercial aquarium salt mix such as Instant Ocean. This mix is nitrate- and phosphate-free, and contains every important major, minor and trace element necessary for aquarium health (Instant Ocean).

Nutrients

Phosphorus, in the form of inorganic phosphate PO_4^{3-}, is essential to life and is the limiting nutrient for many plants. High levels of phosphorus in natural aquatic and marine ecosystems often result in large algal blooms. Nitrogen can have similar effects on algal growth and also has interesting implications for energy storage within the cells. Richardson et al. (1969) found that nitrogen limitation increases lipid synthesis but also greatly reduced carbon dioxide fixation and oxygen evolution in the algae, resulting in an uneconomical production system. However, many studies show that, if not limited by light, CO_2, or other nutrients, cells continue to grow even after the medium is depleted of nitrogen.

Culture media can be made based on the specific requirements of the species in culture. A common culture medium used for growing marine microalgae is F/2 available from NCMA, which contains the following constituents: $NaNO_3$, $NaH_2PO_4 \cdot H_2O$, $Na_2SiO_3 \cdot 9H_2O$, trace metals, and vitamins (Rutgers University 2008). *Dunaliella tertiolecta* thrives in F/2-Si because it is a green algal species and does not require silica. Most algal culturing systems have a continuous feed of fresh medium into the pond or bioreactor, while algal broth is extracted at the same rate to ensure that nutrients are not depleted and become a limiting factor. In large-scale commercial productions, lab-grade

nutrients are too costly, as they are needed at high quantities (1 mL stock solution per L water) and are expensive. Regular plant fertilizers are a more economic option and can produce comparable results; a formula with a N:P:K ratio closest to the Redfield Ratio is favorable (Countway 2011b).

Carbon

During photosynthesis, carbon dioxide is converted into organic compounds using energy from the sun. The rate at which sugars are made in this process increases as available carbon increases, until other factors become limiting. Up to 90% of the dry weight of biomass that algae produce is absorbed CO_2. High purity CO_2 is not necessary for algae culture, which reduces costs and has implications for industrial scale CO_2 capture (Oilgae 2011b). A slight increase in the supply of carbon dioxide can make a large difference in the growth rate of algae (Countway 2011b). Air is only 0.04% CO_2 by volume. An air supply with 1% CO_2 (25x normal air) can greatly increase the amount of biomass produced in the same amount of time (Countway 2011b). Tang et al (2011) grew *Dunaliella tertiolecta* at ten different CO_2 concentrations (0, 0.03%, 0.1%, 2%, 4%, 6%, 10%, 20%, and 100%). The algae grew best at 2%, 4%, and 6% CO_2, and did not grow well with 0% or 100% CO_2 (Tang et al. 2011).

The bioreactor designed for this study was connected to a carbon dioxide tank that could selectively supply the tubes with CO_2 at concentrations between ~0.04 and 100 percent. While the air bubbled into the reactor can be 100% CO_2, it is important to note that not all of this carbon will be dissolved and made available to the algae.

PH

Pure water is neutral, with a pH of 7.0; however, water in natural environments where algae grow can range considerably in pH. The mean pH of the world's oceans is 8.14, which is declining at a significant rate due to increased carbon dioxide in the atmosphere (Countway 2011c). As more CO_2 enters the atmosphere and is absorbed by the ocean, the ocean acidifies as CO_2 dissolves and forms carbonic acid. This process is mimicked when CO_2 is bubbled into algal growth chambers. It is important to monitor the pH of the system and keep it at a constant level. Tang et al. (2011) kept their cultures of *Dunaliella tertiolecta* at a constant pH of 7.8. Subba Rao (2009) found that most *Dunaliella* species grow at >6 pH, and that growth rate is inversely proportional to pH, with 9.2 pH as the

upper boundary for maximum growth rate. Changes in pH of culture media depend on the capacity of the cells to utilize dissolved inorganic carbon during photosynthesis.

Growth Rate

In marine ecosystems, algal growth rates reflect (1) nutrient uptake kinetics among different species, (2) competition among co-occurring algal species, (3) different nutrient ratios and concentrations in the water column, (4) cross membrane nutrient transport rates, and (5) incorporation rates of nutrients into biomass (Countway 2011d). In a controlled environment, growth rates can be a measure of how cells respond to changes in environmental conditions. One objective of algal research in a controlled environment is to study factors that influence growth rate. Higher growth rates result in large amounts of algal biomass from which lipids can be extracted for conversion into biodiesel. Typical algal growth occurs in four distinct phases: (1) initial lag phase after inoculation of a culture, (2) exponential growth phase, (3) a stationary phase when cells attain their maximum volume and do not divide, and (4) death (Subba Rao 2009).

Dense cultures are generally established in about five days after inoculation with a rapidly-growing strain. For temperate climate algae, the exponential phase of growth usually occurs within the first 2 to 4 days of cultivation (Countway 2011b). Population growth will decrease once the stationary phase is reached. However, lipid accumulation per cell and/or stress related pigments might increase during this phase, which can be desirable for commercial applications (Ratha and Prasanna 2012).

Growth rate is expressed as abundance per unit of time, and can be expressed in various ways (i.e., cell number/hour, population doublings/day, generation time, turnover time). Exponential growth rate can be expressed using the equation $N_t = N_0 * e^{\mu t}$, where N is the number of cells, t is time, and μ is growth rate (Countway 2011d). A moderate growth rate for algal populations is between 0.5 and 0.8 day^{-1} (Countway 2011b). The hypothetical algal culture depicted in Figure 3 is growing at a rate of 0.11 $hour^{-1}$, or 2.65 day^{-1} during the exponential growth period. There are other methods used to describe phytoplankton growth rates as well. It is important to be clear about which of the growth rate terms are being expressed and there is often much confusion about the calculation of these terms due to the similarity of their terminology (e.g., ‘turnover’, ‘generation’, ‘doubling’).

Turnover time (days) is the time for a standing stock to be replaced. For the same example used in Figure 3, turnover time, $d = \frac{1}{\mu} = 0.38$ days. Generation time (days) $= \frac{\ln 2}{\mu}$ = 0.26 days means that it will take 0.26 days for the population to double itself during the exponential growth period. Doublings (day^{-1}) $= \frac{1}{\text{Gen.Time}} = 3.82\ day^{-1}$ means that the population will double 3.82 times a day during the exponential growth period.

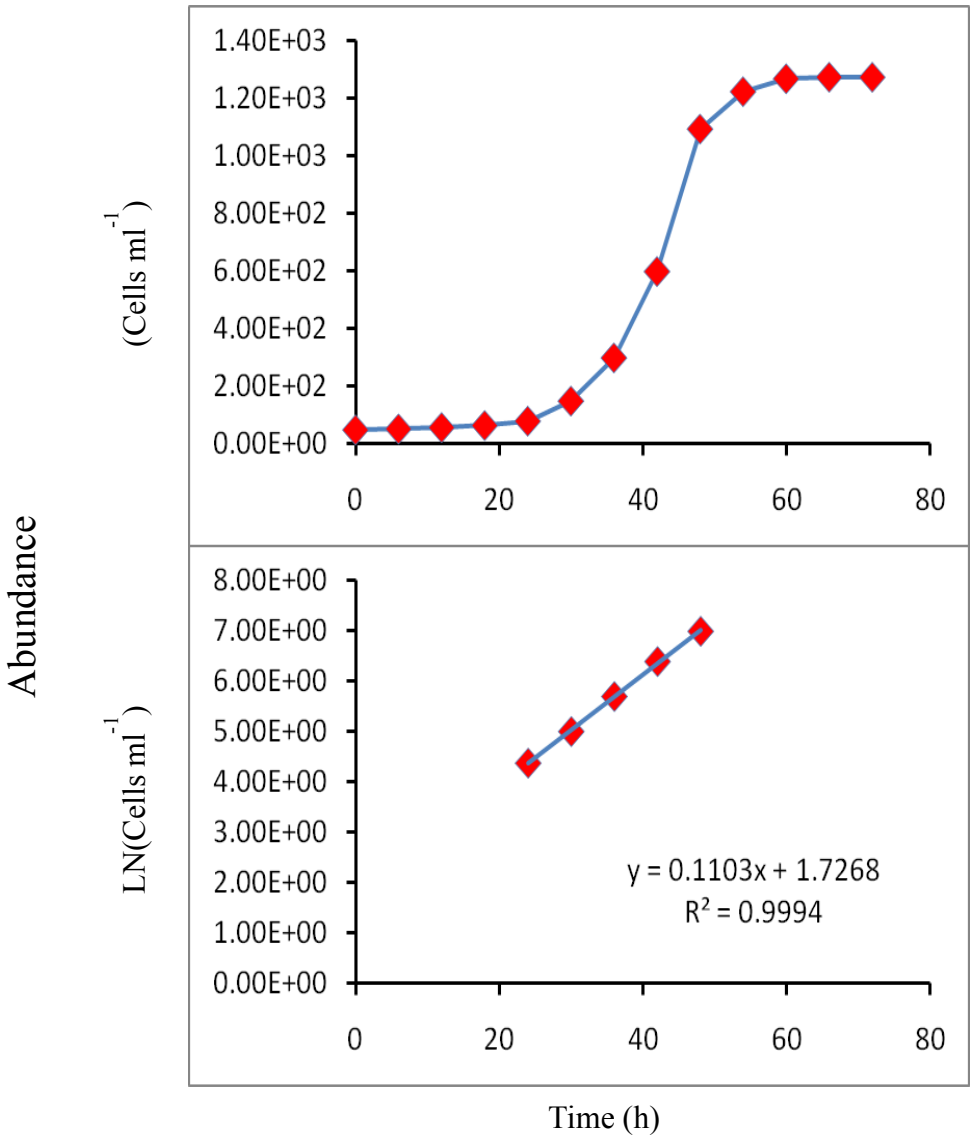

Figure 3. Theoretical Phytoplankton growth rate expressed as abundance vs. time. The top figure shows number of cells per ml vs. hours and the bottom figure uses a log scale for abundance. Regression analysis is used to calculate growth rate during exponential phase = 0.11 h^{-1} = 2.65 d^{-1}. (Countway 2011d)

Lipids

Visualizing Lipids

Cultures with high growth rates coupled with high lipid content will be the most favorable for biofuel production and various methods have been developed to track and measure lipid content. Fluorescence is a common method that allows for quantification, comparison, and sorting of cells and cultures based on the intensity of light emitted by fluorescently stained lipids. For the past 20 years, Nile Red has been the most common fluorophore used for visualizing lipid bodies in microalgae (Cooper et al. 2010). BODIPY 505/515 (4,4-Difluoro-1,3,5,7-Tetramethyl-4-Bora-3a,4a-Diaza-s-Indacene) is a stain that is used as a tracer for oil and lipids within cells (Molecular Probes Inc. 1999). Cooper et al. (2010) report that BODIPY is an excellent vital stain for the oil-containing lipid bodies of live algal cells, and in many ways is favorable to Nile Red. Unlike Nile Red, BODIPY does not damage or harm cells, allowing it to track lipid accumulation over the lifecycle of the cells (Cooper et al. 2010). When BODIPY is added to a culture of live algal cells, the dye stains the intracellular lipid bodies within minutes, by a diffusion-trap mechanism that allows the dye to accumulate in the lipidic intracellular compartments. BODIPY easily crosses cell membranes and organelle membranes due to its high oil/water partition coefficient. The culture can then be observed with epifluorescence microscopy or confocal microscopy, flow cytometry, or detected by a fluorometer. The lipid bodies of the live algal cells stained with BODIPY are distinctly labeled and seen clearly. Fluorescence-activated cell sorters can isolate algal cells with high lipid content, represented by fluorescence (Cooper et al. 2010). Cells with high lipid content can then successfully initiate new "seed" cultures for selected strains. Figure 4 shows the lipid bodies within the microalga, *Mandevilla splendens*. The chromatic separation of BODIPY green fluorescence from the red autofluorescence of chloroplasts can be seen clearly (Cooper et al. 2010).

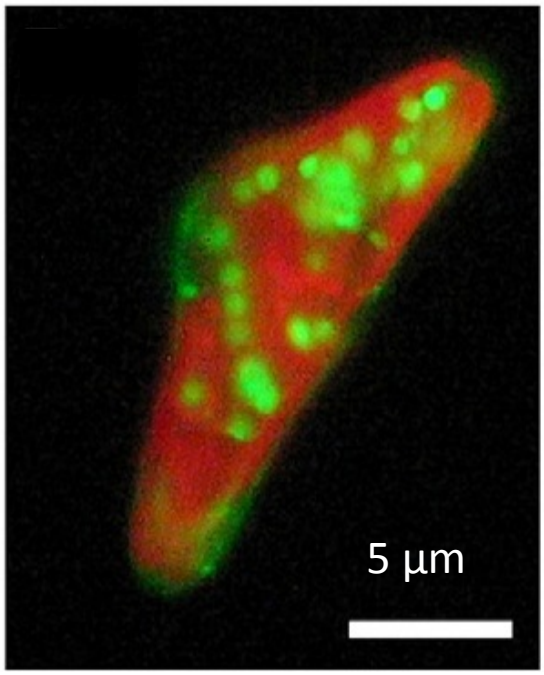

Figure 4. Lipid bodies stained with BODIPY 505/515 within *Mandevilla splendens*. (Cooper et al. 2010)

Stress-induced Lipid Synthesis

Creating stress conditions such as cold, drought, nutrient depletion, and high salinity, is one way of inducing lipid storage in algal cells (Msanne 2011). Nutrient deficiency, nitrogen for green algae and silicon for diatoms, has become the most common approach to increase intracellular lipid content (Huesemann and Benemann 2009). When the nitrogen source is depleted, cell growth and division is halted due to the lack of nitrogen for de novo protein and nucleotide synthesis, and the supplied carbon is converted into a storage lipid (TAG) (Lopes da Silva and Reis 2008). Some green algae store starch instead of lipids, and some can store either, under different conditions. Shifrin and Chisholm (1981) studied 30 green microalgae species during exponential growth and found that on average, nitrogen deprivation increased total lipid content from about 17% to 35%. A study of *Nannochloropsis sp.* by Suen et al. (1987) reported 55% lipid cell content, consisting of 79% TAGs, 9% polar compounds, and 2.5% hydrocarbons under nitrogen-deficient conditions. The challenge with artificially increasing lipid content is that under nutrient limitations cellular growth and photosynthesis rates declines. The goal is to achieve high lipid content and high productivity simultaneously, which is difficult using the 'nutrient stress' approach.

Harvesting

Harvesting microalgae is the process of separating the algal cells from the growth medium to remove the high water content (Francis 2010). Even the densest algal cultures are > 99% water. Microalgae must be concentrated into a thick algal paste to make it available for harvesting. There are four methods used for harvesting microalgae—floatation, centrifugation, filtration, and flocculation (Oilgae 2011a). Settling by gravity is another possible method, but can be extremely slow for small species. The particular method used depends on the strain of algae, and is highly dependent upon the size of the cells and the value of the cell product. High value pigment strains typically utilize rapid (but expensive) centrifugation technology.

Oil Extraction and Conversion

Extraction of various energy products from algae can be done using intensive mechanical methods, chemical methods, or a combination of both (Francis 2010). Many algal oil manufacturers use both mechanical pressing and chemical solvents to extract oil. The first product in the extraction is an algal oil, or "green crude," which is chemically identical to crude oil, but is carbon-neutral, non-toxic, and sulfur free (Aswathanarayana 2010). This product must then be refined through the transesterification process to be converted into biodiesel. The TAGs extracted from algal cells undergo the same transesterification process as other vegetable and animal oils to produce biodiesel; the oil is mixed with a catalyst, such as sodium hydroxide and then an inexpensive alcohol, such as methanol. This produces the fatty acid methylesters that make up the biodiesel (Demirbas and Demirbas 2010). The product will contain a glycerol fraction, a valuable byproduct widely used in pharmeceuticals, which is removed to leave pure algal biodiesel fuel. The extraction process is summarized in Figure 5.

New research is being performed to investigate methods of extracting the desired compounds from the algae without harvesting and killing the cell. This method is called "milking" and has been explored for the extraction of carotenoids like ß-carotene and astaxanthin from algae (Wijiffels et al. 2009). These compounds will be produced again in the same cells after being "milked" from the microalgae, thus reducing the required energy to produce new biomass and reducing the effort and time needed to re-establish new cultures.

New extraction technology is critical to the expansion of algae biofuels because a system that "milks" the desirable compound from the algal cells as opposed to killing the entire cell would increase efficiency and reduce costs. Research has begun studying how to use solvents, such as hydrocarbons, to milk the lipid from the living cell without damaging the cells (Oilgae 2011a).

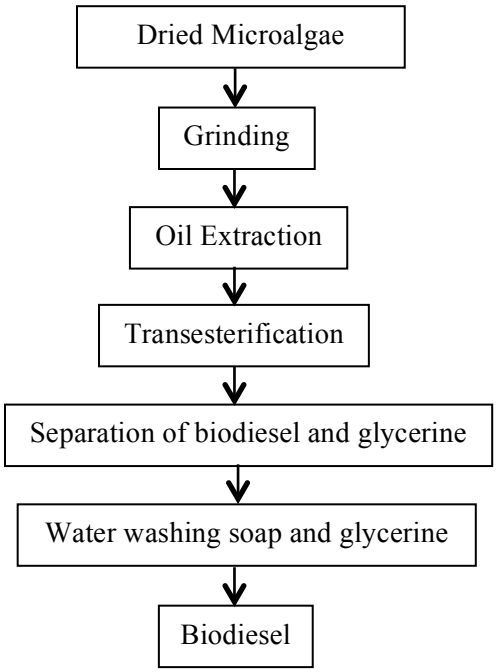

Figure 5. Production stages of biodiesel from algae beginning with harvested and dried microalgal biomass (Demirbas and Demirbas 2010)

RESPONSE OF *DUNALIELLA TERTIOLECTA* TO ENVIRONMENTAL MANIPULATIONS

Introduction

Small-scale laboratory studies on algae-derived biofuels are an essential first step before large-scale applications can be implemented because tests can be conducted to identify highly productive organisms that are robust to environmental changes (Stewart and Hessami 2005). Because microalgal biofuel production is not economically viable at this time, the production process must be streamlined and innovative technologies developed. Coupling high growth rates with high lipid content will allow industrial producers to save valuable nutrient resources and improve efficiency. Inexpensive materials and resources that can be procured in large quantities will also be necessary for the viability of the industry.

The objective of this laboratory study was to investigate growth conditions, lipid induction methods, and harvesting techniques that will continue to increase the economic and environmental sustainability of algal biodiesel production. While this set of experiments was performed in a small laboratory bioreactor, the methods used were developed with the intention of transferability to larger-scale operations.

Growth experiments

The first objective of this experiment was to determine the effects of carbon dioxide on the growth rate of *Dunaliella tertiolecta*. Gaseous carbon dioxide is soluble in water and becomes an available form of carbon for cells to utilize and produce biomass. Carbon dioxide fixation by microalgae is a potential approach to reduce greenhouse gas emissions (Suzuki et al. 1995). In a study of carbon fixation rates by *Dunaliella tertiolecta,* Suzuki et al. (1995) found that the alga was able to grow with up to 24% CO_2-enriched air and without controlling the pH of the culture. The optimal growth rate was achieved with 6% CO_2 aeration, at approximately two doublings per day (Suzuki et al. 1995). Other studies have shown that some species of algae can tolerate 100% CO_2 aeration (Reddy 2002). This finding is significant because exhaust gases from most thermal power plants contain about 10-20% CO_2, all of which could most likely be absorbed by the algae. A study of the microorganism *Synechocystis aquatilis* found that it utilized carbon at a rate of 1.5 g/l/day (Stewart and Hessami 2005). Assuming that this

rate could be sustained for a 4000 m^3 cultivation pond, up to 2.2 kilotonnes of CO_2/pond/year could be sequestered from the atmosphere. Microalgae production presents an opportunity to sequester CO_2 by using the flue gas emissions from industrial sources such as power plants as the CO_2 feed for algae cultivation (Pokoo-Aikins et al. 2010).

Currently, the capture of carbon dioxide produced by combustion of fossil fuels used in electric generation is achieved by amine scrubbing of the flue gases (Stewart and Hessami 2005). Other methods of carbon dioxide sequestration and capture include geologic or oceanic sinks, membrane separation, and molecular sieves. However, these methods are costly and do not address issues of sustainability. Microorganisms capable of photosynthetic reactions are a possible solution to reducing carbon dioxide emissions in both an environmentally and economically sustainable manner (Stewart and Hessami 2005). If algal growth rates are greatly enhanced by increased levels of carbon dioxide, then large-scale algae productions have the potential to act as a carbon sequestration system while simultaneously increasing the biomass available for biofuel production.

Lipid Induction

Lipids are the cellular components that are extracted from microalgal cells for the conversion to biodiesel and new, inexpensive methods to increase lipid content are needed to make microalgae a viable feedstock. Manipulating environmental conditions and genetic modification are two ways to increase the lipid synthesis in the cells. Stress conditions such as cold, drought, high salinity, and lack of nutrients cause microorganisms to adapt, and can affect the cellular functions and structures (Msanne 2011). Under stress conditions, response mechanisms are activated either to prevent damage or to promote the rapid turnover of damaged molecules. Nutrient depletion—nitrogen in green algae and silica in diatoms—to induce higher rates of lipid synthesis has been studied intensively. A study by Msanne (2011) on the abiotic stress responses in two photosynthetic green alga shows that nitrogen depletion under photoautotrophic conditions triggers TAG accumulation due to protein degradation in the nutrient-deprived cells. However, nitrogen deficiency greatly inhibited the cell cycle and production of almost all cellular components other than lipids.

Salinity is an abiotic factor that limits productivity and can cause cellular constituent changes in many environments (Msanne 2011). Salinity stress can destabilize biological membranes and affect the solubility of many important substrates and ions (Katz et al. 2007). Organisms have evolved complex mechanisms to tolerate high salt levels, including the biosynthesis of compatible solutes that stabilize proteins and membranes against the denaturing effect of high concentrations of salts (Msanne 2011).

Algae of the genus *Dunaliella* have an unusually high tolerance for osmotic stresses and are often found naturally in brackish water (Suzuki et al. 1995). *Dunaliella* can undergo rapid changes in shape and volume in response to osmotic fluctuations because they lack a rigid algal wall and are only enclosed by a thin cytoplasmic membrane. Katz et al. (2007) found that dynamic changes in the lipid composition in *Dunaliella* might be involved in the short- or long-term adaptation to changes in salinity. The current study investigated the effects of a "salt shock," a virtually free process, on healthy cultures of *Dunaliella tertiolecta* with the goal of inducing increased lipid synthesis under higher salinity.

Alternative methods to enhance lipid production while not reducing the high growth rates, as stress conditions can, are also being explored. Certain chemicals and treatments can possibly induce lipid synthesis after growth is obtained. Decane ($CH_3(CH_2)_8CH_3$) is a naturally occurring hydrocarbon that is widely used as a solvent, as a component of paints, and in the paper and rubber industries (Technical Resources International 2003). Walker and Pore (1978) determined that the alga *Protetheca zopfii* was able to use hydrocarbons, specifically n-hexadecane, as the sole carbon and energy source, resulting in heterotrophic growth. Lopes de Silva and Reis (2008) studied the impact of n-dodecane additions to *Crypthecodinium cohnii* docoseheaenoic acid (DHA) production. DHA is a polyunsaturated fatty acid that is known to provide health benefits, similar to fish oil (Lopes da Silva and Reis 2008). The study found that the n-dodecane fraction that gave best results in terms of biomass and DHA production was 0.5% (v/v). After the addition of n-dodecane to the culture, glucose uptake volumetric rate increased 2.5-fold and flow cytometry demonstrated that the cell membrane integrity was not affected. Sayre et al. (2011) found that the addition of decane to the alga *Chlorella* resulted in rapid chemical induction of oil accumulation after only a few hours. The cellular

response to hydrocarbons was explored in this study by adding decane (>99% decane obtained from Sigma-Aldrich) to *Dunaliella tertiolecta* cultures during the stationary phase of growth.

Harvesting

Harvesting microalgae is often one of the greatest costs of algae biodiesel, contributing 20 to 30 percent of the total cost of algal biomass production (Demirbas and Demirbas 2010). There is no single best method of harvesting microalgae due to the variability in algae species, growth media, end product, and production cost benefit (Shelef et al. 1984). In industrial applications, the typical cell density achieved in cultures is between 0.3 and 0.5 g dry cells/L, or 5 g dry cells/L at best. Because culture broths are so dilute, large volumes must be concentrated by a factor of 100 to 800 times before bulk harvesting is possible (Demirbas and Demirbas 2010).

Flocculation is the process by which clumps of fine particles aggregate through biological or chemical action to be separated from a medium (Oilgae 2011a). Flocculation prepares the algae particles for subsequent harvesting methods such as filtration, flotation or gravity sedimentation (Brennan and Owende 2010). Autoflocculation requires no additional chemicals or equipment; when the carbon dioxide flow into the algal culture is stopped, the cells accumulate on the bottom of the container. This process is slow and possibly not as effective as other methods at separating a high percentage of cells. The addition of chemicals, such as aluminum chloride or ferric chloride, accelerates the flocculation process. However, the cost to remove these chemicals from both the separated algae and the medium are often too high for commercial application (Oilgae 2011a).

Chitosan is a non-toxic flocculant made from grinding and processing the exoskeletons of crustaceans to acquire the polysaccharide chitin (Lavoie and de la Noue 1983). Chitosan is already used in purification of urban waterways, and has potential for use as an algae flocculant. Brennan and Owende (2010) demonstrate successful use of Chitosan as a bio-flocculant, but note that the efficacy of the method was sensitive to pH, with maximum flocculation at pH 7.0. This study compared the effectiveness of autoflocculation and Chitosan flocculation with samples of *Dunaliella tertiolecta*.

Methods

A 100 mL culture of *Dunaliella tertiolecta* (strain CCMP 1320) was obtained from the National Center for Marine Algae and Microbiota (NCMA) at Bigelow Laboratory in Boothbay Harbor, Maine. The culturing system described earlier in the report was used to inoculate the six 20-liter tubes of the photobioreactor with *Dunaliella tertiolecta* (see Figure 2). The entire inoculation stock was mixed to homogenize the culture and ensure initial consistency among all of the growth chambers. The average starting density was 4 x 10^4 cells/mL.

The light intensity within the environmental chamber ranged from about 300 μE/m^2/s to 500 μE/m^2/s, measured using a LI-COR light meter, model LI-1000. This range is suitable for the growth of *Dunaliella tertiolecta* and did not damage the cells (Neal 2012). Therefore, light was considered a constant variable for all six growth chambers of the photobioreactor in the following experiments. When the bioreactor was first inoculated with *Dunaliella tertiolecta* culture, the side-panel lights were turned off to ease the transition on the algae and avoid light shock damage to the cells.
All six bioreactor growth chambers contained 20 liters of deionized water and approximately 2.6 cups of Instant Ocean, for a final concentration of about 3.5% salinity. Miracle-Gro Liquid All Purpose Plant Food (12N:4P:8K:0.1Fe) was added for final concentrations of 600 μM total nitrogen, 200 μM total phosphorus, 400 μM potassium, and 5 μM iron. The cultures of *Dunaliella tertiolecta* were monitored for pH with a Mettler Toledo pH probe and stayed withing a range of pH 7 to 8.

Growth experiments

The growth experiment was designed to compare the effects of atmospheric levels of carbon dioxide versus increased levels of carbon dioxide on the rate of algal biomass accumulation. Ambient air contains about 0.04% carbon dioxide. A standard aquarium pump circulated ambient air into three of the bioreactor tubes. The remaining three tubes received CO_2-enriched air, bubbled from an identical aquarium pump, to give a final concentration of approximately 1-2% carbon dioxide. Cultures that received added carbon dioxide were labeled Tube 1, Tube 2, and Tube 3; Tube 4, Tube 5, and Tube 6 received ambient air.

Samples were taken from each tube at 12-hour intervals starting at inoculation and continuing until growth reached the stationary phase. Direct cell counts and absorbance were used as proxies for algal biomass. An Improved Neubauer haemocytometer using standard protocol (Abcam 2012) was used to count the cells at each sampling time; the average cell count of three samples from each tube was recorded. A Spec 20 spectrophotometer was also used to measure the amount of visible light absorbed by a sample of the algal broth (Dartmouth College 2000). The absorbance was measured at a wavelength of 465 nm to detect the abundance of chlorophyll *a*, the dominant pigment in green algae such as *Dunaliella tertiolecta*. Chlorophyll *a* can be used as a proxy to estimate production because it is easy to measure and highly correlated with biomass (Countway 2011d). The absorbance of the sample correlates to population density, and growth rates can be calculated in this way.

Lipid Induction

A subset of experiments was carried out using healthy culture of *Dunaliella tertiolecta* in the stationary phase of growth to study the effects of salt and decane treatment on lipid synthesis. Nine liters of culture from the tube containing the densest and healthiest-appearing algae was collected and well mixed before being distributed into nine individual 1000-mL Erlenmeyer flasks. Each flask was fed with an air tube bubbling ambient air and was kept on a 24-hour light period. Flask A contained only algae broth without any additional treatment. Flask B was the "salt shock" culture, and contained algae broth with 30 mL of additional Instant Ocean for a final salinity of about 5%. Flask C was the decane-treated culture, with 10 mL of decane for a final concentration of 1% decane by volume. The salt and decane were added to the culture gradually over the initial six hours of the experiment to avoid causing immediate cell death. The lipid content in each culture flask was observed and quantified three days after the treatment.

BODIPY 505/515 stain was used to visualize lipid bodies within the algal cells to make lipid induction observable and quantifiable. Solid BODIPY was dissolved in dimethyl sulfoxide (DMSO) to make a 5 mM BODIPY solution. DMSO helps the BODIPY permeate cell membranes and enter the lipid bodies (Cooper et al. 2010). 4 µL of this solution was added to 2 mL samples of algae in suspension to achieve a final

concentration of 10 μM. To visualize the lipid cells using BODIPY stain, the sample was excited at 488 nm (blue light) and light was emitted at 530 nm (green light).

Lipids were measured qualitatively using epifluorescence microscopy with a Zeiss Axioscope. Using the BODIPY fluorophore, lipid bodies fluoresced green while chlorophyll appeared red. Lipids were also measured quantitatively using a FLX800 plate Reader. 2 mL of stained algae cells in suspension were centrifuged to concentrate the cells which were then re-suspended in 2 mL of unstained medium to eliminate background fluorescence. One hundred μL of this final solution was placed into the well of a black plate and exited at a wavelength of 485 nm. A filter with an emissions wavelength of 528 nm was used to detect the BODIPY-stained lipid bodies. The fluorescence reading was normalized by cell count for each culture to calculate a comparable fluorescence unit per cell.

Harvesting

The effectiveness of autoflocculation and chemical flocculation with Chitosan were observed in 250 mL samples of *Dunaliella tertiolecta* collected during the stationary phase of the original growth experiment. Three flasks were set up: one with no treatment, the second with 250 mg of powdered chitosan added, and a third with 500 mg of powdered chitosan added. All three flasks were shaken periodically during the first six hours after the initial treatment and then were kept stationary for the remainder of the experiment. To measure the effectiveness of each separation method, 4 mL samples were taken from the surface of the water over a period of three days. Optical density was used as a measure of biomass still in suspension, under the assumption that a reduction in optical density represented the accumulation of algal cells on the bottom of the flask. The spectrophotometer was used to measure absorbance at 465 nm, using chlorophyll a as a proxy for biomass. Haemocytometer cell counts were also used as a secondary measure of flocculation, measured both before treatment and after three days.

Results

Growth Experiment

Growth was monitored over 142 hours (roughly six days) and biomass was quantified using two measures: haemocytometer cell counts and spectrophotometer absorbance at

465 nm. Population growth in all six algal cultures followed the typical algal growth curve consisting of an initial lag phase, followed by exponential growth, and finally stabilizing at the stationary phase (Figure 6). All six cultures began at an initial density of approximately 4 x 10^4 cells/mL and continued at low densities during the lag phase for the first 36 hours. The populations began growing exponentially around 36 hours when the two treatments began to differentiate. After approximately 96 hours of growth, the populations reached the stationary phase. The direct cell counts show divergence between the ambient air-fed cultures and the carbon dioxide-fed cultures, with the CO_2-enriched populations growing at a faster rate and reaching a higher final density after 142 hours. Figure 6 shows the direct cell counts of the cultures in all six tubes throughout the entire growth experiment. The mean cell counts for all three CO_2-enriched cultures can be compared with the mean cell counts for the three ambient air cultures in Figure 7.

Growth rates were determined using linear regression of log-transformed cell densities during exponential growth, estimated to occur between hour 36 and hour 84 (Figure 8). The cultures receiving ambient air grew at a rate of 0.0424 hr^{-1} during exponential growth; this is also expressed as 1.4 doublings per day. The cultures receiving increased carbon dioxide grew at a rate of 0.0511 hr^{-1} during exponential growth, or approximately 2 doublings per day.

Optical density increased at a faster rate in the cultures enriched with CO_2 versus the cultures receiving ambient air (Figure 9). The mean absorbance of the cultures with the same carbon dioxide concentration shows that the cultures with increased carbon dioxide had higher optical density than the ambient air cultures after 72 hours (Figure 10). This trend supports the results of the cell count data. Figure 11 gives a visual representation of the increase in optical density for a single carbon dioxide-fed culture at the 14 sampling times.

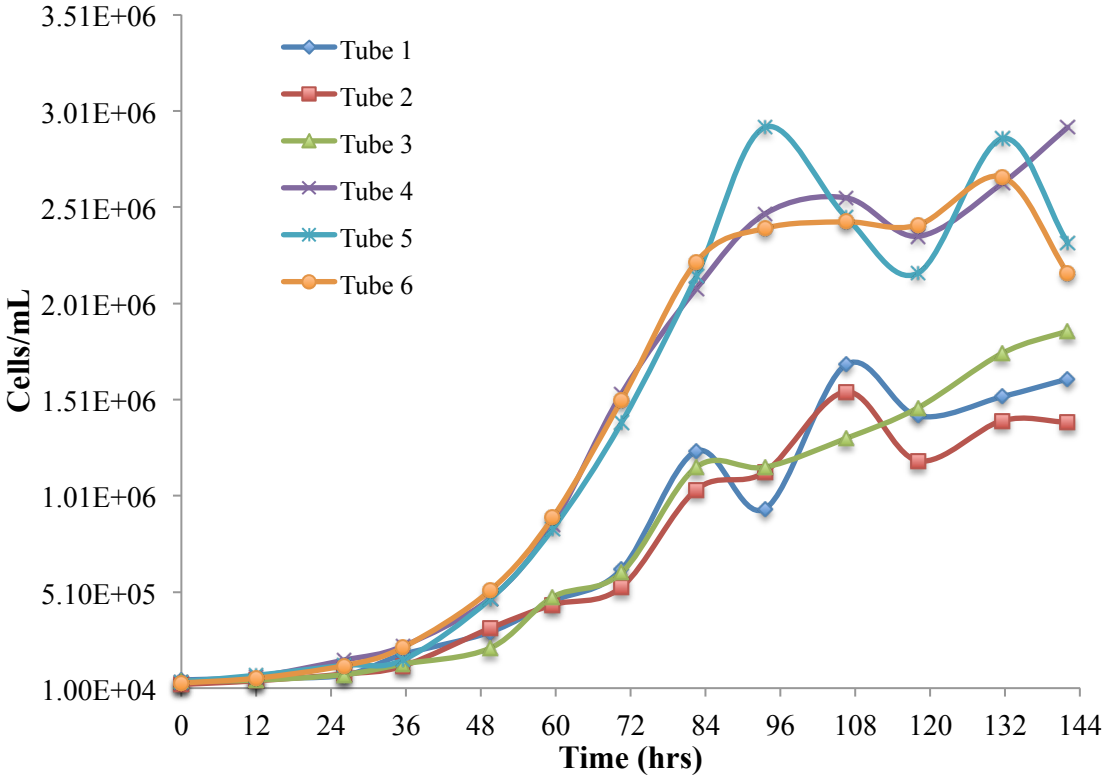

Figure 6. Direct cell counts of all six experimental growth cultures of *Dunaliella tertiolecta* over 144 hours (6 days) after initial inoculation. Tubes 1, 2, and 3 received airflow containing approximately 0.2% carbon dioxide. Tubes 4, 5, and 6 received airflow containing approximately 1-2% carbon dioxide.

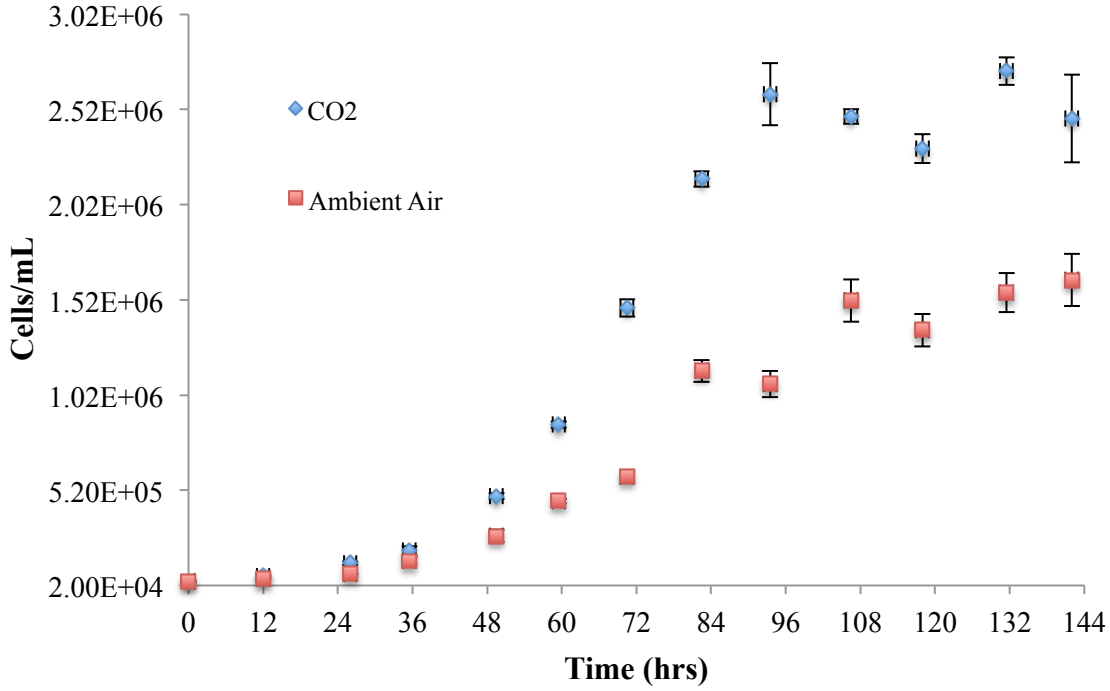

Figure 7. Mean cells mL^{-1} versus time for the three cultures receiving added carbon dioxide and the three cultures receiving ambient air. CO_2-enhanced cultures reached a final mean density of 2.47 x 10^6 cells mL^{-1}. Cultures receiving ambient air reached a final mean density of 1.63 x 10^6 cell mL^{-1}.

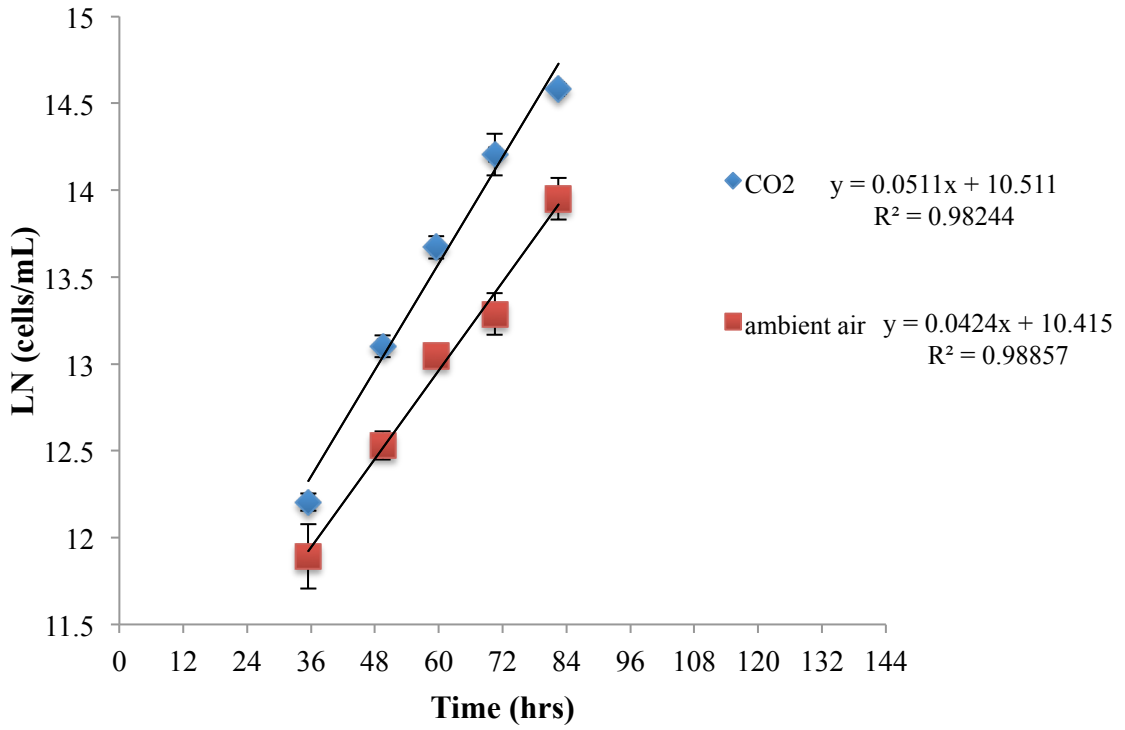

Figure 8. Log scale of mean cell density (cells mL^{-1}) versus time during exponential growth phase for cultures of *Dunaliella tertiolecta*. The growth rates, for the CO_2-enhanced cultures (0.0511 hr^{-1}) and for the ambient air cultures (0.0424 hr^{-1}) were determined by the slopes.

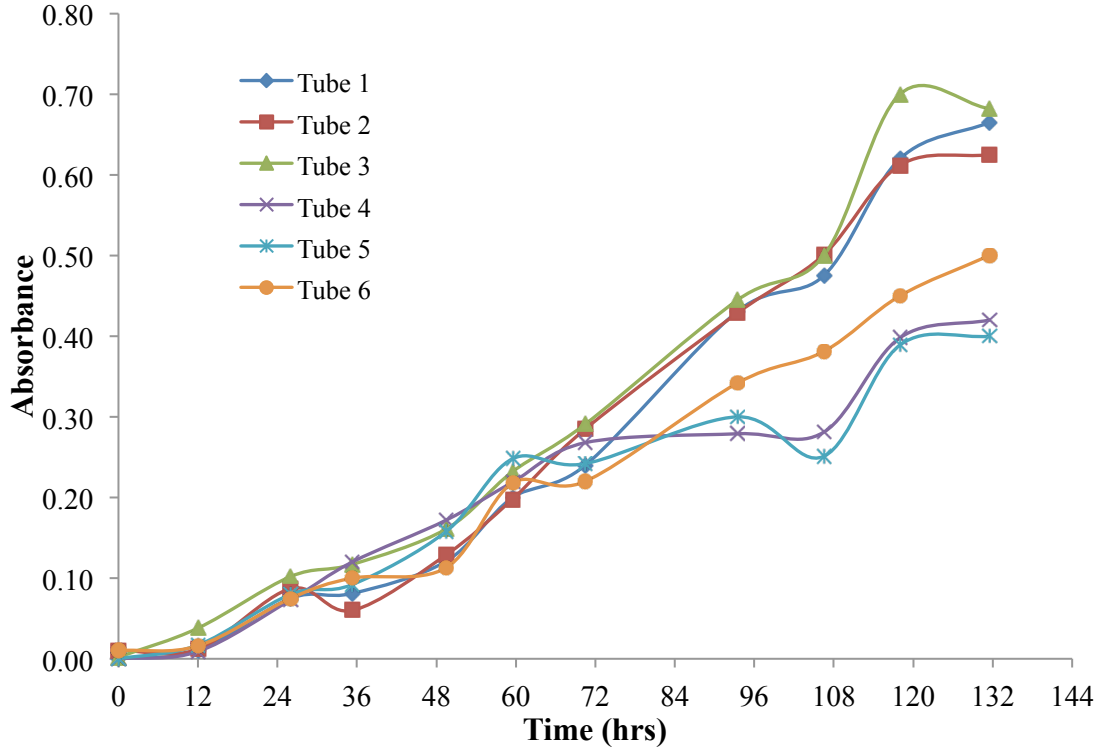

Figure 9. Optical density of all six experimental growth cultures of *Dunaliella tertiolecta*, measured by absorbance at 465 nm by a spectrophotometer, over 144 hours (6 days) following initial inoculation. Cultures with added carbon dioxide (Tube 1, Tube 2, and Tube 3) showed increased biomass production after 72 hours, compared to cultures without added carbon dioxide (Tube 4, Tube 5, and Tube 6).

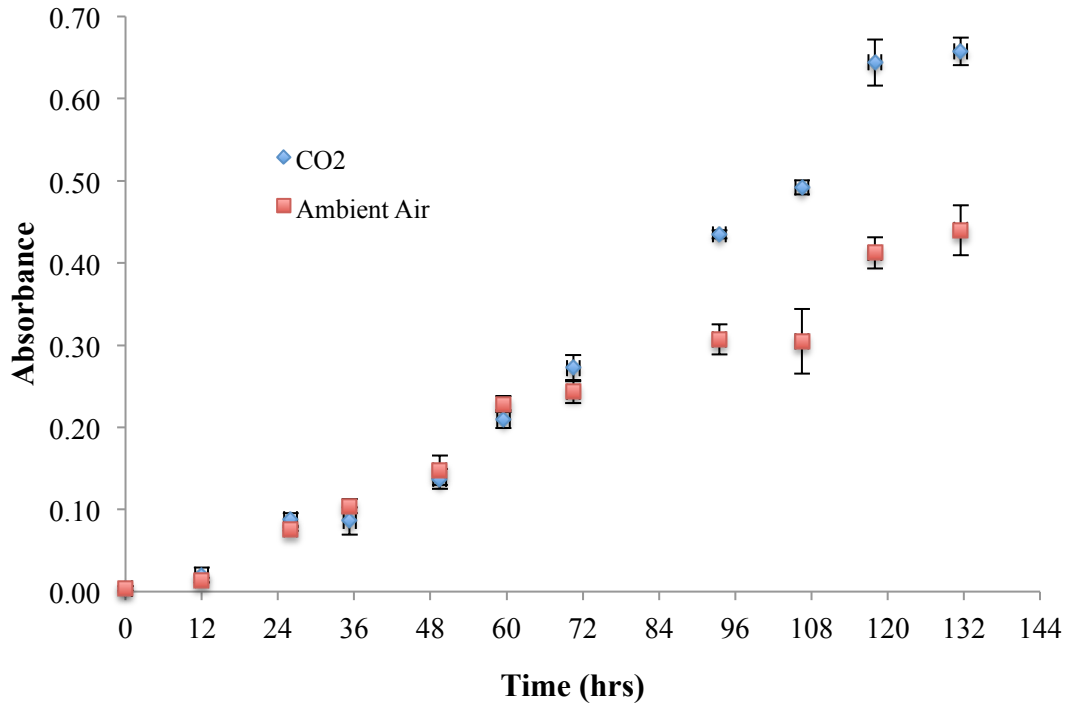

Figure 10. Mean optical density and standard error bars for the three cultures of *Dunaliella tertiolecta* receiving added carbon dioxide and the three cultures receiving ambient air.

Figure 11. Visual representation, moving from left to right, of increasing cellular density over 144 hours (6 days) of a single culture of *Dunaliella tertiolecta.*

Lipid Induction

The salt and decane lipid induction treatments, each performed in triplicate, showed a significant increase in lipid content (Figure 12). The control cultures of algae with no lipid induction treatment had a mean fluorescence reading of 78 fluorescence units. The salt shocked cultures had a mean reading of 120 fluorescence units, and the decane-treated cultures had a mean of 172 fluorescence units.

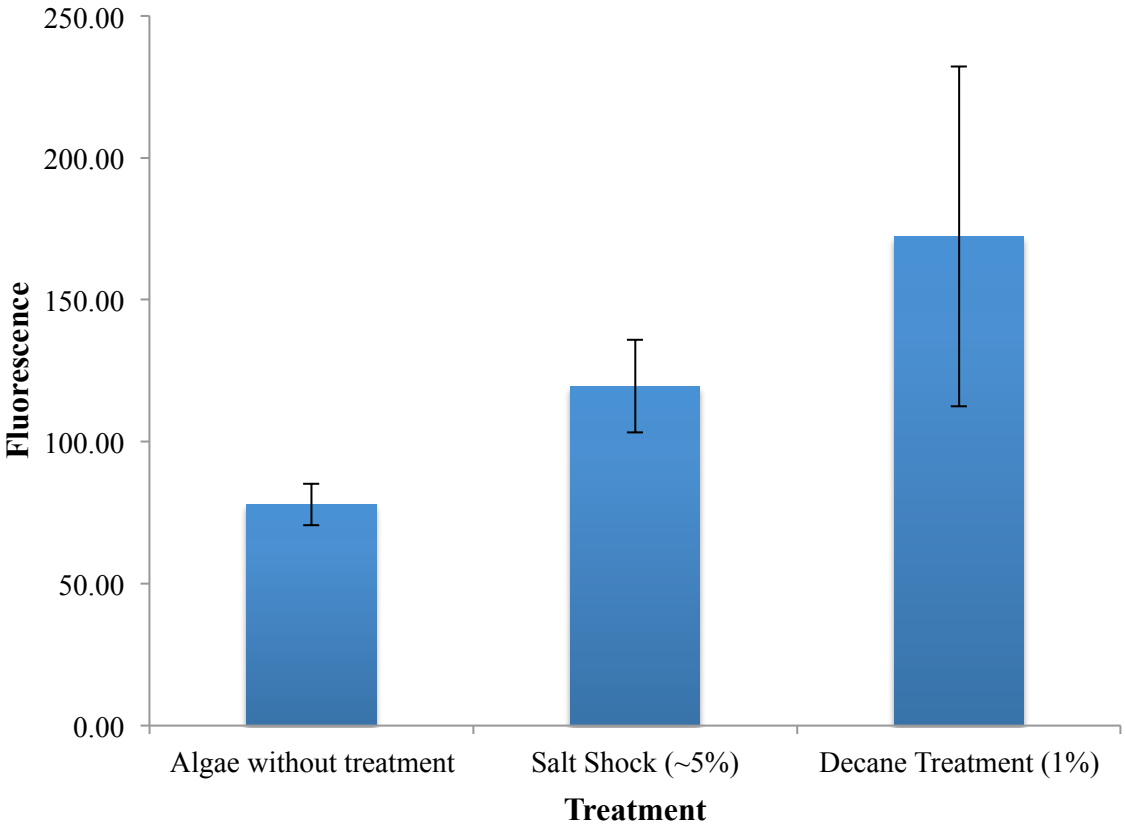

Figure 12. Fluorescence measure of BODIPY-stained lipid bodies three days after treatments in triplicates with standard error bars. The mean fluorescence, measured on an FLX800 plate reader, was calculated for the control (78 fluorescence units), salt shock (120 fluorescence units), and decane treatment (172 fluorescence units) cultures.

Harvesting

The untreated culture that was left to autoflocculate had the slowest rate of algal separation from the media and also resulted in the least amount of algal accumulation on the bottom of the flask. The percent of flocculation determined by optical density indicates that the three treatments separated the algae at a similar rate until 24 hours after treatment (Figure 13) and that flocculation with 500 mg of chitosan was the most effective of the three methods. Based on optical density, the percentages of algae that flocculated and settled on the bottom of the flasks 70 hours after treatment were calculated for the treatments of 500 mg of chitosan (55%), 250 mg of chitosan (33%), and autoflocculation (16%) (Figure 13).

The effectiveness of flocculation methods was also evaluated by direct cell counts. The percent of algal flocculation three days after treatment was calculated using the

direct cell count of the surface water of each flask before treatment, and the direct cell count of surface water of each flask three days after the treatments. These data show the percent flocculation for the autoflocculation (36.36%), 250 mg of chitosan (81.25%), and 500 mg of chitosan (99.27%) treatments (Figure 14).

Figure 15 shows images of the algal cultures three days after flocculation treatments. From left to right, the top image shows the visibility of algae in suspension after the autoflocculation, the 250 mg of chitosan flocculation, and the 500 mg of chitosan flocculation treatments. In the two samples with chitosan added, particles of chitosan remain suspended in solution. The bottom image shows the accumulation of algae on the bottom of the three flasks in the same order.

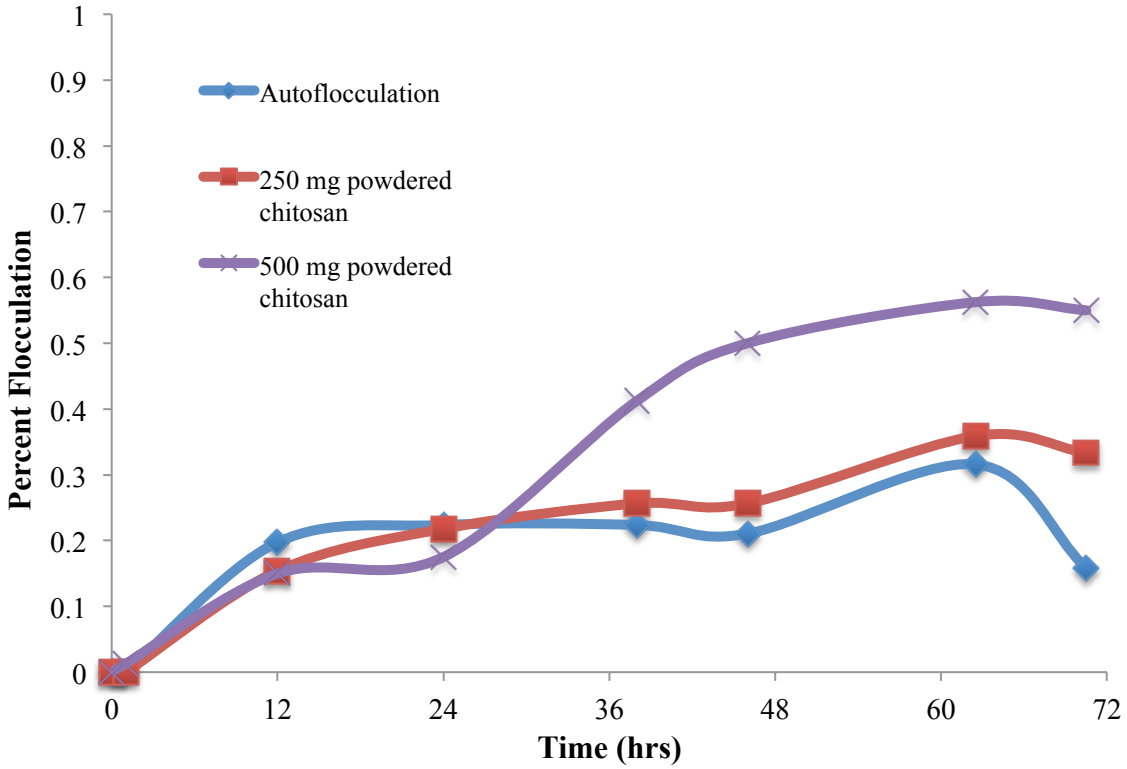

Figure 13. Percent of algal flocculation over 72-hour period after treatment based on optical density of treated sub-samples of Dunaliella tertiolecta. Percent flocculation, calculated by the measured absorbance at 465 nm with a spectrophotometer, showed that after 72 hours 500 mg powdered chitosan was the most effective flocculant (55%), followed by 250 mg powdered chitosan (33%), and finally autoflocculation (16%).

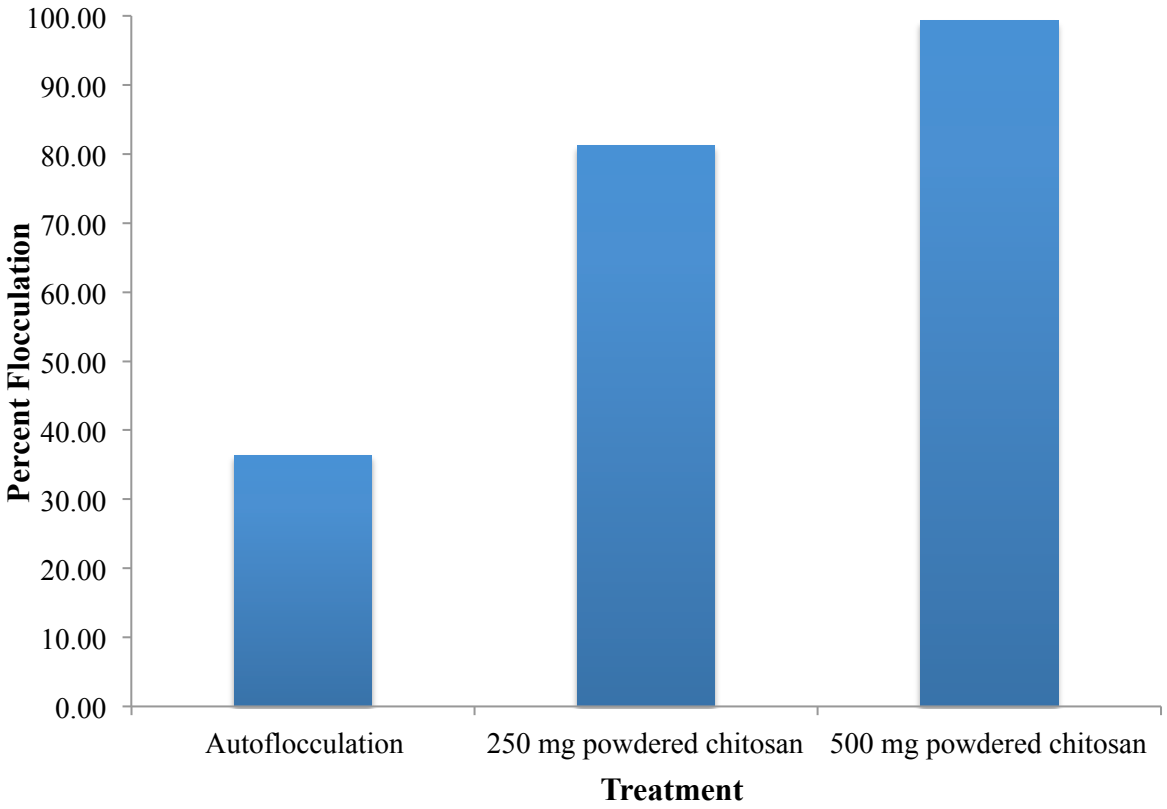

Figure 14. . Percent of algal flocculation over 72-hour period after treatment based on haemocytometer direct cell counts of treated sub-samples of Dunaliella tertiolecta. Percent flocculation, calculated by density of cells remaining in suspension, showed that after 72 hours 500 mg powdered chitosan was the most effective flocculant (36%), followed by 250 mg powdered chitosan (81%), and finally autoflocculation (99%).

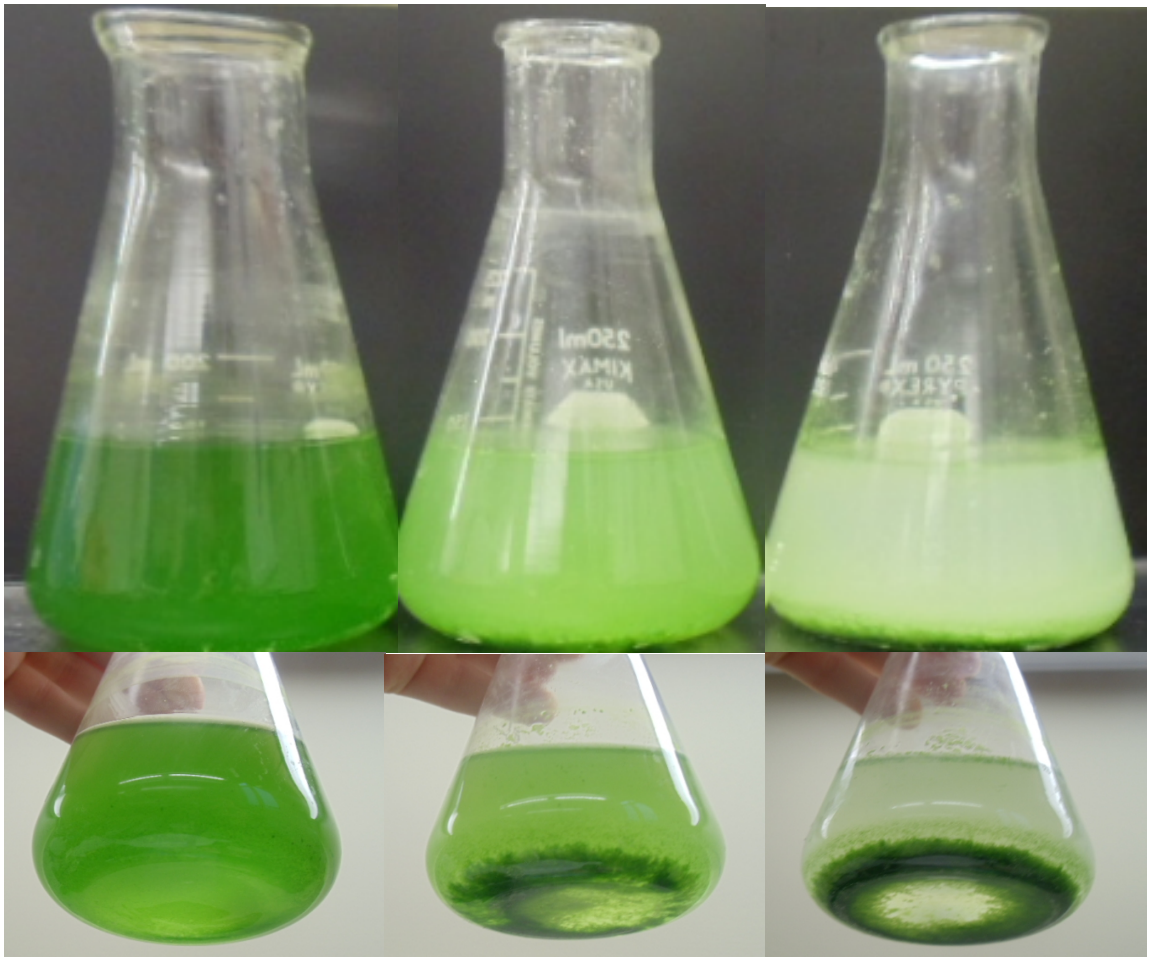

Figure 15. Visual representation of algal flocculation three days after treatment with an initial volume of 250 mL algae broth. Top row shows that dissolved chitosan interferes with optical density. Bottom row shows the coagulation and settling out of the algal biomass. From left to right: autoflocculation, flocculation with 250 mg of chitosan, flocculation with 500 mg of chitosan.

Discussion

The growth, lipid induction, and flocculation experiments have implications for algae biofuel productions and scalability to commercial-size operations. Optimizing biomass production simultaneously with increasing lipid content will result in the highest net biodiesel yields (Packer et al. 2011). Finding effective, affordable, and environmentally-friendly harvesting methods is also an area of important algae biofuel research (Chen et al. 2011). Using innovation and integrating production systems with other industries can help the algal biofuel industry become a viable option for renewable liquid fuel production.

Effects of Carbon Dioxide on Growth Rates

The density of an algal culture must be sufficiently high for viable harvesting and conversion to fuel. However, the algal growth rate is perhaps more important than the final density of the culture in industrial productions (Neal 2012). The more biomass that a company can produce in a given amount of time without compromising lipid storage ability, the greater the overall oil yield will be. The CO_2-enhanced cultures had a growth rate of 2 doublings per day compared to the ambient air cultures with a growth rate of 1.4 doublings per day. This represents a 43% increase in growth rate, which could be the difference between an economically sustainable and unsustainable production system.

The cell abundance data obtained using direct haemocytometer cell counts was the best method to measure algal growth rates. The absorbance of samples measured by the spectrophotometer showed an increasing trend of cell abundance. This method cannot be used to calculate growth rate because it is impossible to isolate chlorophyll *a* in the measure of optical density. Caution should be taken when using optical density as a proxy for biomass because dead cells, salt particles, and other contaminants, as well as slight inconsistencies in protocol such as mixing of samples and equipment preparation time can result in inconsistent measurements. The direct haemocytometer cell counts give an accurate growth rate of the six individual cultures and show the divergence between the CO_2-enhanced cultures and the ambient air cultures.

Stimulation of algal growth rates by elevated CO_2 has been previously documented. The growth rates of some microalgal species have been shown to increase when grown under CO_2 concentrations that are double or triple the present atmospheric values. Different species respond differently to increased concentrations (Fu et al. 2007). A study on the effects of carbon dioxide enhancement on *Dunaliella tertiolecta* showed a 35% increase in growth rate (Beardall and Raven 2004). This enhancement was 100% in the diatom *Skeletonema costatum* (Fu et al. 2007). Other cellular mechanisms may also be affected by the concentration of CO_2 available and could be influential in the identification of ideal biofuel species. For example, photosynthetic sensitivity to CO_2 concentrations differs among species (Raven 2005). Some species, such as the coccolithophore *Emiliania huxleyi* show increased rates of photosynthesis when CO_2 is increased above natural levels (Raven 2005). However, elevated CO_2 concentrations have

also shown to decrease calcification in this organism, which may offset the beneficial effect on photosynthesis and growth. Finding species that can utilize carbon dioxide to greatly increase biomass production should be a focus of algal farms that aim to co-locate with flue gas emitters.

Efficient systems in both time and scale are crucial for algal biofuel projects to be affordable. One way to reduce costs and increase growth rates at the same time is to co-locate algal farms and industrial facilities that emit carbon dioxide. In the past, the Earth's climate has been subject to CO_2 levels much higher than those today, and microscopic algae were adapted to these high concentrations (Beardall and Raven 2004). The approximate concentration of carbon dioxide absorbed by green algae at the time of first appearance (1,000 Mya) was 540 times what it is today (Beardall and Raven 2004). Carbon absorption is not only a valuable resource for the algal biofuel industry, but it could also be an option for carbon sequestration by emitters to reduce their carbon footprint or buy carbon credits.

Other essential resources for algal growth can also be utilized from the wastes of other processes, including nutrient and water inputs from wastewater treatment facilities as well as heat and nitrogen from flue gases (Reddy 2002). Pairing algal biofuel production with other industries can both increase biofuel yields as well as ease the costs of resource acquirement. Olaizola et al. (1991) investigated the effects of increased CO_2 from three different industrial sources and found that the increased productivity more than offset the cost of adding the CO_2.

The pH of the algae culturing system in this study did not experience any significant changes in pH. The absorption of CO_2 in water causes acidification and a low pH can negatively affect algal growth rates. However, when algae perform photosynthetic CO_2 fixation the pH increases (Granum and Myklestad 2002). When large quantities of CO_2 are input into a algal culture, pH should be monitored and controlled (e.g., acid and base titrations) to remain within an acceptable range for the species.

Lipid Accumulation

Increasing lipid content in the algal cells is another important component in maximizing algal biofuel yields. Both a salt shock (5%) and decane treatment (1%) showed an increase in lipid production during the stationary phase of growth. Both

treatments were significantly higher in lipid content after three days – measured by BODIPY fluorescence – than the algae culture without treatment.

Increasing salinity is a form of creating stressful conditions for the algae to induce increased lipid synthesis and storage. Ratha and Prasanna (2012) observed that when salinity was double that of standard seawater, TAG content increased to 67% from the initial 60% in *Dunaliella*. Adding the salt during the stationary phase, as well as in small increments is important to prevent cell death. *Dunaliella* are halotolerant and are found naturally in salt lakes or high salinity seas (Countway 2011b). The natural salinity preferences of the chosen algal species should be known before performing salt shock on the culture to induce lipid synthesis (Ratha and Prasanna 2012). *Dunaliella tertiolecta* cellular vitality was not negatively affected by the increase in salinity, and lipids content increased. Some species, however, might lose cellular functions or die as a response to increased salinity. Alternatively, species that are naturally halotolerant might not be affected by the increase whatsoever and salt shock would not be a good lipid induction method for these species.

A low concentration (1%) of decane also boosted lipid content significantly compared to the culture with no lipid induction treatment. The mechanism for this is unknown, but possibilities include the cells metabolizing the hydrocarbon, or utilizing heterotrophic growth. This treatment may be interesting to perform on an algal species that is known to grow heterotrophically. It was important to use a low concentration of decane because high concentrations can cause cell lysis and can even extract the lipids from the cell bodies.

A two-stage cultivation strategy was important because it allowed cell cultures to reach high densities and biomass, before inducing lipid synthesis. Otherwise, treatments such as nutrient deprivation and increased salinity can limit cellular growth if added during the exponential phase. It is possible that nutrient concentrations and medium composition could be coordinated so that a two-stage lipid production system could be achieved "naturally" as the cells are able to grow exponentially before the exhaustion of the limiting nutrient and then switch to lipid accumulation under nutrient –depleted conditions (Ratha and Prasanna 2012). For large-scale productions, a two-stage strategy could be engineered using both a closed photobioreactor for biomass accumulation and

then expansion into an open pond system for lipid. A low-nutrient pond system would not only be beneficial for lipid induction, but also for contamination control, lower costs, and would be more environmentally friendly (Ratha and Prasanna 2012).

The high variability among the three replicates of salt-shocked cultures, and even more so among decane-treated cultures, could be due to a few factors. Because BODIPY is a relatively new lipid fluorophore for algae research, the best methods for using this stain have not yet been determined. High variability may suggest background noise in the fluorescence reading. One cause for concern is that BODIPY-stained clean medium without algae gives a high fluorescence reading. This outcome indicates that the BODIPY is either staining a component of the Instant Ocean media or auto-fluoresces without attachment to a lipid body. Caution should be taken to eliminate this background fluorescence when using BODIPY. Samples were centrifuged and washed three times to eliminate any BODIPY not incorporated into the cellular lipid bodies, however, a general fluorescence reader is still not the best way to measure lipid content. BODIPY is ideal for identifying and isolating cells with high lipid content using flow cytometry or a fluorescence-activated cell sorter (Cooper et al. 2010). Another advantage of BODIPY that was observed in this study is its ability to stain cells *in vivo*. No increase in cell lysis or death was observed after the addition of BODIPY nor was mobility affected. This fluorophore can be used to monitor lipid synthesis throughout the production process without compromising the health of the cultures. The success of downstream processing is dependent on reliable biochemical and physiological screening tools such as the BODIPY lipid stain (Ratha and Prasanna 2012).

Chitosan Flocculation

Chitosan flocculation at a concentration of 2 g/L was the most effective means of separating the algal cells from the medium, compared with autoflocculation and chitosan at a concentration of 1 g/L. Mixing the powdered chitosan periodically during the first six hours of the experiment was important to prevent the chitosan from immediately settling on the bottom of the flasks. Mixing helped dissolve the powder and effectively bind to the algal cells and over 99% of the cells coagulated on the bottom of the container after three days, making them more available to be harvested. To avoid re-suspension of the cells, the water should be decanted off the top to harvest the remaining biomass. This

preliminary study shows that chitosan is a good algal flocculant and new methods could reduce the amount of chitosan necessary for maximum flocculation.

Other studies have shown various methods to improve the efficiency and efficacy of chitosan flocculation. Lavoie and de la Noue (1983) dissolved the chitosan powder in 1% acetic acid to obtain a 0.5% (w/w) solution at pH 3.5. The solution was then stirred into 1-liter cultures for five minutes. Fifteen minutes after starting the flocculation process over 99% of the algal biomass had flocculated with a chitosan concentration of 10 mg/L (Lavoie and de la Noue 1983).

Chitosan is a good option for a chemical flocculant because it is non-toxic; remaining water can be recycled and expensive separation of the chemical from the algae is not necessary. Chitosan has also been shown to have antibacterial effects against waterborne pathogens (Andres et al. 2007). Depending on location, there is also the possibility of cheaply sourcing crustacean shells, often deposited as waste by seafood processors, for chitosan processing. In Maine, millions of pounds of post-processing discards from lobster, shrimp, and crab fisheries are dumped in the ocean or sent to landfills each year (Skonberg 2003). These discards include empty shells, whole legs, and shells with adhering meat that is not cost-effective for processors to remove. This waste from the seafood industry could be turned into an affordable resource for the algal biofuel industry.

Future Research

Life Cycle Analysis and Cost Reduction

Economic data and life cycle analyses for large-scale algal biofuel production are currently lacking and inconsistent. More research into the feasibility of commercial systems and how integrated systems can reduce costs will be needed for successful business designs. Economic models for algal biofuels vary considerably due to the multitude of model inputs, including plant location; transportation; process, and equipment costs; feedstock costs (CO_2, water, trace nutrients); co-location and integration opportunities (power plants, wastewater treatment facilities, existing petrochemical facilities); and energy, co-production and fuel market variability (Cheng and Ogden 2011).

Pokoo-Aikins et al. (2010) found that the most profitable scenarios for producing biodiesel from *Chlorella* species are those assuming 50% oil content and incorporating heat recovery from outside sources, as well as mass integration for recycling methanol and water. Although this was a laboratory-scale study, the various production and processing methods resulted in an estimated biodiesel selling prices in a range of $3.69 per gallon to $4.20 per gallon (Pokoo-Aikins et al. 2010). As algal production processes become more streamlined and efficient, the price of algal biodiesel is becoming competitive with petrodiesel; the average U.S. on-highway diesel fuel price in March 2012 was $4.057 per gallon (U.S. EIA 2012). The ultimate goal of algal biofuel research is to supply algal oil at $2 per gallon that can be processed to fuel for approximately $0.40 per gallon (Cheng and Ogden 2011).

A review by Chisti (2007) suggests about 1.5-3 times higher productivity is required to reduce costs to desired levels (Packer 2009). A combination of factors can help improve productivity ranging from species selection, condition optimization, co-location with industrial plants, recycling nutrients, and creative sourcing of affordable resources. Genetically modified organisms could also help improve efficiency and reduce costs of algal biofuel production. Genetic modification will most likely be developed in the future if research in algal biofuels continues.

Flue Gas Utilization

Another area of study that needs more research is the ability of algae to grow using flue gases directly from the source. Flue gas from coal combustion produces carbon dioxide as well as sulfur oxides, SO_X (SO_2 and SO_3) and nitrogen oxides, NO_X (N_O and NO_2) (Reddy 2002). Sulfur oxides and nitrogen oxides are air pollutants that can lead to acid rain, forest damage, effects on vegetation, smog formation, and damage to human health (Reddy 2002). Microalgae farms for biofuel production have the potential to help cleanse flue gases of these harmful pollutants. A study by Reddy (2002) found that a gas containing 725 ppm SO_2, 320-400 ppm NO_X, and 320-400 ppm NO with a controlled pH was tolerated by microalgae. NO_X acted as a nitrogen source for the algal photosynthesis and even increased biomass concentration. However, tolerance may vary by species and concentration of flue gases. If carbon sequestration from industrial flue gas is to be used

as a method for growing algae, the choice of algal species should address specific characteristics that ensure tolerance to flue gases (Pokoo-Aikins et al. 2010).

While preliminary studies have shown the flue gases can be used to cultivate microalgae, prolonged, large-scale experiments using recycled water have not been performed. More research will be needed to address questions of how to effectively capture the CO_2 from the flue gas, how to handle heat integration with the power plant, how to control the pH, the effect of trace metals in the flue gas, and how tolerant microalgae are to SO_X and NO_X (Cheng and Ogden 2011).

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