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ACKNOWLEDGEMENTS

The successes of the California Initiative for Large Molecule Sustainable Fuels would not have been possible without the dedication of the co-investigators: Dr. Eric Allen, Dr. Bianca Brahamsha, Dr. Steven Briggs, Dr. Michael Burkart, Dr. William Gerwick, Dr. James Golden, Dr. Susan Golden, Dr. Mark Hildebrand, and Dr. Mark Jacobsen. Each of the investigators led an extensive team of dedicated researchers, technicians and students, many of who are acknowledged in section 2.3. We would also like to thank the scientific coordinators, Dr. Cameron Coates, Dr. James Gregory, Dr. Melissa Scranton, and former Project Manager, Megan Bettilyon. The Division of Biological Sciences at the University of California San Diego also provided support through the ongoing use of facilities at the Biology Field Station. We would also like to thank the members of the Technical Advisory Committee: Jeffrey Jacobs, Todd Peterson, Alex Aravanis, Eric Mathur, Anastasios Melis, Anthony Eggert, Joseph Norbeck, and James Liao. Lastly, we would like to express our gratitude to the Director of Government Relations at University of California San Diego, David Schroeder, for his invaluable support, guidance and insight.
The California Energy Commission Energy Research and Development Division supports public interest energy research and development that will help improve the quality of life in California by bringing environmentally safe, affordable, and reliable energy services and products to the marketplace.

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- Industrial/Agricultural/Water End-Use Energy Efficiency
- Renewable Energy Technologies
- Transportation

*California Initiative For Large Molecule Sustainable Fuels* is the final report for the California Initiative For Large Molecule Sustainable Fuels project (contract number CEC-500-10-039) conducted by the University of California, San Diego. The information from this project contributes to Energy Research and Development Division’s Renewable Energy Technologies Program.

For more information about the Energy Research and Development Division, please visit the Energy Commission’s website at www.energy.ca.gov/research/ or contact the Energy Commission at 916-327-1551.
ABSTRACT

Recent legislative requirements affecting the California transportation sector have emphasized the need to develop sustainable, low-carbon fuels. To meet this demand, the California Initiative for Large Molecule Sustainable Fuels sought to examine the feasibility of using biofuel sources such as algae, Jatropha, and cellulosic biomass to produce low-carbon, drop-in fuels. The researchers examined nine areas of focus within biofuels: 1) Life cycle analysis of potential biofuels organisms and production processes; 2) Road mapping for identifying new technologies and biofuel sources; 3) Development of efficient technologies for rapid biofuel characterization; 4) Development of a genetic toolbox for biofuel organisms; 5) Development of metabolic engineering tools for biofuel organisms; 6) Development of biofuel organism protection strategies; 7) Development of improved harvesting and extraction techniques for biofuels; 8) Development of co-products for improved economic viability of biofuel organisms; and 9) Development of technologies to optimize nutrient utilization and recycling in biofuel organisms. Even though each area of research was successfully addressed, the two categories which saw the most improvement were developing a set of robust genetic tools for commercially relevant strains of algae and cyanobacteria, and developing crop protection strategies for both algae and cyanobacteria species. Development of high throughput technologies, metabolic engineering, and co-product development was especially helpful for the development of products from algae that can reach the commercial market in the near-term. Based on Roadmap input from experts, Technical Advisory Committee input and the research conducted, the researchers concluded that higher value co-product commercialization will enable industry growth and profitability, both of which will be essential to enable the scaling required to reach economic viability for biofuels. Fuel production requires economies of scale that cannot be reached without significant capital expenditure, and this expenditure will only come after smaller, higher value algae-based products have been demonstrated to be economically viable. A two-pronged approach is recommended to establish the most promising of the low-carbon feedstock, algae: 1) immediate and significant investment in the research and demonstration plants for algae based biofuels; and 2) development and approval of pilot demonstration algae energy parks in California.

Keywords: Low carbon fuels, drop-in fuels, algae biofuels, algae biotechnology jobs, greenhouse gas reduction.

Please use the following citation for this report:

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EXECUTIVE SUMMARY

Introduction
Recent legislation has emphasized the need for sustainable, low-carbon fuels that help reduce. For this project, biofuel sources such as algae, Jatropha, and cellulosic biomass were studied to determine their productivity and process feasibility as sustainable low-carbon fuels. The results gathered from the researcher’s findings could potentially affect policy decisions that influence the distribution of resources for future energy development.

To help California and the nation reach legislated requirements for advanced low-carbon fuels, it was necessary to research all aspects of the biofuels production process; these aspects included improving system engineering, developing superior genetic feedstock, developing crop protection strategies, finding cost-efficient processes to refine the biomass into fuels, and recovering and recycling resources like water, nutrients, and carbon dioxide.

To continue progress in biofuel production, which enables California to meet low-carbon fuel goals, collaboration among academic research institutions, industry, and government must increase dramatically. Regulatory uncertainty in California has caused companies to look elsewhere to initiate commercial-scale development, but thoughtful policy measures including pre-permitting, criteria-based exemptions, and pragmatic streamlining of the California Environmental Quality Act process can help to incentivize industry activity and the deployment of pilot and commercial scale facilities in California.

In order to meet the goals of Assembly Bill 32, more capital investment and social commitment will be needed to develop and scale alternative fuels that can leverage available and underutilized resources, such as unused land and brackish water found near the Salton Sea or San Joaquin Valley, or waste water resources, which are presently discharged into oceans or pumped underground. Throughout the development process, a transparent, standardized Life Cycle Analysis system is essential to ensure that any alternative transportation fuel produced for use in California are both environmentally and economically sustainable and scalable, and that these fuels and truly low carbon.

Project Purpose
The California Initiative for Large Molecule Sustainable Fuels was started in 2011 to specifically examine the potential of algae for the production of low-carbon drop-in fuels for the California transportation sector. The Initiative was housed at the California Center for Algae Biotechnology at the University of California San Diego, with a mandate to examine nine research categories: 1) Life cycle analysis of potential biofuels organisms and production processes; 2) Road mapping to identify new technologies and biofuel sources; 3) Development of high throughput technologies for rapid biofuel characterization; 4) Development of a genetic toolbox for biofuel organisms; 5) Development of metabolic engineering tools for biofuels organisms; 6) Development of biofuel organism protection strategies; 7) Development of improved harvesting and extraction techniques for biofuels; 8) Development of co-products for improved economic viability of biofuel organisms; and 9) Development of technologies to optimize nutrient utilization and recycling in biofuel organisms.
Project Results

The California Initiative for Large Molecule Sustainable Fuels (CILMSF) roadmap was developed with participation from biofuels researchers and other stakeholders in the public and private sectors. The roadmap covers the research opportunities and technical barriers for research and development of algae and plant as well as regulatory issues impeding progress. Water, land, and economic resource concerns regarding this green sector are also included in the roadmap. Development of the roadmap also determined that continued Life cycle analysis (LCA) for biofuels is needed to improve the economic efficiency of production decisions and prevent regulatory overlap.

Progress was made in every area of research, and highlights of these results are detailed in the following report, as well as in publications and presentations resulting from the research listed within the report. Most notable was the enormous progress made on developing a set of robust genetic tools for commercially relevant strains of algae and cyanobacteria, and on developing crop protection strategies for both algae and cyanobacteria species. Progress on developing high throughput technologies and in metabolic engineering and co-product development has laid the foundation for commercial deployment of near term economically viable production strains.

Project Benefits

A number of jobs have already been created in the San Diego area as algae biotechnology companies begin the commercial deployment of high value algae-based products based on the discoveries from California Initiative for Large Molecule Sustainable Fuels research. These high value co-products will enable growth and profitability of the industry, both of which will be essential to achieving the scale required to reach economic viability with biofuels. Fuel production will require economies of scale that cannot be reached without significant capital expenditure, and this expenditure will only come after smaller, higher value algae-based products have been demonstrated to be economically viable.

Timing is critical for California to take action on securing its energy future and remaining a leader in the quickly growing algae biotechnology industry. If California is going to meet its obligations under Assembly Bill 32 and the Low Carbon Fuel Standard, additional new low-carbon feedstock needs to be developed and deployed. The research team now has the opportunity to establish the most promising of this low-carbon feedstock, algae, through a two-pronged approach: 1) immediate and significant investment in the research and demonstration plants for algae based biofuels; and 2) development and approval of pilot demonstration algae energy parks in California. The creation of these parks and subsequent industry partnerships will not only provide industry a roadmap and pilot demonstration experience that will enable them to roll out commercial plants in California, which they are currently reluctant to do, but it will do so on a much faster timeframe than industry would be able to alone.
CHAPTER 1:  
Introduction

1.1 Background and Overview

Conventional petroleum-based transportation fuels (gasoline, diesel, jet) are nonrenewable and contribute to air quality degradation and climate change through carbon release. Reliance upon them stands in the way of California’s energy independence. Large molecule drop-in biofuels represent a promising and revolutionary set of technologies for not only reducing California’s petroleum dependence, but also safeguarding the environment and supporting the continued success of California’s economy. These fuels are engineered to be fungible with conventional fuels, i.e., indistinguishable to the end users. As such, they are also compatible with existing infrastructure, obviating massive and disruptive investments in new bulk transportation, storage, and distribution systems. However, before biofuels can compete with petroleum economically, more research and development has been required to not only reduce the cost of production, but also to identify value-added co-products, and co-benefits that can help make biofuels economical and bring them to market. Green chemistry processes, pharmaceuticals, nutraceuticals, bioplastics, etc., all need to be taken into account, as they can all be part of the processes that go into creating economic alternative fuels.

Investments in university-led basic research and multidisciplinary collaboration are essential to developing the next generations of large molecule biofuel technologies and related materials. A diverse pool of well-trained technical personnel and professional scientists is also required. In 2011, the California Energy Commission Public Interest Energy Research (PIER) program created a new research initiative, the California Initiative for Large Molecule Sustainable Fuels (CILMSF), to address the research, development and deployment required for large molecule sustainable fuels (LMSF); inform stakeholders including policy makers, industry professionals, and the community about sustainable drop-in fuels; and support California’s nascent bioenergy industry and green collar job training and creation. Through these efforts, the CILMSF was designed to help foster California to meet its transportation fuels demand with in-state production and lead the nation and world in the development of a sustainable, environmentally favorable sustainable fuels industry.

Led by Principal Investigator (PI) and Director of the California Center for Algae Biotechnology (Cal-CAB), Dr. Stephen Mayfield, the CILMSF team is comprised of nine faculty from the University of California, San Diego and the Scripps Institution of Oceanography (SIO), as well as students, postdoctoral fellows, staff and volunteers working across disciplines and providing valuable training experiences for young scientists and engineers.

The CILMSF operates from a platform of technological neutrality, involving research scientists from the fields of biology, chemistry, bioengineering, chemical engineering and economics, with a primary mission to develop the knowledge, tools, and industrial practices that will allow the production of economically viable and fungible large molecule fuels from sustainable renewable sources. The research projects have comprised multi-disciplinary research teams that
address the most significant barriers to economic viability of large molecule biofuels, while training the next-generation scientific and technical workforce essential to the future success of a sustainable renewable liquid transportation fuels industry in California.

The CILMSF has partnered with regional and statewide economic and workforce development organizations, as well as university and community colleges, to provide access to a robust curriculum to train scientists and technicians for the green collar jobs being created by the emerging biofuel industry. It has facilitated the transfer of technology to the commercial sector, and worked to foster interaction and collaboration with regional and state policy makers regarding the development of large molecule fuels for economic and environmentally sustainable energy production.

1.2 Project Objectives

The objectives of the CILMSF were divided into nine research categories: 1) Life cycle analysis of potential LMSF organisms and production processes; 2) Road mapping to identify new technologies and biofuel sources; 3) Development of high throughput technologies for rapid LMSF characterization; 4) Development of a genetic toolbox for biofuel organisms; 5) Development of metabolic engineering tools for biofuels organisms; 6) Development of biofuel organism protection strategies; 7) Development of improved harvesting and extraction techniques for LMSFs; 8) Development of co-products for improved economic viability of biofuel organisms; and 9) Development of technologies to optimize nutrient utilization and recycling in biofuel organisms. All of these research objectives are aligned with the Energy Commission’s overall goal of bringing sustainable, drop-in large molecule fuels to commercial viability as rapidly as possible.

A fundamental limitation for the production of large fuel molecule fuels from energy crops, algae, or any other sustainable biomass feedstock lies in the scarcity of state-of-the-art molecular and genetic tools for many of these species, as well as the high throughput industrial practices required to develop these organisms into efficient fuel-producing organisms. In comparison to modern industrial organisms and agricultural crops, most biomass organisms that can be cultivated at large scale on non-arable land lack a strong foundation in fundamental biological information and all but the most basic genetic tools and industrial practices. By developing these tools and protocols, the CILMSF has helped to accelerate the rate at which these organisms become an economically viable source of sustainable large molecule fuels.

1.3 Technical Advisory Committee

A Technical Advisory Committee (TAC) for the CILMSF was created to provide strategic guidance to the CILMSF through meetings, calls and participation in the CILMSF Roadmap meeting. The TAC was composed of researchers, academic experts, algae biotechnology industry representatives, biofuels industry representatives, and policy experts (see Table 1).
Table 1: Technical Advisory Committee

<table>
<thead>
<tr>
<th>Name</th>
<th>Title</th>
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Source: Cal-CAB.
CHAPTER 2: Project Tasks

The following is a summary of the technical tasks that were performed under this project. More detailed descriptions and technical information is contained in the Progress Reports as well as the project deliverables, as described below.

2.1 Task 2.0 - Life Cycle Analysis

*PI: Jacobsen*

The full life cycle of a biofuel determines its effectiveness in reducing the emissions of greenhouse gases into the atmosphere. Fuels that require a large amount of energy or fertilizer in the production process, for example, will have higher life cycle emissions than others and will not be as effective in helping California meet the goals of its biofuels regulation. As a result, California has introduced the novel use of life cycle analysis (LCA) into the requirements of the Low Carbon Fuel Standard (LCFS). Fuels with low life cycle emissions are rewarded under the standard. The research team studied the economic incentives that this places on producers and considered the efficiency of LCA-based policy relative to more traditional regulatory standards and taxes.

One of the key technical advantages of LCA-based regulation is in the ability to regulate multiple sources of greenhouse gas emissions within the confines of a single requirement. Rather than separate laws on the use of fertilizer in corn production and natural gas and electricity in the processing of algal biofuel, for example, the entire process can be reduced to a single value representing carbon intensity of a fuel. Fuel blenders and retailers can select a portfolio of fuels with different carbon intensities in order to meet the average standard. In the framework developed for the LCFS the process for producing each fuel is specified in a state-certified “pathway” and assigned a particular carbon intensity value.

How do these pathways create economic incentives to improve efficiency in the upstream use of inputs (embodying the types of scientific advances described below in this report) and downstream use of the fuel? Figure 1 provides a graphical overview of the analysis conducted. The axes represent two broad dimensions of choice in the production of a final good \( Y \), which is defined here as transportation services. The vertical axis then shows the ratio of an intermediate \( Z \) (fuel) to the production of the final good. This is a “downstream” decision in the sense that the choice of fuel efficiency is made after a regulation like the LCFS has already been applied. The horizontal axis represents decisions made any distance upstream, for example the amount of electricity used in an algae biofuel production process. The upstream carbon content \( X \) per unit of fuel is given in the ratio \( X/Z \). An economically efficient policy will pass incentives both upstream and downstream, reducing carbon intensity at every point in the process. The classic example of an efficient policy in the economics literature is a carbon tax: it incentivizes producers to emit less carbon at every phase in production, and also raises the price of the final fuel, incentivizing users of the fuel to be more efficient with it as they produce transportation services. In the figure, this appears as the point labeled \( \tau \).
This ideal point is first compared to direct regulation levied upstream, for example a hard limit on the amount of electricity that an algal biofuel plant can use per unit output or on the amount of natural gas that a corn ethanol plant can consume. These policies will generally produce outcomes like the point \( s \) on the figure: The fuel is produced cleanly in compliance with the regulation, so \( X/Z \) is low, but the true cost of the pollution is not passed through. Too much fuel is used per unit of transportation services. A regulation or tax levied just on fuel, not shown, has a similar difficulty in terms of missed incentives: fuel use will be reduced as consumers switch away to avoid the higher price, but the remaining fuel will not be produced as cleanly as it could be. Neither of these alternative policies produces the efficient outcome \( \tau \).

In contrast, a downstream LCA-based policy, even though no specific rules are placed on upstream production technologies, is able to reach the efficient point along both dimensions. Even though all of the emissions at points upstream have been aggregated into a single value (the life cycle carbon intensity of the fuel), each individual upstream will separately act as though a tax were placed on the pollution itself. In the full algebraic model the incentive placed by the LCA exactly lines up with the incentive that would be placed by an efficient tax levied directly on the pollution. The blue curved lines in Figure 1 show the iso-profit sets from the perspective of fuel producers. A theoretical model demonstrates that the highest profit point, subject to the requirements of an LCA-based rule (the curve labeled “downstream LCA regulation”), will fall right at the economically efficient point \( \tau \). Interestingly, even the most comprehensive collection of direct regulations (placed on each aspect of biofuel production upstream, the vertical “upstream regulation” line in the figure) cannot reach efficiency along both dimensions.

**Figure 1: Comparison of LCA-Based Policy, First-Best Tax, and Traditional Regulation**

![Figure 1: Comparison of LCA-Based Policy, First-Best Tax, and Traditional Regulation](source: Cal-CAB)
There are considerable advantages to LCA-based policy rules there also remain some important caveats. The limits of the policy become most visible in the boundaries that must be drawn around any engineering-based LCA. For example, the opportunity cost of water used in an algae fuel production process might greatly exceed the price of agricultural water in a particular region. If this is true, and the marginal value of the water is not considered by the LCA, there will be an incentive to switch away from inputs covered by the LCA (for example electricity inputs) at the cost of greater use of inputs not covered by the LCA. Along these lines, the research team also pursued a secondary project that mapped out water resources across the southwestern U.S. and considered the likely pressure that biofuels production might place on these resources.

Extensions of the basic model also consider a variety of key complications informed by the existing economics literature. Information flows and the speed of regulatory updates (accounting for changes made in production efficiency), for example, will act to reduce the efficiency of the LCA-based policy relative to a tax. Cost heterogeneity and overlapping federal policy (not based on life cycle emissions) also create a unique set of challenges for California’s LCFS policy. The research team studied how this potential mismatch in incentives operates and how the policies might be better harmonized.

2.1.1 Highlights
Life cycle analysis has not traditionally been employed in energy regulation. Its introduction into California rules for low carbon fuels presents both a unique opportunity to improve the economic efficiency of decisions made throughout the production process and the potential for harmful regulatory overlap.

Under ideal conditions, LCA-based policy has the potential to exactly mimic the least-cost tax on carbon. Each actor in the system has an incentive to cut back on carbon emissions and by the same proportion that would be achieved with the tax. The research team also studied the limits of this equivalence, and cases where simpler rules like direct regulation on processes and emissions might be preferred. The presentation to the Energy Institute at Haas highlights the key theoretical contribution made, and additional details of the analytical work appear in the report to the Energy Commission.

2.1.2 Outputs


2.2 Task 3.0 – CILMSF Roadmap

PI: Mayfield
The CILMSF held a meeting of biofuels researchers and other stakeholders in the public and private sectors to produce a roadmap report, which defines the challenges and the opportunities for the bioenergy industry in this country, and specifically, the unique opportunities and challenges that exist in the State of California. Beyond solving scientific, economic and logistical hurdles, the goal was to develop a long-term strategy for the identification and development of new research areas and technologies that may be useful for the sustainable production of LMSFs, and ultimately to keep California as the leader in this field.

The CILMSF Roadmap meeting was held on October 18, 2012, in Atkinson Hall at UC San Diego. Twenty experts from academic, industry and government groups addressed prepared presentations and participated in panel discussions (see Table 2). Over 150 faculty, researchers, students, industry representatives, government representatives, non-governmental organizations (NGOs), and members of the community attended the event. An Executive Summary of the Roadmap Meeting was written and edited by presenters.

Follow-up meetings with faculty, NGOs, and industry representatives were integrated into regular Cal-CAB advisory meetings, annual Food & Fuel for the 21st Century Symposia and a ribbon cutting event for the CILMSF algae raceways at UC San Diego. A final review and update of the Roadmap Report took place at the end of the grant. The report will become available on the Energy Commission and Cal-CAB websites when finalized.
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<td>Tim Olson</td>
<td>California Energy Commission</td>
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Source: Cal-CAB

The CILMSF roadmap addresses the technical challenges that impact algal and plant research and development, as well as commercialization of different biomass feedstock. The roadmap addresses government policies and regulations pertaining to alternative fuel development with proposed solutions from industry and government representatives. Water, land, and economic resource issues regarding the biofuels sector are also addressed.
2.2.1 Outputs

*California Initiative for Large Molecule Sustainable Fuels Roadmap Meeting*. Held at Atkinson Auditorium, UC San Diego, on 18 October 2012.


2.3 Task 4.0 – High Throughput (HT) Processes

*PIs: Mayfield, Briggs*

2.3.1 Subtask 4.1 - High throughput screening to improve chloroplast gene expression

*PI: Mayfield*

Microalgae have significant potential for mass production of industrial molecules such as biofuels, therapeutic proteins, or industrial enzymes, due to their ability to produce biomass at large scale in a rapid and cost-effective manner. However, current levels of recombinant protein accumulation in algae are well below the levels achieved in other production hosts, making only the most valuable products economically suitable for algal production. The highest yields achieved to date in the model alga *Chlamydomonas reinhardtii* are around 10% of total soluble protein, but most recombinant proteins accumulate to less than 1%. To fully realize the potential of algae as a biotechnology platform, the research team needed robust and controlled gene expression in both the nuclear and chloroplast genomes.

Although progress has been made understanding gene expression in algae, the regulatory processes that govern gene expression and protein accumulation in the chloroplast remain an area of active investigation. A better understanding of the regulatory processes that govern protein accumulation may enable the design of custom regulatory regions that overcome the current limitations in producing recombinant proteins.

Protein expression in *Chlamydomonas* chloroplasts is regulated primarily during translation, governed by regulatory sequences in the untranslated regions (UTRs) of mRNAs and by trans-acting factors that interact with elements in these UTRs. For chloroplast gene expression, most research has focused on the 5’ UTRs of plastid mRNAs, as these have been shown to exert significant control over protein accumulation, impacting both translation rates and mRNA stability. Using a more systematic approach to characterize many chloroplast UTRs may explain recurring structures or sequences, leading to a more comprehensive understanding of plastid gene regulation. In addition to gaining a better understanding of native mRNA elements involved in gene regulation, the systematic analysis of UTR regulatory elements will allow the design of truly synthetic UTRs for driving expression of exogenous genes in transgenic algae. A notable characteristic of some of the most commonly used endogenous chloroplast regulatory regions is that they exhibit autoattenuation, and therefore, can only be used effectively in a
strain in which the native gene has been deleted. For example, the well-studied psbA regulatory regions are capable of high levels of recombinant protein production, but the psbA gene product inhibits expression of additional psbA transcripts. Therefore, these regulatory regions are only useful for driving exogenous gene expression in a nonphotosynthetic psbA knockout strain, eliminating the energetic benefit of using a photosynthetic organism to produce recombinant proteins at large scale. By identifying regions useful for strong positive regulation across many UTRs, the research team may be able to mix and match sequence elements from many UTRs to create novel synthetic elements that evade the negative feedback mechanisms involved in autoattenuation while maintaining high expression of the transgene of interest.

2.3.1.1 Highlights
In this study, the research team developed a high throughput systematic synthetic biology approach for identifying important 5' UTR sequence elements involved in modulating chloroplast gene expression (Specht et al., 2012). Large-scale oligonucleotide synthesis was used to create libraries of variant UTRs (Figure 2) and cloned these libraries into vectors driving the expression of a luciferase reporter gene. By selecting pools of transformants that have high,
medium, or low expression of the reporter, followed by next-generation sequencing of these different pools, the research team was able to quantitatively identify effector elements from each of the pooled groups. The HT method was validated by confirming previous partially characterized elements within the psbD 5’ UTR, though these elements were found to extend far beyond the region initially identified through site-directed mutagenesis, highlighting the superiority of a comprehensive, unbiased approach. Researchers then extended the analysis to psaA to identify novel regulatory regions within its 5’ UTR. Finally, the predictive power of the method by creating a synthetic UTR, based on the aggregate data from the psaA 5’ UTR, that outperforms the wild type version (Figure 3).

This high-throughput systematic approach was used to not only gain understanding of the gene regulation within the algae chloroplast, but also to design a better UTR to drive gene expression of non-native proteins. In the future, this synthetic regulatory DNA can be used to increase the expression of wide range of non-native proteins such as therapeutic proteins, industrial enzymes or enzymes involved in the production of biofuels.

2.3.1.2 Outputs

2.3.2 Subtask 4.2 - Proteome responses to nitrogen starvation

**PI: Briggs**

The research team developed and applied high-throughput proteomics methods for algae to obtain profiles of the proteome of *Chlamydomonas reinhardtii* before and during nitrogen starvation to discover changes that are associated with a metabolic transition toward lipid accumulation. Understanding this transition may enable algae to be engineered to have enhanced lipid levels under normal growth conditions. The lipid levels of algae are proportional to the biofuel potential of crude oil extracted from algae. Therefore, this project aimed to lay the knowledge foundation required to increase the biofuel potential of algae.

2.3.2.1 **Highlights**

High lipid accumulating, fast-growing algae have high potential in large-scale biodiesel production without having to competing for arable land. Although nitrogen limitation is the single most critical macronutrient affecting lipid production in algae, most efforts made so far to characterize signaling components of nitrogen-induced responses have used genetic tools and monitored changes in gene expression. Previous data show that *Chlamydomonas reinhardtii* cells respond to nitrogen deprivation by changing protein content and their post-translational modification status. An iTRAQ-based quantitative proteomic approach was employed to investigate proteome-wide changes in the model alga *C. reinhardtii* in response to nitrogen starvation. A time course analysis of quantitative proteomics changes in *C. reinhardtii* cells during nitrogen starvation treatment was used to detect changes that may be transitory. The aim was to analyze the overall cellular response to nitrogen starvation and to find proteins involved in this response that might be useful targets in metabolic engineering design of algal cells to facilitate high lipid yields.

For each condition, N depleted and N deprived, three independent biological replicates are collected. The total proteomics and phospho-proteomics analysis of all biological and technical replicates was finished. For acetylome, the first two stages have been tested. A total of 1936 non-redundant differentially expressed proteins and 350 differentially expressed phosphoproteins with fold changes greater than 1.5 (p < 0.05) were quantified during nitrogen stress. 208 acetylated proteins quantified in *C. reinhardtii* cells during the first two stages of treatment. In Figure 4, the Venn diagram shows the distribution of all the differentially regulated proteins found in the total proteome and phospho-proteome category, pointing out the presence of specific proteins characterizing each single stages, as a well as the shared ones. Interestingly, a total of 871 (out of 1,936 total differentially protein; 44.99%) proteins and 116 (out of 1,936 differentially phosphoproteins; 33.14%) phosphoproteins were observed only in a single time point. Figure 5 shows the expression kinetics of the differentially expressed proteins.

Many of the identified proteins were involved in metabolism, cellular component organization, localization and biological regulation processes, confirming that these pathways are highly
stressed by nitrogen starvation. From 6 hours until 72 hours, the number of differentially expressed proteins increased resulting up to 1253 differentially expressed proteins at the 72 hour timepoint (Figure 6A). Within them, 740 proteins were upregulated and 513 were downregulated, suggesting that in response to nitrogen starvation, *C. reinhardtii* cells regulated both by actively upregulating the signaling pathways and driving survival under stress by shutting down the interfering proteins.

**Figure 4: Distribution of the Differentially Regulated Proteins Found in the Total Proteome, Phospho-Proteome and Acetylome in *C. Reinhardtii* Cells at Different Time Points After Nitrogen Depletion**
The proteins regulated during nitrogen starvation. a) Differentially expressed proteins at all timepoints studied; b) Plot of the biological process classifications of up-regulated and down-regulated proteins during the nitrogen starvation.

Figure 6B shows a plot of the main biological processes of the upregulated and downregulated proteins that met an average fold change cutoff threshold of 1.5. In the response to nitrogen starvation over time, reduced levels of ribosomal and photosynthetic proteins were observed, pointing that the turn-over of abundant proteins such as RuBisCo (Ribulose-1, 5-biphosphate carboxylase/oxygenase) may provide carbon/energy for lipid accumulation in the nitrogen deprived cells. The major biological processes categories such as nitrogen compound metabolic process are exhibiting a more dynamic regulation. Several proteins like Glycerol-3-phosphate acyltransferase, catalyzing the initial and committing step in glycerol-lipid biosynthesis, increased at protein abundance under nitrogen starvation; on the contrary, enzymes for de novo
fatty acid synthesis, such as 3-ketoacyl-ACP-synthase, were downregulated. These results suggest that a more controlled method of lipid induction than gross nutrient manipulation would be necessary for development of sustainable bioprocesses. Results show that proteins in a complex have similar dynamic pattern in C. reinhardtii cells during nitrogen starvation, such as proteins in Rubisco and GAPDH/CP12/PRK complex related to CO₂(carbon dioxide) assimilation.

Figure 6: Proteins Regulated During Nitrogen Starvation

A) Differentially expressed proteins at all timepoints studied. B) Plot of the biological process classifications of upregulated and downregulated proteins during the nitrogen starvation.

Source: Cal-CAB
Figure 7: Rubisco Complex in C. Reinhardtii Grown Under Nitrogen Starvation Conditions

A) Acetylated lysine of Rubisco and rubisco activase identified in C. reinhardtii. B) Changes in acetylation of Rubisco and rubisco activase in C. reinhardtii in response to nitrogen deprivation. Source: Cal-CAB

The results also showed that protein acetylation is widely regulated by nitrogen starvation. Some of the nitrogen starvation induced changes in protein acetylation do not accompany changes in protein abundance. So, artificial anti-acetyllysine antibodies were used to investigate acetylation changes in C. reinhardtii cells after nitrogen starvation treatment. 208 acetylated proteins have been identified. Figure 7A shows the identified acetylated lysine sites of Rubisco and rubisco activase in C. reinhardtii cells. Changes in lysine acetylation of Rubisco and rubisco activase did not synchronize with changes in those proteins' abundance (Figure 7B) in C. reinhardtii cells grown under nitrogen starvation conditions. For Rubisco, the protein acetylation is a transient modification that is lower occupancy, meaning that only a fraction of a particular protein may be acetylated on a given site at any particular time. Therefore, specific and efficient acetylated peptide-enrichment methods along with sensitive and accurate mass-spectrometric instrumentation are useful for analyzing complex protein mixtures, such as complete cell lysates. To study whether the changes in acetylation sites cause increases or decreases in protein activity, the role of changes were selectively tested by mimicking and blocking these modification changes using site-directed mutants. At this point, 6 RuBisCo large subunit mutant strains were constructed against two acetylated lysine sites (rbcl K164 and K201).

To test the effects of acetylation on the RBCL catalytic domain, the research team collaborated with the Mayfield Lab and substituted both glycine and arginine for lysine 201 (K201). Glycine (Q) mimics acetylated lysine whereas arginine (R) removes the carbonyl but preserves the charge and shape of the side-chain. As expected, no K201Q mutant strains could be recovered but slow-growing K201R strains were obtained. Similar amino acid substitutions were made at
K164 and, except for K164R which grew at WT rates, slow-growing strains were obtained with the following transgenic compositions: K164Q, K164Q-K201R, K164R-K201R.

2.3.2.2 Outputs:
Dr Ying Lin, a post-doctoral associate, and Dr. Zhouxin Shen, a Senior Research Associate, conducted the experiments. Dr. Lin presented the results at Cal-CAB symposia. A manuscript is under revision.

2.4 Task 5.0 – Genetic Toolbox
PIs: J Golden, S Golden, Burkart, Hildebrand, Allen

2.4.1 Subtask 5.1 - Genetic toolbox for cyanobacteria
PIs: J Golden, S Golden

Exogenous DNA can be introduced into cyanobacteria by transformation, conjugation, or electroporation, and can be propagated in a strain if carried on a replicating plasmid, or if integrated into the host chromosome. Genetic tools have been developed for a select group of model cyanobacterial strains, including autonomously replicating vectors, integration sites, selection markers, reporter genes, and promoters. These molecular tools were originally developed to study fundamental cellular processes, whereas there is an increasing interest in using cyanobacteria as cell factories for production of small molecules. Most research has been on a few genetically manipulable model strains, primarily *Synechococcus elongatus* PCC7942 and *Synechocystis* sp. PCC6803. However, industrial-scale production is likely to require the use of strains with greater potential for practical use such as large-scale production in outdoor ponds. Depending on the growth conditions and the products to be made, different production strains and compatible well-suited advanced genetic tools will be needed.

In comparison to *Escherichia coli* or *Bacillus subtilis*, engineering cyanobacterial strains requires special considerations because of their oligoploidy or polyploidy, the presence of different restriction/modification systems, the presence of interacting differentiating cell types in some species, and their circadian rhythms. The cyanobacterial phylum is diverse and even the current model organisms differ from one another by their morphology, ecology, physiology, and genomic content. Depending on these variations and their ability to undergo natural transformation, different protocols and culture conditions need to be applied for different strains. Genome size, genome content, and codon usage can also be strikingly different from one strain to another. Such variations may affect the ability of a particular strain to properly express an introduced gene of interest.

In order for cyanobacteria to be developed into superior biotechnological platforms, the process of engineering them must become streamlined. An underlying goal of synthetic biology is to make this engineering process easier by defining and developing standards and by characterizing parts and devices. A DNA fragment that performs a defined function is often referred to as a *part*. Multiple parts associated together to provide a higher order function is called a *device*, which is available as a specific module, and several devices linked with each other can be used to create a *circuit*. A few genetic parts and devices are known to work in a few
cyanobacterial strains but many parts and devices are strain-specific. The research team has focused on producing broad-host-range parts and devices for synthetic biology research and development.

An integrated and expandable platform was developed for the efficient construction of vector systems from design to laboratory protocols. An assembly strategy, many biological devices (76 as of Jan 2014), bioinformatics tools, and improved protocols were devised to provide the necessary tools to construct a wide range of vectors to allow engineering of different strains of cyanobacteria. 42 shuttle plasmids were assembled and introduced into select cyanobacteria to characterize 27 modules or devices. In addition, the research team constructed 14 different destination vectors harboring a cloning site, different antibiotic resistance markers, and different chromosome neutral sites or a broad-host-range replicon variant of RSF1010. 55 different devices were tested (as of Jan 2014). The combinatorial construction of shuttle vectors was shown to be very efficient. Adding new biological devices that carry the proper GC-adaptor overlapping sequences can easily expand this platform.

Inducible negative regulator devices were designed and constructed for turning off an expressed gene. The strategy is to evaluate known repressors under control of the inducible riboswitch parts. Two repressors and corresponding promoters (lacI-Ptrc and vanR-PvanA) driving the expression of yellow fluorescent protein (YFP) are being tested. The repressors were placed under the control of a PconII promoter followed by a riboswitch activated upon addition of theophylline. For both repressor systems, 3 riboswitch variants are being tested (B, C, and F). Overall, for different strains, the Pptrc promoter should result in high expression levels whereas the PvanA promoter should result in low expression levels when the promoters are not repressed by addition of theophylline. The riboswitch variants (B, C, and F) have different minimum and maximum levels of expression as well as different activation ratios. The table below summarizes the plasmids and strains that were constructed. Preliminary experiments suggested that the PconII promoter with the riboswitch F caused baseline expression levels of the repressor that were too high and therefore resulted in very low level of YFP whether the riboswitch was activated or not. These results were observed in S. PCC7942 with both the lacI-Ptrc and the vanR-PvanA systems. Similar results were obtained with the riboswitch B for the vanR-PvanA system. Based on preliminary data for S. PCC7942, the deactivation ratios, 3 days after addition of theophylline, reached 0.34 using the vanR-PvanA system with the riboswitch C and 0.53 using the lacI-Ptrc system with the riboswitch B. In the future, it will be useful to construct plasmids and strains to evaluate these inducible negative regulator devices in a variety of cyanobacteria strains.

To expand the currently available genetic toolkit, the research team has demonstrated the utility of synthetic theophylline-responsive riboswitches for effective regulation of gene expression in four diverse species of cyanobacteria, including two recent isolates. The research team evaluated a set of six riboswitches driving expression of a YFP reporter in Synechococcus 20longates PCC 7942, Leptolyngbya sp. BL0902, Anabaena sp. PCC 7120, and Synechocystis sp. WHSyn. The evaluations demonstrated that riboswitches can offer superior regulation of gene expression compared to the commonly used IPTG induction of a lacIq-Pptrc promoter system. In addition, the expression of toxic protein SacB can be effectively regulated, demonstrating utility
for riboswitch regulation of proteins that are detrimental to biomass accumulation. This work demonstrates the easily accessible utility of riboswitches in the context of genetic engineering and synthetic biology in diverse cyanobacteria, which will facilitate development of algal biotechnology.

2.4.1.1 Highlights
Inspired by the developments of synthetic biology and the need for improved genetic tools to exploit cyanobacteria for the production of renewable bio-products, we developed a versatile platform for the construction of broad-host-range vector systems. This platform includes the following features:

- An efficient assembly strategy in which modules released from 3 to 4 donor plasmids or produced by PCR are assembled by an isothermal assembly reaction guided by short GC-rich overlap sequences.
- A growing library of devices categorized in 3 major groups.
  - replication and chromosomal integration
  - antibiotic markers
  - expression cassettes, reporter cassettes, and promoter-reporter modules
- A web service, the CYANO-VECTOR assembly portal, which was built to organize the various modules, facilitate the in silico construction of shuttle vectors, and encourage the use of this system.

This work also resulted in the construction of an improved broad-host-range replicon derived from RSF1010, the characterization of 9 antibiotic cassettes, 4 reporter genes, 4 promoters, and a ribozyme-based insulator in several cyanobacterial strains. Genetic devices were tested for function in several of the following cyanobacterial strains: *Synechococcus 21longates* PCC7942, *Synechocystis* sp. WH5yn, *Synechocystis* sp. PCC6803, *Anabaena* sp. PCC7120, *Leptolyngbya* sp. BL0902, and *Nostoc punctiforme* ATCC29133.

A set of synthetic theophylline-responsive riboswitches provided effective regulation of gene expression in four diverse species of cyanobacteria. Because this regulation does not require translation of a protein transcription factor, these riboswitch regulation modules can be expected to function in a broad range of cyanobacteria and provide an important tool for gene regulation for metabolic engineering and synthetic biology.

2.4.1.2 Outputs
This task resulted in 1 patent application, 3 publications, and 8 presentations.

James Golden and Amy Ma. REGULATION OF GENE EXPRESSION IN CYANOBACTERIA. U.S. Provisional Application Serial No. 61/775,28 filed on March 8, 2013.


Arnaud, T. San Diego, California, December 8-11, 2013. Pacific Rim Summit on Industrial Biotechnology & Bioenergy. “Improved broad host range molecular tools for synthetic biology and biotechnology in cyanobacteria.” Invited as a speaker for the Life Technologies’ workshop entitled “Advances in Algal Synthetic Biology Technologies”

Arnaud, T. La Jolla, California, November 15, 2013. Cal-CAB Student and Postdoc Symposium Series. “Improved broad host range molecular tools for synthetic biology and biotechnology in cyanobacteria.”

Arnaud Taton, Amy Ma, You Chen, Federico Unglaub, Tyler Swinney, Edward King, Ron Cook, Nicole E. Wright, Susan S. Golden, and James W. Golden. 2013 “Improved genetic tools for cyanobacteria.”


2.4.2 Subtask 5.2 – Metabolic engineering of prokaryotic and eukaryotic algae for crop protection and high value products

*PI: Burkart*
Heterologous expression of synthases in eukaryotic or prokaryotic hosts, can lead to interesting production hosts for high value products. These synthases also often produce compounds with anti-microbial activities, possibly opening the door for herbicide-free crop protection. In the process of engineering the eukaryotic green microalgae *C. reinhardtii* in close collaboration with the Mayfield lab, the research team found that heterologous expression of these large proteins (1200 amino acids and larger) in green eukaryotic microalgae is more difficult than initially expected, and therefore, it is still a work in progress. Many synthases require post-translational modification with a 4’-phosphopantetheine arm to be active. To prepare green microalgae for the introduction of these synthases, the research team engineered the enzyme that performs this modification, a so-called phosphopantetheinyl transferase, successfully into *C. reinhardtii*.

A simpler expression host for these large synthases is a cyanobacterium, which offers the benefits of photosynthesis but does not have the complexity of a eukaryote. Also in cyanobacteria however, these large synthases have to date not been heterologously expressed. But since the genetic toolbox for cyanobacteria is further developed and the species are more amenable to genetic manipulation, rapid progress is being made in the engineering of eukaryotic algae. In close collaboration with the Golden laboratory, the research team prepared suitable expression constructs of these large synthases and are in the process of optimizing their heterologous expression.

### 2.4.2.1 Highlights

The engineering of a foreign phosphopantetheinyl transferase (PPTase) in *C. reinhardtii* was successful and prepares the eukaryotic algae strain for metabolic engineering with synthases. The research team is in the process of investigating expression levels and activity of the PPTase, depending on expression in chloroplast or nucleus, and vector used. The fitness of the strain will be assessed for introduction of a synthase.

A preliminary screen with the products of both synthases, 6-methylsalicylic acid and in-house isolated and purified blue pigment indigoidine, showed activity against pathogenic chytchyds (in collaboration with the Briggs lab), fungi and bacteria. The research team is currently extending this assay, to facilitate a fast screening pipeline for engineered algae strains. PPTase-overproducing strains will also be included. These strains show significant changes in their fatty acid and lipid profiles, possibly leading to anti-grazer phenotypes.

### 2.4.2.2 Outputs


2.4.3 Subtask 5.3 – Development of genetic tools for diatoms

PI: Hildebrand

2.4.3.1 Overview

This subtask’s goals were to develop highly useful genetic manipulation tools for diatoms to allow understanding of metabolic processes, and manipulation to improve production of desired molecules. The research team focused on developing new promoter systems, characterizing the intracellular location of proteins using fluorescent tagging, developing knockdown and knockout approaches, and developing a new antibiotic selection marker that also was able to improve the expression of a protein fused to it.

2.4.3.2 Highlights

Characterization of novel gene expression control elements: To facilitate metabolic engineering of diatoms, promoters that can be used to control expression of introduced genes are highly useful. Prior to this work, only one inducible promoter, for nitrate reductase, was identified in diatoms. The research team characterized several more diatoms in detail, such as the silicon transporter (SIT) promoter from the SIT1 gene in *T. pseudonana*. This promoter was not active under growth conditions with sufficient silicon in the growth medium but became induced at the transcript level by 4 hours, and at the protein level by 6 hours, after silicon starvation with 90+ percent of cells expressing. This promoter has proven to be highly useful for protein expression work. A lack of metabolic drain on the cell is related to growth in silicon replete conditions, because the promoter is off. This promoter will be useful for difficult to express proteins that may adversely affect the cell.

Screening of transcriptomic data identified four other silicon-starvation-induced promoters from the diatoms *Thalassiosira pseudonana* and *Cyclotella cryptica*, which have now been characterized. All four show minimal expression under uninduced conditions and high expression in terms of fluorescence intensity of GFP per cell and percentage of cells expressing under induction. Three of the four promoters express at higher levels than any previous promoter described from diatoms.

Protein tagging and localization: Due to the complex intracellular compartmentation of diatoms, and the multi-compartmental nature of metabolic pathways involved in carbon flux (Smith et al. 2012), determination of protein localization in the cell is critical to understand its metabolic processes. The research team successfully demonstrated the use of CFP, eGFP, mWasabi, bflo1, eYFP, RFP, and TagRFP in *T. pseudonana* and *C. cryptica* as fluorescent tags.
Codon optimization was not required for any of them. In the investigations, the researched team targeted the plasma membrane, cytoplasm, ER, periplastid compartment and membrane, chloroplast membrane, pyrenoid, mitochondria, chrysolaminarin vacuole, and potentially two types of peroxisome. This analysis is shedding new light on the role of compartment-specific processes in diatoms.

Knockdowns and knockouts: RNAi and antisense knockdown approaches for centric diatoms were previously demonstrated (Shrestha and Hildebrand, submitted). To push the technology further, multiple knockdowns were attempted using a single construct with antisense multiple genes. The team was unable to isolate viable transgenic lines expressing multiple antisense knockdowns. One explanation was that the knockdowns were lethal, and hence no growth resulted. The approach has not been further pursued, because identification of appropriate genes that will enable evaluation of the knockdown phenotype is required.

The research team also experimented with the TAL nuclease approach in *T. pseudonana* in collaboration with Life Technologies, Inc. eGFP was cloned into a Gateway Entry vector supplied by Life Tech, which was subsequently cloned into a destination vector and transformed into *T. pseudonana* cells. The TAL-eGFP was successfully transcribed under the transcriptional control of the constitutive promoter fucoxanthin chl a/c binding protein, fcp. It appears that although the TAL was functional in *T. pseudonana*, the expression level could be enhanced with codon optimization for *T. pseudonana*. Further analysis never resulted in a clear knockout phenotype using the TAL approach. Experimentation with the CRISPR system began recently, attempting to knockout nitrate reductase.

The research team evaluated the relation between codon usage and expression using the Sh ble zeocin resistance gene as a test case. *T. pseudonana* has not been amenable to zeocin selection because expression levels of the Sh ble gene are too low for the required stoichiometric binding of the protein to the antibiotic. Analysis of rare and common codons showed an unfavorable bias, which when corrected, enabled the use of zeocin as a selectable marker. This analysis also revealed that eGFP codon usage could be improved, and in fact, using native eGFP as a reporter has underestimated possible expression levels. Researchers have experimented with antibiotic-mediated forced expression by fusing the expressed protein (eGFP) to the Sh ble gene and subjecting cells to increasing doses of zeocin. At the highest zeocin concentrations, GFP fluorescence is 2-3 times higher than at low concentrations. There is an apparent “memory” for the response, cells previously treated with high zeocin which is subsequently removed, retain higher expression levels for at least 2 days.

2.4.3.3 Outputs

Shrestha RP, Hildebrand M. Evidence for a regulatory role of diatom silicon transporters in cellular silicon responses. Submitted to *Eukaryotic Cell*.

Davis AK, Abbriano RM, Smith SR, Hildebrand M. The intracellular location of malic enzyme in diatoms and clarification of its roles. *In preparation*.

Presentations:


2.4.4 Subtask 5.4 – Recombinant synthesis of long-chain omega-3 polyunsaturated fatty acid products in heterologous microbial hosts

PI: Allen

Long chain ω3-PUFAs such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) are essential components of metazoan membranes (Figure 8). An extensive body of literature citing the importance of ω3-PUFAs for human health, proper development, and disease prevention has accelerated research into the role ω3-PUFAs play in human physiology and disease-state prognoses. PUFA consumption has been linked to the prevention and stasis of
several disease states including atherosclerosis, thrombosis, hypertriglyceridemia, rheumatoid arthritis, mental disorders, and cancer, as well as playing an essential role in proper eye and brain development. In addition to the beneficial effects of a diet rich in ω3-PUFAs, the availability of prescription ω3-PUFAs marketed under the brand name LOVAYZA® (GlaxoSmithKline), principally EPA and DHA in the form of ethyl-ester derivatives derived from marine fish sources, serves to underscore the effectiveness of ω3-PUFAs as a lipid-regulating therapeutic to specifically reduce triglyceride levels in patients with severe hypertriglyceridemia. Because of their importance in human health, numerous ω3-PUFA nutraceutical products are available for human consumption in the form of gel capsules or oils derived from marine fish extracts or marine microalgae.

**Figure 8: Structure of EPA and DHA.**

The primary source of ω3 oils for human consumption or aquaculture feed remains wild-caught fish. However, the stability and sustainability of natural fish stocks cannot support the increased demand for ω3 products. Consequently, the identification and development of sustainable replacements for these products is an industry priority. Moreover, concerns that oil extraction and purification methods from marine fish include heavy metals and other bioaccumulated contaminants, including PCBs and dioxin-related chemicals, further motivates the exploitation of new sources. The production of ω3-PUFAs in native and recombinant microbial hosts represents a tractable solution to meet the global demand for cleaner and renewable ω3-PUFA products.

Several species of marine gamma-proteobacteria are capable of de novo synthesis of EPA and DHA using a “PUFA synthase” mechanism encoded by the pfaEABCD operon. This pathway has been successfully cloned and expressed in *E. coli*. PUFA synthase-related gene clusters have also been identified in diverse marine bacterial species and the eukaryotic marine 27longat, *Schizochytrium* sp. ATCC-20888, suggestive of horizontal gene transfer. *Schizochytrium* is a heterotrophic stramenopile 27longat (“slime net”) and a member of a larger class of protists known as the Labyrinthulomycetes (family Thraustochytridae). Many labyrinthulids have been shown to accumulate ω3-PUFAs, principally DHA in the form of triacylglycerides that can account for greater than 60% of cellular dry weight. A number of labyrinthulid strains are currently being used in food supplements as a source of commercial DHA (Martek). Importantly, the efficiency of the Pfa Synthase mechanism (pfa gene products) is responsible for the high DHA accumulation in these strains.
The research team analyzed all available genome sequence data from labyrinthulid strains and identified the presence of three PUFA-synthase genes in three of four organisms analyzed. Unlike the bacterial operon structure, the PUFA-synthase genes are fragmented and present on separate chromosomes in the labyrinthulid genomes, with each gene presumably under its own native regulatory control. A large collection of marine labyrinthulids was isolated in the vicinity of a laboratory at the Scripps Institute of Oceanography in La Jolla, California. Based on chemical analysis of lipid products and growth properties of the cultured isolates collection, one strain, *Thrustochytrium* sp. Strain LLf1b, was promoted as the model labyrinthulid for genetic characterization. LLf1b produces high levels of DHA, accounting for approximately 45% of total cellular fatty acids, with a doubling time of approximately 2 hours at room temperature (23°C). Using two sets of transcriptomic data, the researchers were able to identify three PUFA synthase ORFs in LLF1b and reconstruct gene models most closely corresponding to the canonical *pfaA*, *pfaB*, and *pfaC* genes in other labyrinthulid species and marine bacteria (Figure 9). The gene models are well curated and have served as genetic templates for cloning and heterologous expression as described below.

**Figure 9: Pfa Gene Cluster Responsible for the Synthesis of DHA from the Marine Isolate *Thraustocythrytrium* Sp. Llf1b. the Complete Three Gene Cluster is Approximately 23 Kbp.**

An ideal heterologous host should have a sequenced genome with advanced genetic tools, a degree of relatedness to the native host, and a track-record for industrial production. *Saccharomyces cerevisiae* has been used for millennia in the production of products for human consumption, and continues to be utilized extensively in the production of bread, beer, wine and other fermentative products. It is also the quintessential eukaryotic model organism, being widely employed in life science research. While the expression of bacterial operons in other bacteria is not trivial, expression of multi-gene biosynthetic pathways in eukaryotic organisms such as yeast presents an interesting challenge. Yeast are not known to process bacterial operons without modification, since each gene must have its own promoter and terminator sequence for efficient expression. The efficiency of the yeast homologous recombination machinery can be used to manipulate and recombine multiple large genetic pieces with relative ease.

**2.4.4.1 Highlights**

With the availability of a native, complete, and self-contained pathway for the production of ω3-PUFAs (Pfa Synthase) from a unique isolate (LLf1b) in a tractable heterologous host (*Saccharomyces cerevisiae*), the research strategy involved a two-phase approach for the engineering of yeast strains capable of producing the ω3-PUFA DHA (22:6n-3).

**Phase I: Isolation and mobilization of PUFA synthase genes from LLf1b and their heterologous expression in S. cerevisiae**
PUFA synthase genes were amplified from LLf1b genomic DNA using high-fidelity PCR. While the genomic context of \textit{pfaA}, \textit{pfaB}, and \textit{pfaC} is unclear, each gene must be placed under the control of its own promoter and terminator for downstream expression in \textit{S. cerevisiae}. An additional gene for the full pathway to be active is the phosphopantetheinyl transferase (PPTase), encoded by the \textit{pfaE} gene, which is involved in the post-translational modification of the acyl carrier protein (ACP) domain repeats contained in PfaA.

To mobilize these four genes (PfaA-D, PPTase) for downstream applications, the high copy 2\textmu- based yeast-expression shuttle plasmid was used as the expression vector (Figure 10). This vector contains a cloning site flanked by ADH2 promoter and CYC1 terminator sequences, along with \textit{E. coli} and yeast replication machinery and appropriate selectable markers. Using Gibson cloning, each gene has been integrated between the promoter and terminator sites on the vector, producing gene cassettes for yeast expression.

\textbf{Figure 10: Schematic of 2\textmu-Based Yeast-Expression \textit{E. Coli} Shuttle Plasmid (Pxp842) to be Used for Construction of Gene Cassettes}

\begin{center}
\includegraphics[width=0.8\textwidth]{fig10.png}
\end{center}

Source: Nancy DaSilva, UC Irvine.

\textit{Phase II: Assemble PUFA synthase genes into a single genetic construct in \textit{S. cerevisiae} to reconstitute full pathway}

Following confirmation of successful individual gene expression, the research team attempted reconstruction of the full PUFA synthesis pathway in a single genetic construct. Due to the size of the construct and the number of pieces for assembly, the integration strategy took advantage of the \textit{in vivo} homologous recombination machinery of \textit{S. cerevisiae}. Using a second round of high-fidelity PCR, each cassette (Promoter\textsubscript{ADH2}-Gene\textsubscript{pfaX}-Terminator\textsubscript{CYC1}) will be amplified from its vector with additional 25-50bp homology arms to facilitate seamless \textit{in vivo} recombination. The four gene cassettes, along with a yeast artificial chromosome/bacterial artificial
chromosome (YAC/BAC) vector with appropriate selectable markers, were transformed in a one-step reaction. This should have resulted in a single construct, \( \text{pfaA}_{1-3} \text{pfaB}_{1-3} \text{pfaC}_{1-3} \text{pfaE}_{1-3} \) on a YAC/BAC mobile backbone however attempts to build this genetic construct were unsuccessful to date.

To circumvent these cloning difficulties, a similar strategy could be employed for direct chromosomal integration of the PUFA synthase pathway. While chromosomal expression often reduces protein expression levels, it greatly increases long-term stability of heterologous genes. Additional linkers with homology to yeast chromosomal sites would be employed on either end of the integrated pathway for a one-step chromosomal insertion strategy. Many target sites for chromosomal integration have been characterized for optimal expression levels. Additionally, repetitive sigma elements are found all over the yeast genome next to tRNA genes, providing a potential location for multi-copy stable integration into the yeast chromosome.

On-going research is aimed at the successful expression of the full PUFA synthesis pathway in recombinant yeast. While heterologous expression offers many benefits, it may be desirable to optimize the production of PUFAs within the native labyrinthulid strain L. llf1b. A first step would include manipulation of growth conditions, such as temperature, salinity, and culture media. GC/MS analysis would permit selection of conditions producing the greatest amount or preferred profile of \( \omega-3 \) PUFAs in wild-type L. llf1b. Further efforts would include selection of natural variation through colony picking or Fluorescence Activated Cell Sorting (FACS) coupled with a neutral lipid dye such as BODIPY or Nile Red. Random mutagenesis could be employed to enhance natural genetic variation. More targeted strategies rely on developing genetic tools for L. llf1b, but could include site-directed mutagenesis of pfa genes or mutagenesis through error-prone PCR. Genetic manipulation of upstream and/or competing metabolic pathways can also be targeted to improve metabolic flux towards \( \omega-3 \) PUFA synthesis and accumulation.

2.4.4.2 Outputs
With support from the Energy Commission, tremendous progress has been made in exploring new technologies for the sustainable production of commodity nutraceutical molecules. Although no research publications have resulted from the above studies, the CILMSF is optimistic that on-going research efforts in this area will be submitted for publication in the near future. Future research will be aimed at optimizing the genetic constructs for heterologous expression of PUFA biosynthetic genes in heterologous microbial hosts and assess the quantity and quality of enhanced PUFA production.


2.5 Task 6.0 – Metabolic Engineering

*Pis: J Golden, S Golden, Gerwick, Hildebrand, Mayfield*
2.5.1 Subtask 6.1 – Metabolic engineering in cyanobacteria

Pis: J Golden, S Golden

Omega-3 fatty acids EPA and DHA are long-chain polyunsaturated fatty acids (PUFAs) found in fish oils. They show substantial health benefits to the human body such as supporting brain function and reducing inflammation. Production of fish oil is struggling to meet the increasing demand for omega-3 fatty acids, which is projected to reach $34.7 billion in 2016 globally. Alternative sources of omega-3 fatty acids, such as marine microalgae and marine proteobacteria, as well as heterologous production in transgenic plants and microorganisms, could help meet the increased demand. CILMSF researchers are exploring the renewable production of PUFAs in cyanobacteria.

Researchers introduced a 20-kb gene cluster (pfaE-ABC-D) from the marine bacterium *Shewanella pealeana* that is responsible for EPA biosynthesis into cyanobacterial strains. Using a GC-MS method for EPA (C20:5)/DHA (C22:6) analysis with extended running time, substantial amounts of the C22:5 fatty acid were detected in both *Synechococcus* and *Anabaena* EPA-producing strains. Repeated EPA production assays of *Anabaena* PCC7120 RSF1010-EPA strains showed that EPA and related PUFAs accounted for up to 6.0% of total fatty acid methyl esters (FAME). Although PCR analysis confirmed the presence of EPA genes in transgenic *Synechocystis* WHSyn, the preliminary EPA production assay, however, showed no accumulation of EPA or related PUFAs in *Synechocystis* WHSyn RSF1010-EPA strains.

In an attempt to increase biosynthetic gene expression levels, two plasmids with a rearranged EPA gene cluster (pfaE-pfaRABC-pfaD) designed to replace native promoters with the trc promoter were constructed. Serials transformation assays were conducted for EPA strains of *Synechococcus* PCC7942, *Anabaena* PCC7120, and *Synechocystis* WHSyn to make the final production strains via homologous recombination. However, increased production levels were not observed. There could be many reasons for this result that could be tested in future studies.

It is possible that strains expressing EPA would be healthier and therefore produce more EPA in a desC mutant, which would not produce delta-9 desaturase. However, this gene is important because only single recombinants or “merodiploid” double recombinants could be obtained when a specific Tn5 transposon insert (8S14-K2) was used to disrupt the delta-9 desaturase gene (desC) in *Synechococcus* PCC7942. Introduction of a copy of riboswitch-controlled desC into NS2 of the “merodiploid” 8S14-K2 strains did not facilitate the segregation of the desC locus in the presence of 2 mM theophylline. The EPA gene cluster has been introduced into the “merodiploid” 8S14-K2 strains with or without the presence of the riboswitch controlled desC gene. In the future, EPA production assays could be performed for these strains. A different desC knock-down construct using Arnaud Taton’s inducer-repressor concept was built, in which desC is driven by the trc promoter and the lacI gene is under the control of a riboswitch. The native desC was replaced by this new allele via double recombination. In the future, production assays of EPA-producing strains carrying the desC allele in the presence of different concentrations of theophylline could be performed.
The pANS plasmid has been shown to have higher copy number than that of the chromosome, which may serve as a better platform for EPA production. The 20-kb EPA gene cluster was cloned into pANS-based shuttle vectors and then introduced into both WT and pANS-cured *Synechococcus* PCC7942 strains. EPA production assay for these strains have been conducted. EPA samples have been collected and processed for GC-MS analysis.

The research team also attempted to engineer HGL (heterocyst glycolipid) biosynthesis in cyanobacterial strains. The 19-kb HGL gene cluster, with its native promoter or with the petE promoter, was PCR amplified and cloned into a RSF1010-derived shuttle vector. The HGL genes were introduced into heterologous cyanobacterial strains and HGL aglycone production could be determined in future work.

### 2.5.1.1 Highlights

The researchers introduced a 20-kb gene cluster (*pfaE-ABC-D*) from the marine bacterium *Shewanella pealeana* that is responsible for EPA biosynthesis into two cyanobacterial strains. Significant amounts of EPA and related PUFAs were accumulated in these strains at 18°C: 6.5% of total FAME in unicellular *Synechococcus* PCC 7942 and 6.0% in filamentous *Anabaena* PCC 7120. Manipulating membrane lipid fluidity is a possible route to enhance EPA accumulation. Because the sole membrane lipid desaturase gene in *Synechococcus*, desC (*synpcc7942_2561*), is essential, the researchers developed a desC knock-down system, in which the desC gene is under the control of a theophylline-dependent riboswitch. This work will provide a sustainable platform for heterologous biosynthesis of omega-3 fatty acids in photosynthetic cyanobacteria.

### 2.5.1.2 Outputs


### 2.5.2 Subtask 6.2 – Fuel precursor pathway identification and modification in cyanobacteria and diatoms

**PI: Gerwick**

*Characterization and modification of cyanobacterial hydrocarbon biosynthesis* – Cyanobacteria exhibit the rare capacity to produce hydrocarbons using lipid precursors. In order to metabolically engineer these pathways an investigation was undertaken into the natural capacity for hydrocarbon production. This work will constitute a major reference point for hydrocarbon biosynthesis in cyanobacteria and includes not only an in-depth structural analysis of hydrocarbon compositions of 32 cyanobacterial strains but it also includes an in-depth investigation of the evolutionary history of hydrocarbon biosynthetic pathways in cyanobacteria. The analysis performed for this investigation identified multiple aspects of hydrocarbon biosynthesis that can be leveraged to not only produce biofuels but also other high...
value chemicals. In particular the olefin synthase (OLS) found in a small group of cyanobacteria exhibits a unique sulfotransferase and thioesterase domain that could be used to produce terminal alkenes. Not only are terminal alkenes applicable to diesel and jet fuel applications, they can also be used a variety of applications including plastic precursors and bioactive secondary metabolites. Additionally, the identification of uniquely branched hydrocarbons in a variety of different cyanobacteria suggests some variability in the pathways involved in their biosynthesis. Although focus has been on the methyltransferase involved in branched hydrocarbon biosynthesis in \textit{Anabaena} sp. PCC 7120, there are likely novel substrate preferences, and therefore, novel branching patterns in subsequent products of methyltransferases in some of the strains we investigated (including \textit{Fischerella} sp. PCC 7414).

\textit{Investigation of methyltransferases involved in branched chain alkane biosynthesis} – Some cyanobacteria produce branched alkanes in addition to the straight chain alkanes and alkenes found in all cyanobacteria. This investigation sought to identify the methyltransferase responsible for branched alkane biosynthesis in cyanobacteria. Branched hydrocarbons can be used as gasoline precursors or diesel fuel directly. This investigation sought to identify the methyltransferase responsible for branched hydrocarbon biosynthesis and modify this pathway to increase production of these fuel relevant hydrocarbons. Using bioinformatics all of the methyltransferases in the branched chain alkane producing model, cyanobacterium \textit{Anabaena} sp. PCC 7120 was targeted for investigation. Three target methyltransferases (all2121, all3016 and alr3038) were bioinformatically identified as targets for further investigation. Heterologous expression in \textit{E. coli} was used to evaluate the specificity of the three target methyltransferases. No branched fatty acids were detected, suggesting that none of the three methyltransferases exhibits this biochemical activity in \textit{E. coli}. Purified proteins derived from all3016 and alr3038 were used for \textit{in vitro} analysis; however branched fatty acids were not produced, indicating that these methyltransferases do not exhibit methyltransferase activity under the conditions tested.

A knockout of all3016 was generated by homologous recombination in \textit{Anabaena} sp. PCC7120. However, branched hydrocarbon production was not abolished in the resulting mutant strain, suggesting that all3016 is not involved in branched hydrocarbon biosynthesis, or that additional methyltransferases contribute or can compensate to maintain branched hydrocarbon production. All2121 was transformed into two cyanobacterial strains that do not naturally produce branched hydrocarbons and were subsequently tested for production of branched hydrocarbon production. No branched hydrocarbons were observed in the transformed strains, suggesting that all2121 may not be involved in branched hydrocarbon biosynthesis.

\textit{Lipase knock-down in Thalassiosira pseudonana} – The research team has achieved the first targeted knock down of a lipase in the diatom \textit{Thalassiosira pseudonana}, resulting in the production of strains exhibiting increased triacylglycerol (TAG) and total lipid accumulation without compromised growth. The \textit{T. pseudonana} homologue of the human enzyme CGI-58, Thaps3_264297, was found to be downregulated during lipid accumulation. Functional characterization of the enzyme revealed lipase, phospholipase and acyltransferase activities. Targeted knock down of Thaps3_264297 using antisense RNA resulted in strains exhibiting wild-type-like growth, increased and earlier TAG accumulation during stationary phase of growth, and increased TAG and total lipid accumulation during silicon-limited conditions.
Analysis of two transgenic strains under silicon-limited conditions showed a greater than 2-fold increase in TAG and greater than 3.5-fold increase in total lipid per cell compared to wild-type. Because the growth of the transgenic strains is unaltered, almost identical increases were also found in overall lipid yields. Quantitative analyses of fatty acids, individual lipid classes and membrane stability revealed an increase in both TAG and phospholipids in the transgenic strains analyzed, suggesting a role for Thaps3_264297 in membrane turnover. These manipulations show that inhibiting lipid catabolism through metabolic engineering represents a realistic and novel method for increasing lipid yields in eukaryotic microalgae.

2.5.2.1 Highlights
The highlights of this research task include the following accomplishments:

- Characterization of the hydrocarbon production capacity of a wide range of cyanobacteria.
- Identification of a wide range of targets for future manipulations to cyanobacterial hydrocarbon biosynthesis.
- Achievement of the first targeted knock-down of a lipase in the diatom *Thalassiosira pseudonana*, resulting in the production of strains exhibiting increased triacylglycerol (TAG) and total lipid accumulation without compromised growth.

2.5.2.2 Outputs
This task resulted in 2 publications, 1 patent and 3 presentations to date:


2.5.3 Subtask 6.3 – Characterization of fatty acid and LCPUFA metabolic pathways in diatoms and metabolic engineering to improve triacylglycerol and LCPUFA yields

PI: Hildebrand

The research team focused on the characterization and improvement of triacylglycerol (TAG) and fatty acid (FA) synthesis genes in *T. pseudonana*. In terms of FA, they specifically focused on the high value product of long chain polyunsaturated fatty acids (LCPUFA). The process of LCPUFA and TAG synthesis is poorly characterized in diatoms, and this investigation included identification of genes involved in these processes, and overexpression and knockdown of genes to evaluate their roles. For both processes, manipulations led to higher yields of products.

In addition to this focus, subsequent analyses contributed to two publications related to carbon flux and its manipulation in diatoms and other algae.

2.5.3.1 Highlights

*Characterization and manipulation of TAG biosynthesis*: The intracellular location of TAG biosynthesis in diatoms is unclear. Freeze fracture SEM analysis of *T. pseudonana* and *C. cryptica* during TAG accumulation revealed a large intracellular vacuole called the chrysolaminarin vacuole or Cv (diatoms store carbohydrates as β-1, 3-linked glucans called chrysolaminarins) in close proximity to developing lipid droplets. In other organisms, FA synthesis occurs in the chloroplast and TAG synthesis in the endoplasmic reticulum (ER), but the association of diatom lipid droplets with the Cv suggests that TAG enzymes may be associated with that organelle. Initial data localizing a MGAT protein suggests a possible Cv localization. If this is the case, then stored carbohydrate would be broken down in the cytoplasm, imported into the chloroplast, where Fas are synthesized, which would then be exported to the Cv for TAG synthesis.

The research team identified all of the genes encoding proteins involved in TAG synthesis in *T. pseudonana*, and determined their predicted intracellular targeting (Table 3). The analysis revealed that early steps in TAG synthesis involved proteins targeted to the chloroplast, and for later steps no targeting was predicted, indicative of cytoplasmic localization, or at least localization to a membrane system.
Researchers generated fluorescent protein fusion constructs to several of these enzymes to directly demonstrate localization. They focused on determining the location of MGAT and DGAT enzymes, and the effect of overexpression of them. To date, transgenic lines of three MGAT-GFP fusions and two DGAT-GFP fusions have been generated. Results suggest an at least peripheral association with membrane systems (likely the ER) around the chrysolaminarin vacuole with some cytoplasmic localization. This is consistent with previous predictions of targeting, which indicate either ER or cytoplasmic localization.

One enzyme, a DGAT2 homolog, has been characterized in detail. It appears to alter its location depending on the stage of growth, during exponential growth, it is chloroplast localized, but during the stationary phase, it becomes ER associated. This may be consistent with the location of formation of large lipid droplets. Researchers tested this directly by colocalization of a DGAT2/TagRFP fusion protein with staining of lipid droplets using BODIPY, and found that they were distinctly located. This indicates that TAG is synthesized in membrane systems distinct from lipid droplets, and must be transported to the droplets. Overexpression of this DGAT resulted in a 1.2-1.5 fold improvement in TAG accumulation. A manuscript is in preparation describing this work.

The research team is in the process of completing the analysis of intracellular targeting, and will prepare a manuscript detailing the process of TAG synthesis in *T. pseudonana*.

**Characterization and manipulation of LCPUFA biosynthesis:** Researchers completed a 36longates36tics analysis of all enzymes involved in fatty acid (FA) and long chain polyunsaturated fatty acid (LCPUFA) synthesis in *T. pseudonana* to determine their predicted intracellular location. Previous transcriptomics analyses identified mRNA expression patterns for their genes, which was coupled with an analysis of changes in FA composition over time. After 24 hrs of silicon-limitation triggered TAG accumulation, C16 Fas increased substantially (2-5 fold), C18 Fas decreased substantially (4 fold), and LCPUFAs increased 2-3 fold.
Researchers identified three elongase genes in the *T. pseudonana* genome, which make natural targets for knockdowns and overexpression followed by monitoring the effect on FA and LCPUFA levels. A single KASII gene, whose encoded enzyme should be involved in elongating C16 Fas to C18 Fas, has also been identified. This gene is only moderately upregulated, and overexpression could greatly increase the proportion of longer-chain Fas.

Researchers focused on the characterization of the fatty acid elongases. All three elongase genes were analyzed, Elo1 (ID 3741), Elo2 (ID 93), and Elo3 (ID 728). Transgenic lines overexpressing all three elongases were generated, in both GFP-fused, and native form. The growth rate as well as maximum cell density of several transgenic lines were not affected by the over-expressed genes and appeared similar to wild-type *T. pseudonana*. TAG (assessed by BODIPY staining) accumulation in transgenic lines was tested at late exponential phase, early stationary phase and later stationary time points in nutrient-replete medium. TAG levels in all transgenic lines were 2-10 times higher than wild type. Intracellular location analysis using GFP-tagged proteins indicated that Elo1 and Elo2 are ER localized and Elo3 displayed punctate staining in the cytoplasm.

Researchers fully analyzed fatty acid profiles in the overexpression transgenic lines. In the Elo1 and Elo3 overexpression lines and during exponential growth and stationary phase, EPA and DHA levels were increased 1.5-2 fold over wild type in two separate transgenic lines for each. No increase over wild type was seen after 24 hr silicon starvation. For Elo2, a 2.75-fold increase specifically in DHA occurred in stationary cultures. The data are consistent with the proposed step Elo2 catalyzes, which is the conversion of EPA to DHA. This is an important finding because diatom DHA levels are approximately 10% those of EPA, and enabling increased accumulation of DHA would have health and economic benefits.

### 2.5.3.2 Outputs

To date, this task has resulted in 2 publications, 1 submitted manuscript, 2 manuscripts in preparation, and 8 presentations.


Manandhar-Shrestha K, Hildebrand M. Overexpression of a DGAT2 leads to increased TAG accumulation in *Thalassiosira pseudonana*. *In preparation*.
Cook O, Beld J, Abbriano RM, Burkhart, M, Hildebrand M. Characterization of LCPUFA elongases in *Thalassiosira pseudonana* and manipulation to improve EPA and DHA levels. *In preparation.*


**2.5.4 Subtask 6.4 – New genetic tools for increased non-native protein expression in the C. reinhardtii nucleus**

*PI: Mayfield*

Microalgae are a diverse group of photosynthetic microorganisms with considerable biotechnological potential. Algal products are currently used in the animal and fish feed
industries, and for cosmetics, pigments, and nutraceuticals. Furthermore, microalgae have the potential to be a valuable source of bioenergy. Genome engineering in algae offers the potential for improved product yields and crop protection, as well as the potential to modify metabolic pathways to produce unique products. Also, transgenic microalgae also have the potential to be low-cost bioreactors for commercially valuable recombinant proteins such as therapeutic proteins and industrial enzymes. However, genetic engineering of microalgae is still far behind other microorganisms.

A major obstacle remains low transgene expression levels from the nuclear genome of many microalgae. A more significant roadblock to nuclear engineering in *C. reinhardtii* is transgene silencing. Reports have demonstrated transgene silencing at both the transcriptional and post-transcriptional levels. Thus, it is necessary to screen large numbers of transformants to identify individual clones that demonstrate the desirable, or sometimes even detectable, level of protein expression.

### 2.5.4.1 Highlights

In this study, researchers utilized the foot-and-mouth-disease-virus (FMDV) 2A peptide to link transgene expression to that of a selection marker in *C. reinhardtii*. The FMDV 2A peptide encodes a short 20 amino acid sequence that mediates a self-cleavage reaction. It is believed that during translation elongation of the 2A sequence, a peptide bond fails to form between the last two amino acids of the 2A sequence. Thus, when 2A is fused between two genes in a single open reading frame (ORF), the resulting protein is processed to yield two discrete proteins, with the short 2A sequence fused to the C-terminus of the first protein product. FMDV 2A and 2A-like sequences have been used for heterologous gene expression and biomedical applications in many eukaryotic systems including mammalian cell culture, retroviral gene therapy, and transgenic plants.

Researchers constructed a *C. reinhardtii* nuclear expression vector in which GFP or the industrial enzyme xylanase 1 (xyn1) from Trichoderma reesei was fused to the bleomycin antibiotic resistance gene sh-ble via the 2A sequence. The 2A peptide is correctly processed in the algal cytoplasm, resulting in cleavage of GFP or Xyn1 from Ble-2A (Figure 11). GFP and Xyn1 are stably expressed and functional. By linking the Xyn1 to Ble-2A researchers improved xylanase activity by approximately 100-fold compared to unlinked Xyn1 expression. Finally, by fusing an endogenous secretion signal between Ble-2A and Xyn1, researchers were able to target Xyn1 for secretion, resulting in significant accumulation of highly active xylanase enzyme in the culture media (Figure 12).

Researchers generated constructs that allow greater and more consistent non-native protein expression from the *C. reinhardtii* nuclear genome. They have also shown that these proteins can be correctly targeted for export out of the cell. These tools will be essential for future metabolic engineering in algae to allow expression and selected targeting of enzymes involved in metabolic pathways throughout the cell.
Figure 11: FMDV 2A Peptide ‘Cleavage’ in C. Reinhardtii

A) SDS-PAGE in-gel fluorescence analysis of total protein isolated from transgenic lines expressing ble-GFP or ble-2A-GFP. Labeled bands represent in-gel fluorescence signals of the respective heterologous proteins. B) Microscopy images of GFP signals from transgenic cells expressing ble-GFP or ble-2A-GFP.

Photo Credit: Cal-CAB

Figure 12: Insertion of A Secretion Signal Peptide Between FMDV 2A And Xyn1 Leads To Robust Accumulation of Functional Xyn1 in the Culture Media

Comparison of intracellular and extracellular xylanase activity for the ble2A-xyn1 (Xyn1) and ble2A-SP-xyn1 (SP-Xyn1) transgenic lines. Intracellular (cells, grey) and extracellular (media, blue) xylanase activities are represented as a percent of total activity for each strain.

Source: Cal-CAB

2.5.4.2 Outputs


### 2.6 Task 7.0 – Protection of Biofuel Organisms from Pests and Pathogens

*Pis: J Golden, S Golden*

#### 2.6.1 Subtask 7.1 – Protection of biofuel organisms from pests and pathogens: cyanobacterial antifungal genes

*PI: J Golden*

A fungal/cyanobacterial model system was developed. It has been shown that some cyanobacteria produce antifungal chitinase enzymes. Researchers cloned 4 potential antifungal chitinase genes: a cho gene from *Anabaena* sp. PCC7120, its homolog from *Anabaena variabilis* ATCC29413, a chitinase gene from *Nostoc punctiforme* ATCC29133, and a synthetic codon-optimized chitosanase cho gene from *Anabaena fertilissima* that previously has been demonstrated to have antifungal activity. The chitosanase from both strains of *Anabaena* have been inserted into expression vectors for *Synechococcus 41longates* PCC7942, *Anabaena* sp. PCC7120, *Leptolyngbya* sp. BL0902, and *E. coli*. These expression vectors were transformed into the 3 cyanobacterial strains and *E. coli*.

Researchers performed a PAGE (polyacrylamide gel electrophoresis) procedure followed by Coomassie staining and immunoblotting to verify the expression of the cloned chitinase and chitosanase genes in *E. coli* from the expression vectors. All three chitosanase genes were expressed by *E. coli* cells. The chitosanase from *A. fertilissima* and *Anabaena* sp. PCC7120 appear to be secreted. The chitinase from *N. punctiforme* ATCC29133 is not expressed in *E. coli*. This chitinase has two domains, a glycoside hydrolase family-18 domain and a phospholipase domain. We truncated the chitinase gene to include only the glycoside hydrolase domain, and the truncated version is expressed within *E. coli* cells but is not secreted.

Several previously tested several fungal strains obtained from stock collections in bioassays with different cyanobacterial strains did not observe antifungal activity with these fungal strains. Therefore, researchers isolated natural strains of fungi from locations where they would interact with cyanobacteria. Petri plates containing cyanobacterial BG-11 medium supplemented with 5% LB were opened and placed outside to collect airborne fungal spores that may fall into ponds. Surface layer water from 6 of San Diego’s reservoirs was also plated to isolated fungi. Using these approaches, researchers isolated 6 fungi that are susceptible to the antifungal compound benomyl. Bioassays for antifungal activity against these fungi have not detected any antifungal activity.

The research team tested the lysates and concentrated supernatants of the transformed *E. coli* strains expressing cyanobacterial chitin and chitosanases against fungi by spotting the lysates
and supernatants on fungal lawns, with wild-type E. coli as a negative control and benomyl as a positive control. Antifungal activity was not detected in these experiments, so the Task was ended.

2.6.1.1 Highlights

Algal farmers must look ahead to obstacles such as nutrient challenges and crop protection that impede commercialization of large-scale algal pond farms. Instances of outdoor algal pond crashes due to fungal parasites have already been reported.

A defining characteristic of fungi is the presence of a chitinous cell wall and is a potential target for antifungal molecules. There has been a cyanobacterial chitosanase reported to have antifungal activity against plant pathogens. This chitosanase along with two homologs in two different cyanobacterial strains as well as an uncharacterized chitinase in another cyanobacterial strain were investigated as possible antifungal molecules for algal crop protection. Though all the proteins were found to have catalytic activity, they did not exhibit antifungal activity against the tested fungi. Additionally, 10 strains of cyanobacteria were screened to look for new antifungal molecules. No antifungal activity was observed from the cyanobacteria against the tested fungi.

2.6.1.2 Outputs


2.6.2 Subtask 7.2 – Protection of biofuel organisms from pests and pathogens: mutations in cyanobacteria that confer resistance to amoebal grazing

(Cost-share with U.S. DOE funding)

PI: J Golden, S Golden

Algal species growing in open ponds are subject to predation by a variety of organisms. Grazing by zooplankton such as rotifers, and protozoa such as amoebae, ciliates and flagellates, can be the cause of rapid pond collapse. The goals of this task were to identify grazers of cyanobacteria invading model outdoor ponds, isolate a suite of grazers of a variety of cyanobacterial strains, develop grazer/cyanobacterium model systems for laboratory manipulation, isolate grazing-resistant mutants, and identify the genes and pathways responsible for conferring grazing resistance.

2.6.2.1 Highlights

A model system was established, consisting of the unicellular cyanobacterium Synechococcus 42longates PCC7942 and the clonal amoeba HGG1. The system was used to isolate grazing-resistant mutants. Researchers screened a gene knockout library of PCC7942 for resistance to HGG1 and identified one resistant mutant. The mutation was in a gene required for the production of the O-antigen of the lipopolysaccharide (LPS) component of the outer membrane. Through a combination of bioinformatics predictions and phenotype screening, the catalog of mutations that confer resistance was expanded to a total of 10 genes. Eight of these genes are responsible for individual steps involved in the synthesis, transport, or ligation of O-antigen to
lipid A in the process of generating LPS and two of these impair synthesis of the sugar core of lipid A. Mutations in genes involved in O-antigen synthesis may upregulate the production of high MW sugars. Researchers are testing the hypothesis that the upregulation of these molecules, rather than the lack of O-antigen itself, may be the true mechanism of resistance. Interestingly, mutations in genes involved in O-antigen biosynthesis do not impair the growth of PCC7942 relative to wild-type and they confer an autoflocculation phenotype. Both of these traits are advantageous for production conditions.

2.6.2.2 Outputs

This task, which is a DOE Cost share task, has resulted in 1 patent application, 3 publications (1 in preparation), and 7 presentations.

Susan S. Golden, James W. Golden, Bianca Brahamsha, Ryan Simkovsky, Emy Daniels, and Brian Palenik. CYANOBACTERIAL STRAINS RESISTANT TO GRAZERS AND CAPABLE OF AUTOFOCCULATION. U.S. Provisional Application Serial No. 61/635,814 filed on April 19, 2012.


S. Golden: Speaker, Developing cyanobacteria as platforms for food and fuel production, Food and Fuel for the 21st Century Symposium, La Jolla, CA.

S. Golden: Speaker, Our Energy Futures public lecture series, La Jolla, CA, April 19-20, 2013.

S. Golden: Speaker, Cyanobacteria: the other algae, Keystone conference on Biofuels, March 1-6, 2011, Singapore.


2.7 Task 8.0 – Harvesting and Extraction

PI: Mayfield

2.7.1 Raceway and Harvest System Construction

To examine large-scale cultivation, harvesting and extraction, two raceway ponds and a harvesting station were constructed. The team took further advantage of the design and construction of these facilities as a training opportunity by having over 30 staff, researchers, students and volunteers assist by digging the trenches, laying the plumbing, building the walls, installing the liner, and designing the airlifts and automated systems.

The two 8,000-L raceways allow Cal-CAB to grow algae at a large scale, similar to commercial systems. The raceways use forced-air lifters to create a current in order to mix and propel the aquaculture in a safe manner. Bubblers at each end automatically dissolve CO$_2$ into the raceway to regulate pH and growth (Figure 13 and Photo Credit: Cal-CAB, 2014).

Figure 13: 8,000-L Air Mixed Raceway With *Nannochloropsis* Culture

Photo Credit: Cal-CAB, 2014.
Figure 14: PVC Array for Mixing

Photo Credit: Cal-CAB, 2014.

Figure 15: Harvest Slab Set Up, Fixed With Pump and Plumbed to Raceway for Easy Harvest

Photo Credit: Cal-CAB, 2014.
The harvesting station (Photo Credit: Cal-CAB, 2014. Figure 15) was designed to enable researchers and technicians to efficiently harvest large volumes from the raceways and the outdoor stock ponds. Large 900-L conical tanks allow for an initial settling step, which reduces the volume that needs to be centrifuged. Base flocculation methods described below are also used for small cell size strains such as *Nannochloropsis*. Underground plumbing allows for easy transfer of liquid from raceways and ponds to the tanks, and effluent is eventually transferred to an evaporation pond.

The systems constructed are designed with training and education in mind, as they are relatively inexpensive to build and operate. Information on the design, materials and productivity of the pilot scale facilities has been published to make this information available to other institutions and researchers (Schoepp et al. 2014).

2.7.2 Large Scale Harvests using chemical flocculation

The process of the dewatering microalgal biomass is very energy intensive and time consuming due to the minute size of microalgal cells varying from 5-50µm and the limited maximal density of a healthy culture due to photo inhibition. Flocculation proves to be a novel method to concentrate biomass in a dense suspension prior to centrifugation to reduce energy and time costs for large-scale cultivation. Different methods of flocculation involve ultrafiltration, which uses a polyvinylchloride ultrafiltration membrane and constant flow and pressure of harvest media to concentrate microalgal cells (Zhang et al, 2010). Micro flotation uses microbubble generation by a fluidic oscillator to aggregate cells based on the idea that hydrophobic particles stick to buoyant micro bubbles (Hanotu et al, 2011). Electro-coagulation-flocculation involves the uses a small voltage generated by nickel electrodes to concentrate microalgal cells (Shuman et al, 2014) and chemical coagulation using ammonia (Chen et al, 2012), multivalent cations (Wu et al, 2012) and acid (Liu et al, 1998). A paper is currently in preparation that will explore methods of chemical coagulation for large-scale harvests of 8,000L of varying microalgal production strains, including wild type *Nannochloropisis salina*, using chemical coagulation through pH increase.
Nannochloropsis salina (Ns) was grown, scaled up and monitored according to the methods presented in Schoepp et al, 2014 paper. For flocculation of Ns by pH increase, a 1M NaOH solution was made using DI and Fisher Scientific hydroscopic sodium hydroxide pellets. Six 250-mL aliquots of Ns in supplemented natural seawater (SNSW) were dosed with the 1M sodium hydroxide to pH values ranging from 9.0-11, increasing in increments of 0.5 pH units. In addition to the six experimental tests, a control test was prepared with 500mL of N.Sali/SNSW. Optical density readings were recorded using a Bausch and Lomb Spectronic 20 spectrometer set at 750nm using 13 X 100mm glass test tubes as cuvettes. The appropriate pH was achieved through distributing aliquots of 1M NaOH solution using a P1000 micropipette until desired pH was reached. pH measurements were conducted by Milwaukee SMS120 pH meter. Upon dosing with NaOH, the samples were thoroughly mixed, capped and left to sit for approximately 1 hour at room temperature. Optical densities at 750nm of tests and control were assessed every hour over a six-hour time period. Readings were taken at approximately middle of effluent layer. Pictures were taken to make qualitative assessments of the efficiency of flocculation at each pH increment with respect to the control (Figure 16).

The mechanism of flocculation is a function of pH increases, which was conducted using sodium hydroxide as a coagulant. Due to the pH increase of the effluent, multivalent cations, particularly magnesium and calcium, precipitate out of solution with hydroxide ions. A plausible mechanism of flocculation would be that the magnesium and calcium intrinsically mask the negative charge of the microalgal cells (Wu et al, 2012), allowing them to aggregate. When grown in artificial seawater, Nannochloropsis salina has a high magnesium content of 2312ppm. Therefore, as seen in Figure 16, the higher pHs (9.0-11.0) flocculated significantly,
resulting in a milky-green flocculated layer composed of algal cells and magnesium hydroxide precipitate. In order to figure out an optimal dose, both qualitative and quantitative assessments were made. At too high of a pH, the amount of magnesium hydroxide was significant, masking almost all algal cells but creating a large flocculated layer. Whereas, at too low of a pH, the amount of precipitate formed was not enough to mask algal cells and cause them to flocculate. In order to assess the magnesium concentration of the effluent layer, flame atomic absorption spectrometry (FAAS) was used in order to determine how much magnesium is present in the flocculated layer. The results from the FAAS indicate that the magnesium was in higher flocculated layers and increased with increasing pH, suggesting possible involvement in the mechanism of flocculation and allowing a determination for optimal dosing at pH 10 due to the flocculation efficiency of 89% and magnesium content utilized.

For 8000-L harvests, a Nannocloropsis salina culture in natural supplemented seawater was scaled up and grown in an open raceway. The pH was controlled with an Arduino automation system, which is described in the Schoepp et al. 2014 paper. Once an optical density of 1.000AU measured at 750nm was achieved, 3600L were harvested into four 250-gal conical tanks (Photo Credit: Cal-CAB, 2014. Figure 15). Each tank was dosed with 875g of sodium hydroxide pellets dissolved in 2 gallons of DI water. Upon dosing with concentrated base, the tanks were thoroughly mixed using a submersible pump. The tanks were left to sit for 4 to 6 hours in order to flocculate. The concentrated biomass was separated from effluent layer by opening a valve fixed to the bottom of container and gravity draining it into a 300L black tub. Once concentrated biomass was collected from all tanks into the black tub, the contents were centrifuged continuously using a peristaltic pump over a four to five hour time period, approximately 8-10kg of biomass was collected per harvest. This methodology was employed continuously over 53 days, harvesting approximately every 7 to 10 days.

Using this method of chemical flocculation for 3600-L continuous harvests of Ns to concentrate biomass prior to centrifugation decreased time and energy costs by approximately half with an overall efficiency of 89%, which was calculated from the flocculation efficiency over a 6-hour period.

2.7.3 Continuous Solvent Extraction

Extraction of lipids and hydrophobic metabolites from microbial sources remains an obstacle in the production of these compounds at the laboratory and industrial scale. Analytical techniques for the total extraction of non-polar metabolites from biological material are well established, but rely exclusively on expensive and time consuming processes. This makes these techniques unsuitable for direct translation to continuous or large volume systems, unable to move beyond proof-of-concept studies, and leaves a major gap in the translation of new bio-products requiring a purified extract (Table 4). The ideal biomass extraction system must be able to handle a wet feedstock, be amenable to process enlargement and automation, require minimal pretreatment of the biomass, and produce a crude extract that has not been significantly affected by the extraction process. Continuous solvent extraction can meet all of these requirements. We worked to bridge these gaps by demonstrating the use of a continuous liquid-
liquid extraction system capable of bulk lipid extraction from wet, untreated biomass, and simultaneous concentration of the unmodified extract in a “lipid trap”.

CILMSF researchers have designed a straightforward, scalable, continuous liquid-liquid extraction system, and demonstrated its effectiveness in generating an unmodified crude hydrophobic extract from a range of biomass slurries with no pretreatment, with the hope that this system will serve as a unit for processing a variety of natural metabolites regardless of the host organism. The described system utilizes readily available materials, equipment, and solvents, and can be scaled by orders of magnitude without changing the fundamentals or efficacy of the system. A 1.8-L version of the system was used to evaluate system dynamics with bacterial (Rhodococcus opacus PD630), fungal (Saccharomyces cerevisiae), algal (Scenedesmus dimorphus UTEX 1237), and plant feedstock (Glycine max), prior to scaling the system by an order of magnitude to demonstrate large-scale viability and a 100-fold increase in capacity over standard techniques (Table 4). Dry weight, lipid content, and lipid trap quantifications for each extraction experiment were measured gravimetrically, in quadruplicate, using an analytical balance readable to 0.1 mg. Extraction experiments were performed in triplicate. All solvents were reagent grade.

Results from this research have been submitted for publication and are currently in review (see Outputs section below). Overall, extraction efficiency was above 90% for each feedstock. Following scale-up, extraction was performed on upwards of 4 kg of slurry (660 g dry weight), yielding an average efficiency of 96%, and allowing generation of a crude extract at a scale not previously possible. The resulting system allows for direct and high-throughput extraction of biomass sources without pretreatment, specialized instrumentation, or intensive user input.

### 2.7.4 Outputs

This task has resulted in the following publications to date, as well as several oral and poster presentations (see Workforce Development):


<table>
<thead>
<tr>
<th>Method</th>
<th>Organism</th>
<th>Scal e (g)</th>
<th>Pretreatm ent</th>
<th>Preservation of Lipids</th>
<th>Possible in Lab Setting</th>
<th>Demonstrated Scalability</th>
<th>Reference</th>
</tr>
</thead>
</table>

Source: Cal-CAB
The extraction vessel (left) is initially charged with 500 mL of biomass slurry, 500 mL of hexane, and 650 mL of isopropanol as the transfer solvent, while the lipid trap is charged with 500 mL hexane (right) (1). The extraction vessel is heated to 45˚C to increase the rate of extraction, while the lipid trap is heated to reflux. Upon heating, extraction begins, and the condensed solvent from the trap causes the organic phase of the extraction vessel to overflow, carrying with it extracted lipid (2). As extraction continues, lipids become concentrated in the trap while extraction continues (3). Upon completion, the delipidated slurry remains in the extraction vessel, with concentration of the lipid fraction in the trap (4).

Source: Cal-CAB

2.8 Task 9.0 – Co-Products

PI: Mayfield

Vaccine antigens and therapeutic proteins are potentially high impact and/or high value co-products that can be produced in conjunction with biofuels. Infectious and parasitic diseases killed more than 1.7 million children and adolescents in low-income countries in 2008, which represents nearly half of all deaths in that age bracket. Much of the inequality in malarial treatment is the result of disparate vaccine coverage in the developing world, mainly brought about by the high cost and logistical difficulty of large-scale vaccination campaigns in countries with underdeveloped health infrastructures. Heat-stable oral vaccines could overcome the largest obstacles that deter widespread vaccination in low-income countries. Thermostability
would eliminate the need for cold-chain storage, and oral delivery would be safer, simpler, and cheaper than injectable vaccines.

A major challenge for oral vaccines is overcoming antigen degradation by commensal bacteria, proteases, and the acidic stomach environment in order to safely deliver intact antigens and mucosal adjuvants to the gut-associated lymphoid tissue. Algae delivery systems are particularly attractive for this purpose for two reasons. First, several algae species are generally regarded as safe (GRAS) for human consumption. Unlike conventional organisms used for recombinant protein production, GRAS organisms pose little risk of harmful viral, prion, toxin, or bacterial contaminants. Second, algae cells have a rigid cell wall that can protect encapsulated vaccine antigens, allowing ambient temperature storage. The cell wall can also provide a layer of protection from proteolysis in the gastrointestinal tract. Finally, previous studies have shown that \textit{C. reinhardtii} can be rapidly transformed into stable transgenic strains and grown in enclosed bioreactors or outdoor ponds. \textit{C. reinhardtii} can also produce complex proteins such as antibodies, human therapeutics, and immunotoxins.

\subsection*{2.8.1 Highlights}

Over the course of this study, the Mayfield lab has constructed \textit{Chlamydomonas reinhardtii} strains that produce Pfs25, a malaria transmission blocking vaccine candidate (Gregory et al., 2013). The Pfs25 vaccine antigen was genetically fused to a mucosal adjuvant protein, which facilitates an immune response through the gut mucosa. The Pfs25 vaccine antigen was shown to be stable in freeze-dried algae stored at room temperature for over 5 months (Figure 18). Feeding mice freeze-dried algae was ineffective at generating the prerequisite Pfs25-specific antibodies to block malaria transmission. However, it was able to stimulate production of antigens to the cholera toxin B subunit (CtxB), which was used as the mucosal adjuvant. Thus, it could be used to protect against pathogens like Salmonella or Clostridium that invade mucosal surfaces. This work has shown that algae are effective at producing thermally stable vaccine antigens. Future work will now focus on a better understanding of mucosal immune responses. If successful, this work on whole-cell oral vaccines from algae would greatly simplify vaccine delivery and eliminate costly purification steps, both of which would enable affordable vaccination in poor countries.
Figure 18: Stability of alga-produced CtxB-Pfs25 in lyophilized algae

Soluble protein extracts from JAG101 (both pre- and postlyophilization) and ΔpsbA C. reinhardtii strains were serially diluted and applied to GM1 ganglioside-coated ELISA plates in triplicate. Binding was detected with anti-CT antibodies. B) Lyophilized JAG101 C. reinhardtii was stored at 4°C, 22°C, and 37°C; lyophilized ΔpsbA C. reinhardtii was stored at 4°C. Soluble protein extracts were prepared on the indicated days, serially diluted, and applied to GM1 ganglioside-coated ELISA plates in triplicate. Binding was detected with anti-CT Abs. Error bars shown are 1 standard deviation. Binding endpoints were defined as the last dilution with an absorbance greater than that of the ΔpsbA strain at 10 µg/ml plus 3 standard deviations.

Source: Cal-CAB

2.8.1.1 Outputs


2.9 Task 10.0 – Water and Nutrient Recycling *(Cost-share with DOE funding)*

*Pis: J Golden, S Golden*
The goal of this project is to engineer a nitrogen-fixing cyanobacterium, *Anabaena* PCC 7120, to supply a nitrogen-rich compound to designated recipient cells that are engineered to utilize it, while not feeding non-target species. CILMSF researchers chose octopine as the nitrogen source, which contains four amine functional groups that can be reduced to ammonium. Octopine is an amino acid derivative formed by the condensation of arginine and pyruvate. Its biosynthesis and metabolism have been well studied in the soil bacterium, *Agrobacterium tumefaciens*. In nature, octopine-type *A. tumefaciens* have the genes for synthesizing and utilizing octopine as a carbon and nitrogen source. Octopine synthesis requires the *ocs* gene, which is found in the T-DNA region of the native tumor inducing (Ti) plasmid of octopine-type *A. tumefaciens*. *A. tumefaciens* genetically engineers infected plant tissue by transfer of the T-DNA, where the *ocs* gene is expressed and plant cells produce octopine. The *occ* operon and *ooxAB* genes are needed for octopine uptake and catabolism respectively. These genes are found in the *occ* catabolic region of the Ti-plasmid, and allow *A. tumefaciens* cells in the vicinity of infected plants to utilize octopine. To demonstrate that bioengineered cyanobacteria can supply nitrogen-rich molecules for their co-cultured neighbors, Researchers worked to develop an octopine donor and recipient pair of cyanobacteria, in which the *ocs* gene was cloned into *Anabaena* sp. PCC 7120, while the *occ* operon and *ooxAB* genes were designed to be cloned into *Synechococcus* 54longates PCC 7942. The goal was to engineer *Anabaena* (octopine donor) to synthesize octopine and change *S. 54longates* (octopine recipient) so that it can express the necessary utilization genes to take up and metabolize octopine and use it as a nutrient source for growth.

### 2.9.1 Highlights

*Anabaena* PCC 7120 has been engineered to express the *Agrobacterium tumefaciens* octopine synthase gene (*ocs*) and produces octopine. This was demonstrated by use of a bioassay for octopine using *Agrobacterium tumefaciens* octopine reporter strains that express GFP or B-glucuronidase in the presence of octopine. A potential octopine-recipient strain, *Synechococcus* PCC 7942, was engineered to express the octopine import (*occQMPJ*) genes along with an endogenous reporter of octopine to indicate whether the compound is taken up by the target cells. Although the approach is valid and worth pursuing, several technical difficulties were encountered, and the more rapid progress in other tasks led the team to concentrate efforts elsewhere.

#### 2.9.1.1 Outputs

This DOE Cost-share task has resulted in 1 patent application and two presentations to date.

A provisional patent application based on this project and titled “TARGETED DELIVERY OF NUTRIENTS TO RECIPIENT ORGANISMS” was submitted through UC San Diego.


CHAPTER 3: 
Workforce Development

Cal-CAB has a strong history of contributing to workforce development for the biofuels and coproducts sectors, from providing hands-on training to graduate and undergraduate students in research laboratories to developing and running formal courses and certificates, including the EDGE Biofuels Certificate Program at UC San Diego Extension. Although no financial support was provided through this initiative, the CILMSF leveraged the groundwork and training culture at Cal-CAB to provide not only formal training and work experience opportunities, but also new industry-relevant research and training facilities. During the grant period, students were able to participate in the construction of these facilities, which will now serve as a training ground for many students in the future.

3.1 Formal Education

Biofuels-related education opportunities through UC San Diego, SIO and UC San Diego Extension include undergraduate courses, undergraduate volunteer and student employee positions in Cal-CAB labs, graduate research positions in Cal-CAB labs, and EDGE Biofuels Certificates at UC San Diego Extension. The EDGE (Educating and Developing Workers for the Green Economy) Biofuels Certificate Program was developed under a California Department of Labor grant to create a training program for the new biofuels industry, and is now run on a tuition basis through UC San Diego Extension.

In total, more than 640 UC San Diego undergraduates have taken biofuels related courses at UC San Diego during this period. During this time, 175 EDGE students have enrolled in an EDGE Biofuels Certificate with 135 certificates completed, and a number of students enrolling in individual EDGE courses without applying for the certificate. A full list of biofuels related courses developed by Cal-CAB faculty, staff and graduate students is listed in Table 5.

<table>
<thead>
<tr>
<th>UC San Diego Courses</th>
<th>UC San Diego Extension Courses - EDGE Biofuels Certificate Program</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction to Biofuels (BIBC 140)</td>
<td>Introduction to Biofuels (BIOL-40261)</td>
</tr>
<tr>
<td>Advanced Topics in Modern Biology – Bioenergy (BIMM 194)</td>
<td>Downstream Processing and Refinement of Biofuels (BIOL-40268)</td>
</tr>
<tr>
<td>Organic Chemistry II – Biofuels (CHEM 145)</td>
<td>Biomass Production (BIOL-40278)</td>
</tr>
<tr>
<td></td>
<td>Biomass Production Lab (BIOL-40291)</td>
</tr>
<tr>
<td></td>
<td>Genetics and Physiology of Photosynthetic Microorganisms (BIOL-40262)</td>
</tr>
<tr>
<td></td>
<td>Photosynthetic Microorganisms Molecular Biology Laboratory (BIOL-40265)</td>
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<tr>
<td></td>
<td>Aquatic Ecology (BIOL-40264)</td>
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<td></td>
<td>Aquatic Microbiology Laboratory (BIOL-40266)</td>
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<tr>
<td></td>
<td>Chemistry and Biochemistry of Biofuels (BIOL-40263)</td>
</tr>
<tr>
<td></td>
<td>Analytical Chemistry Laboratory (BIOL-40267)</td>
</tr>
</tbody>
</table>

Source: Cal-CAB
3.2 Public Outreach

Cal-CAB and CILMSF faculty and staff have also been engaged in a number of successful and high profile public outreach initiatives around biofuels during this time, including the CILMSF Roadmap Meeting, annual Food & Fuel for the 21st Century Symposia, Cal-CAB Student and Postdoc Symposia, the Our Energy Future Public Lecture Series, the development of two Massive Open Online Courses (MOOCs), invited public lectures, and high school outreach and summer training programs.

3.2.1 Our Energy Future

The Our Energy Future Public Lecture Series was held in October 2013 at UC San Diego to connect with University as well as local San Diego communities, bring awareness to energy issues, and showcase local research and solutions, including biofuels. This lecture series was attended by over 100 people on each of its four nights, and is now available on YouTube. It also forms the core of both Our Energy Future MOOCs produced by the PI, Stephen Mayfield and supported by a gift from Google.

These highly successful MOOCs (Table 6) were aimed at introducing not only students, but also the general public and global community, to the issues of energy in the 21st century – including food and fuels – which are inseparably linked. It discusses energy production and utilization from the biology, engineering, economics, climate science, and social science perspectives. The MOOC on Google CourseBuilder is a shorter, more general overview for those without a strong science background. The full length Coursera MOOC is an in-depth look at current production and utilization of energy, as well as the consequences of this use, examining finite fossil energy reserves, how food and energy are linked, impacts on the environment and climate, and the social and economic impacts of present energy and food production and use. Lectures are prepared and delivered by over 30 leading UC San Diego and SIO faculty, as well as industry professionals.
Table 6: Enrollment and Reach of Our Energy Future MOOCs

<table>
<thead>
<tr>
<th>Our Energy Future course</th>
<th>Enrollments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Google CourseBuilder</strong></td>
<td><a href="https://ourenergyfuture-ucsd.appspot.com/001">https://ourenergyfuture-ucsd.appspot.com/001</a></td>
</tr>
<tr>
<td>Winter 2014 – Pilot</td>
<td>130 total students registered</td>
</tr>
<tr>
<td></td>
<td>10 completions with a passing grade</td>
</tr>
<tr>
<td>Continuous enrollment</td>
<td>37 total students registered as of Jan. 2015</td>
</tr>
<tr>
<td></td>
<td>4 completions with a passing grade as of Jan. 2015</td>
</tr>
<tr>
<td><strong>Coursera</strong></td>
<td><a href="https://www.coursera.org/course/ourenergyfuture">https://www.coursera.org/course/ourenergyfuture</a></td>
</tr>
<tr>
<td>Summer 2014</td>
<td>15,968 total students registered</td>
</tr>
<tr>
<td></td>
<td>8,755 active learners who have watched lectures</td>
</tr>
<tr>
<td></td>
<td>663 statements of completion earned</td>
</tr>
<tr>
<td>Fall 2014</td>
<td>11,554 total students registered</td>
</tr>
<tr>
<td></td>
<td>5,536 active learners who have watched lectures</td>
</tr>
<tr>
<td></td>
<td>408 statements of completion earned</td>
</tr>
</tbody>
</table>

Source: Cal-CAB

### 3.2.2 Training for Future Teachers

As it can be difficult to find teaching opportunities for young scientists, Cal-CAB places great importance on creating teaching and mentorship opportunities for students and postdoctoral scholars as part of their training. There are opportunities to mentor undergraduate volunteers in each CILMSF lab, as well as instructor and teaching assistant opportunities in UC San Diego and UC San Diego Extension biofuels-related courses. During this grant, more than 11 undergraduates, 10 graduate students and 8 postdoctoral scholars took on instructor and teaching assistant roles in 1 UC San Diego course, 7 UC San Diego Extension courses and 1 MOOC.

### 3.2.3 Symposia

Cal-CAB helped to organize an annual public research symposium, Food & Fuel for the 21st Century Symposium, to foster collaboration between faculty, as well as bring together academic and industry researchers and facilitate the transfer of research and technology from the lab bench to the field. These symposia attended by between 150 and 250 people annually. They are also aimed at helping to inform policy makers at the local, state and federal level. CILMSF researchers have presented their research at these symposia each year. These symposia provide students and young researchers the opportunity to present their research in talks or poster presentations (see Student Outputs section below).

To provide additional opportunity for students and postdoctoral scholars to practice presenting their research and identify potential collaborations, Cal-CAB hosts a monthly Student and Postdoc Seminar series at UC San Diego and SIO. This series attracts 30-60 attendees each.
month. CILMSF research has been presented extensively by students and postdoctoral scholars at these seminars and is listed in the Student Outputs section below. These seminars are also open to the public and provide an excellent networking opportunity for young researchers and alumni.

3.2.4 High School Outreach

Outreach to high school students is not only key to attracting talented new students to study biofuels and algae biotechnology for the future workforce, but also to help educate students and parents about energy and fuel production and use, so they are able to make informed decisions in their own lives. High school tours are hosted at the Cal-CAB facilities at the Biology Field Station throughout the year. Cal-CAB students and staff often talk at local high school and middle school classrooms. More formal training is also provided to local underrepresented minorities through the California State Summer School for Mathematics and Science (COSMOS) program and the Institute of the Americas summer program, where small groups of talented high school students are able to do a two-week workshop where they learn to grow algae at scale and make biodiesel.

Cal-CAB also regularly assists high school teachers and students with information and advice for biofuels science projects. The Gerwick Lab created an AP Environmental Science lesson plan on Biomass Energy and Algal Biofuels, which is publically available for use (http://earthref.org/SCC/lessons/2010/biomass/). Two publications have also been made available to provide instruction on how to set up inexpensive algal growth facilities for education and on how to convert algal biomass to biofuel.


3.3 Hands-on Experience

Cal-CAB provides numerous opportunities for undergraduate students and EDGE alumni to get hands-on experience in research labs, including volunteer positions, lab tech positions, summer internships and volunteer Cal-CAB-wide working days. Despite the Energy Commission contract not allowing for payment of undergraduate employees, nearly all CILMSF labs hosted at least three student researchers each year, with some labs mentoring as many as 15 volunteers at a time (Table 7). A more intensive hands-on training initiative is the Cal-CAB Summer BioEnergy Research Program, which enables talented undergraduates to spend 10 weeks conducting research in a lab, including many CILMSF labs. Interns also get the opportunity to tour industry sites and present their research findings at a symposium. During this grant period, 36 undergraduates participated in the Summer BioEnergy Research Program. Working days at the Cal-CAB Algae Research & Development Facility at the UC San Diego Biology Field Station also provide unique hands-on training. For the CILMSF, more than 30 students, staff and volunteers from a range of disciplines came together during designated
working days to help build the Energy Commission-funded algae raceways and harvesting system, providing a rare training opportunity to learn about the engineering, construction and maintenance of industry-like production facilities.

**Table 7: A Total of 84 Students and EDGE Alumni Received Hands-on Training Through CILMSF Research from 2011-2014**

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Student Researchers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jacobsen Lab</td>
<td>Benjamin Miller, Graduate Student</td>
</tr>
<tr>
<td></td>
<td>William Leung, Graduate Student</td>
</tr>
<tr>
<td></td>
<td>Narayan Gopinathan, Undergraduate</td>
</tr>
<tr>
<td>Allen Lab</td>
<td>Ben Auch, Graduate Student</td>
</tr>
<tr>
<td></td>
<td>Jillian Freese, Undergraduate</td>
</tr>
<tr>
<td></td>
<td>Marissa Quijano, Undergraduate</td>
</tr>
<tr>
<td>Burkart Lab</td>
<td>Jillian L. Blatti, Graduate Student</td>
</tr>
<tr>
<td></td>
<td>Jennifer Michaud, Graduate Student</td>
</tr>
<tr>
<td></td>
<td>Yuan Pu, Masters Student</td>
</tr>
<tr>
<td>J. Golden Lab</td>
<td>Federico Unglaub, Graduate Student</td>
</tr>
<tr>
<td></td>
<td>Ben Auch, Graduate Student</td>
</tr>
<tr>
<td></td>
<td>Eammon Riley, Graduate Student</td>
</tr>
<tr>
<td></td>
<td>Katrina Nguyen, Graduate Student</td>
</tr>
<tr>
<td></td>
<td>Wei Yue Zeng, Graduate Student</td>
</tr>
<tr>
<td></td>
<td>Linda Truong, Masters Student</td>
</tr>
<tr>
<td></td>
<td>Calvin Schmidt, Undergraduate</td>
</tr>
<tr>
<td></td>
<td>Lindsey Pieper, Undergraduate</td>
</tr>
<tr>
<td></td>
<td>Michaela Go, Undergraduate</td>
</tr>
<tr>
<td>S. Golden Lab</td>
<td>Anthony Daulo, Masters Student</td>
</tr>
<tr>
<td></td>
<td>Ben Auch, Graduate Student</td>
</tr>
<tr>
<td></td>
<td>Ben Rubin, Graduate Student</td>
</tr>
<tr>
<td></td>
<td>Don Nguyen, Graduate Student</td>
</tr>
<tr>
<td></td>
<td>Stacey Huynh, Undergraduate</td>
</tr>
<tr>
<td></td>
<td>Karen Tang, Undergraduate</td>
</tr>
<tr>
<td>Gerwick Lab</td>
<td>R. Cameron Coates, Graduate Student</td>
</tr>
<tr>
<td></td>
<td>Emily Trentecoste, Graduate Student</td>
</tr>
<tr>
<td></td>
<td>Jonathan Tram, Graduate Student</td>
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<tr>
<td>Hildebrand Lab</td>
<td>Sarah Smith, Graduate Student</td>
</tr>
<tr>
<td></td>
<td>Raffaela Abbriono, Graduate Student</td>
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<td></td>
<td>Emily Trentecoste, Graduate Student</td>
</tr>
<tr>
<td>Mayfield Lab</td>
<td>Elizabeth Specht, Graduate Student</td>
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<tr>
<td></td>
<td>Daniel Barrera, Graduate Student</td>
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<tr>
<td></td>
<td>Javier Gimpel, Graduate Student</td>
</tr>
<tr>
<td></td>
<td>Miller Tran, Graduate Student</td>
</tr>
<tr>
<td></td>
<td>Nathan Schoepp, Masters Student</td>
</tr>
<tr>
<td></td>
<td>Christina Van, Undergraduate</td>
</tr>
</tbody>
</table>

|                      | Nathan Schoepp, Masters Student                                                   |
|                      | Wilson Wong, Masters Student                                                      |
|                      | Michael Lay, Undergraduate                                                        |
|                      | Nicholas Ridout, Undergraduate                                                    |
|                      | Edward King, Undergraduate                                                        |
|                      | Tyler Swinney, Undergraduate                                                      |
|                      | Ron Cook, Undergraduate                                                           |
|                      | Kanav Jain, Undergraduate                                                          |
|                      | Ross London, High School Student                                                  |
|                      | Matthew Paddock, High School Student                                              |
|                      | Joshua Kenchel, Undergraduate & EDGE Alumni                                      |
|                      | Mallika Malleswaran, Undergraduate                                                |
|                      | Keval Desai, Undergraduate                                                         |
|                      | Emily Effner, EDGE                                                               |
|                      | Nicholas Bearmar, EDGE                                                           |
|                      | Minh Khuc, Undergraduate                                                          |
|                      | Rujing Shi, Undergraduate                                                         |
|                      | Mike Wolinsky, EDGE                                                             |
|                      | Syh-shiuan Chao, Undergraduate                                                    |
|                      | Matthew Krause, Undergraduate                                                    |
|                      | David Doerner, Undergraduate                                                      |
|                      | David Carruthers, Undergraduate                                                   |
|                      | Teo Pier, Undergraduate                                                          |
|                      | Bryn Taylor, Undergraduate                                                       |
### Laboratory | Student Researchers
---|---
Karen Wang, Undergraduate | Melissa Keller, Undergraduate
Josephine Huang, Undergraduate | Lydia Tusakul, Undergraduate
Joanna Chiu, Undergraduate | Vyvy Tran, Undergraduate
Ryan Setten, Undergraduate | Daphne Li, Undergraduate
Greg NewKirk, Undergraduate | Edward Lin, Undergraduate
Aaron Topol, Undergraduate | Prema Karunanithi, Undergraduate
Chesa Cox, Undergraduate | Rong Sang, Undergraduate
Lauren Tomosada, Undergraduate | Rosalie Ellis, Undergraduate
Kevin Hoang, Undergraduate | Jody Tu, Undergraduate
Tiffany Luong, Undergraduate | Kevin Fong, Undergraduate
Austin Hallgren, Undergraduate | Lexandra Quigley, Undergraduate, EDGE
Amy Hoang, Undergraduate | Vincent Sun, Undergraduate, EDGE
Sara Pennebaker, Undergraduate | Ryan Stewart, EDGE
Emily Fu, Undergraduate | Chris Mahn, EDGE
Nikki Bose, Undergraduate | Riley Peterson, High School Student
Cara Sun, Undergraduate | Mitchell Hooper, High School Student
Susan Park, Undergraduate |  

Source: Cal-CAB

### 3.4 Student Outputs

As part of their training, undergraduate and graduate students were given the opportunity to assist with and take the lead on a number of CILMSF outputs, including publications, presentations, posters and patents. These students, as well as postdoctoral scholars, were also given the opportunity to not only train and mentor others, but to teach for the EDGE Biofuels Certificate program at UC San Diego Extension.
CHAPTER 4: Conclusions

The goal of the California Initiative for Large Molecule Sustainable Fuels was to advance the science and technology development of fungible alternative transportation fuels in order to address California’s energy challenges. In doing this, the CILMSF also set out to support drop-in fuel demonstrations; spur California’s bioenergy industry growth; and inform policy makers, industry professionals, and consumers with regard to the importance of commercially viable biofuel alternatives to meet the State’s liquid transportation fuel demand.

Over the span of three years, the CILMSF’s researchers and students have generated more than 30 published articles and patents, with a number of others currently under review or awaiting publication. Advancements were made in all areas of the project, ranging from metabolic engineering of algae for altered fatty acid accumulation for fuel to crop protection and coproduct production – all important foci critical to helping advanced alternative transportation fuels become economically viable. These improvements demonstrate the rapid progress being made toward environmentally and economically sustainable drop-in fuels produced from algae, and represent research accomplishments of which the Energy Commission can be proud.

Advancements in synthetic biology have been instrumental in designing algal and cyanobacterial bio-factories for the creation of biofuels, and high value bio-products. For example, the work done in Task 4.0 in developing high throughput screening to understand and design a better untranslated region to drive gene expression of non-native proteins in green algal chloroplasts shows significant potential for mass production of industrial molecules. Additionally, work done in Task 5.0 in creating genetic tools for green algae, diatoms, and cyanobacteria has made the genetic engineering process easier, faster, and more efficient.

With the advanced genetic tools and methods created in Task 5.0, significant progress was made in identifying and modifying: metabolic pathways to create biofuels; advantageous phenotypes for protection against pests and pathogens like fungi and amoebae; and high value products. Task 6.0 researchers found promising results with metabolic engineering for the production of polyunsaturated fatty acids (PUFA) for the production of omega-3 fatty acid nutraceuticals, EPA and DHA, and the biodiesel precursor, triacylglycerol (TAG).

Additionally, Cal-CAB is also proud of the biofuel industry workforce training opportunities it has developed on behalf of the State. According to a 2014 analysis, conducted by the San Diego County of Governments’ (SANDAG) economic bureau, direct employment in the algae biotechnology industry in the San Diego region has nearly doubled since 2009, with direct, indirect, and induced economic impact of the algae biotechnology research and manufacturing industry currently generating a total of approximately 1,020 total jobs, $80 million in wages, and over $175 million of economic output to the San Diego regional economy. During the grant period, Cal-CAB has provided workforce development training and research experience for 84 students and postdoctoral researchers working on CILMSF projects, as well as biofuels courses for more than 700 UC San Diego and UC San Diego Extension students, and 15,000 MOOC
students world-wide. The CILMSF is also proud of the student- and staff-built raceway ponds and harvesting station, which provided a unique learning opportunity in the design and production of algal production facilities, and gives Cal-CAB the capacity to continue and expand its scaled up outdoor growth research. The project team can confidently say that through the CILMSF, the science, technology, public information, and trained labor force have come closer to the realization of commercialized advanced algal biofuels in California.

4.1 CILMSF Opportunities Moving Forward

On January 19, 2007, California enacted the Low Carbon Fuel Standard (LCFS). The LCFS is a key element of the State’s plan to implement AB32, the California Global Warming Solutions Act, and requires that the mix of transportation fuels sold in California be at least 10 percent lower in carbon intensity by 2020. This is a reduction of CO$_2$ equal to 1.4 billion gallons of gasoline equivalent per year, requiring that about 3 billion gallons of low carbon fuel be produced and used annually.

Over the eight years that these targets have been in place, California has produced a total of about one hundred million gallons of low-carbon transportation fuels, mainly from green waste and recycled cooking oils – enough to supply about one week’s requirements of low-carbon fuels under the LCFS. To achieve the mandates of the LCFS by 2020 – only five years away – the deployment of low-carbon fuels will need to increase 100 times over what it is today, and this increase cannot be achieved using recycled waste feedstocks, as those resources are limited and already heavily utilized. Over the past three years, California has invested approximately $90 million dollars to establish feedstocks, including waste oils and green waste, which cannot scale to meet the volume of fuel required. California has not been as aggressive in investing in scalable feedstocks such as algae (Table 8, Figure 19). Since the start of the CILSMF grant, only one new algae biofuels project has been funded by the Energy Commission. As that project returned the funding prior to starting, there has effectively been no new Energy Commission funding for algae biofuels research since 2011.
## Table 8: Comparison of Recent California Energy Commission Feedstock Investment Through the Alternative and Renewable Fuel and Vehicle Technology Program

<table>
<thead>
<tr>
<th>Alternative and Renewable Fuel and Vehicle Technology Program Grant Solicitation</th>
<th>Waste Oil</th>
<th>Green Waste</th>
<th>Other</th>
<th>Algae</th>
</tr>
</thead>
<tbody>
<tr>
<td># Prop. Awards</td>
<td># Prop. Awards</td>
<td># Prop. Awards</td>
<td># Prop. Awards</td>
<td># Prop. Awards</td>
</tr>
<tr>
<td>Grant Solicitation PON-11-601 Biofuels Production Facilities (2012)</td>
<td>4</td>
<td>$ 5,772,187</td>
<td>6</td>
<td>$ 22,999,064</td>
</tr>
<tr>
<td>Grant Solicitation PON-13-601 Commercial Scale Advanced Biofuels Production (2013)</td>
<td>2</td>
<td>$ 9,904,375</td>
<td>0</td>
<td>$ -</td>
</tr>
<tr>
<td>Grant Solicitation PON-13-609 Pilot-Scale and Commercial-Scale Advanced Biofuels Production Facilities (2014)</td>
<td>3</td>
<td>$ 9,183,421</td>
<td>4</td>
<td>$ 15,450,000</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>$ 24,859,983</td>
<td>10</td>
<td>$ 38,449,064</td>
</tr>
</tbody>
</table>

* This project withdrew and forfeited funding before starting; Three other algae proposals passed review but were not funded.


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**Figure 19: Breakdown of New California Energy Commission Funding by Feedstock Through the Alternative and Renewable Vehicle Technology Program from 2012-2014**

- **Waste Oil**: 27%
- **Green Waste**: 42%
- **Other**: 30%
- **Algae**: 1%*

* This project withdrew and forfeited funding before starting; three other algae proposals passed review but were not funded.

If California is to meet its obligations under AB32 and the LCFS, additional new low-carbon feedstocks must be developed and deployed here in California, and these feedstocks must have the potential to scale to the significant volumes mandated by law. There are a number of ways to stimulate this development, and several of those are outlined below.

Developing dependable, economically competitive, and environmentally sustainable low-carbon fuels is essential to continued economic growth and responsible stewardship of the environment. Beyond reduction of greenhouse gases (GHG), developing renewable energy sources in California has the potential to drive unprecedented economic growth, and the regions that develop and deploy these technologies are expected to have significant jobs creation. Today, third generation biofuels with greatly improved GHG emission reduction are now under development, with hydrocarbons from algae seen as an area of research with high potential for success. In large part due to the efforts over the past 5 years by the CILMSF and its industry partners, San Diego has emerged as the leading cluster of excellence in algal biofuels R&D and scale up efforts. Continued development of algal biofuels must involve larger scale pilot facilities using algae strains that are tolerant to saline or seawater and various sources of degraded water, such as wastewater effluent and agricultural drainage that carries excess nutrients that currently threaten natural systems. This ability to utilize non-potable water in the course of producing “drop-in” alternative transportation fuels is of particular interest to California, given the increasing demand for and costs of potable fresh water.

Algae offer multiple advantages over other potential sources of alternative transportation fuels. First and foremost, algae can produce “drop-in” fuels that are compatible with existing petroleum refining and distribution infrastructure. Algal biofuels can help reduce CO$_2$ emissions associated with transportation fuels, as algae consume CO$_2$ from the atmosphere in the course of the production process. Algae require simple mineral nutrients, water, and land, as do all photosynthetic organisms, but algae offers significant advantages over other photosynthetic feedstocks, as the water utilized can be brackish, seawater or hypersaline, or even partially processed, nutrient-rich effluent from various wastewater sources. Importantly, the land used can be non-arable, thus preserving valuable agricultural lands for continued food production. A full and comprehensive life cycle analysis (LCA) of algae production has demonstrated that its overall resource input needs are far superior – economically and environmentally – to all other known feedstocks. As a result, algae offer the most promising option for producing low-carbon transportation fuels, and indeed multiple public demonstrations to date of such fuels – by both the private sector and the Department of Defense – have proven this. However, several economic challenges remain before algae biofuels can compete as a cost-effective commercial scale alternative to fossil fuels. These challenges need to be addressed by advanced research, followed by pilot scale demonstration, and then commercial demonstration. The CILMSF, along with their commercial partners, are poised to provide both the research and demonstration of economically viable low carbon fuels.

4.2 Recommendations

Today, many news headlines proclaim U.S. energy independence coming soon, and talk about the dropping price of crude oil. Additional analysis beyond these headlines reveal facts that
portend something very different; crude oil is becoming much more difficult to find and ever more expensive to extract. Additionally, energy independence with increased domestic crude oil production does nothing to reduce greenhouse gas emissions. Dropping crude oil prices may provide the U.S. a few years of relief from increasing energy costs; however, sooner or later, oil prices are going to increase again and, when they do, the California economy is going to suffer.

Now is the time to focus on establishing a sustainable system for the production of low-carbon renewable fuels in California – for California. The State has the opportunity to do just that, with the assistance of the CILMSF, through a two-pronged approach. Immediate and significant investment in the research and demonstration plants for algae based biofuels is one key area, which will create both economic and environmental benefits that can sustain California for decades to come. The second area in which California needs to provide leadership and funding is the development and approval of pilot demonstration algae energy parks in California. The creation of these parks in close collaboration with industry will not only provide industry a roadmap and pilot demonstration experience that will enable them to roll out commercial plants in California, but it will do so on a much faster timeframe than industry would be capable of alone. To date, algal biofuels companies have avoided setting up demonstration sites in California, due to perceived and real expenses and risks associated with regulatory permitting in the State. Instead, these companies are choosing to set up sites in New Mexico, Arizona, Texas, Hawaii and Florida. Pre-approved algae energy parks will address these issues and attract industry. Superior to many areas already hosting pilot facilities, sites near the Salton Sea and in the southern San Joaquin Valley have the right mix of resources, including sunlight, flat land, and saline water that could ideally sustain significant algae production facilities. In the case of the Salton Sea, an algae farm could also provide the cover required to mitigate the environmental and health issue that are going to arise as the Salton Sea recedes and exposes the playa allowing the polluted sediment to become airborne. The Pacific Institute estimates that the long-term social and economic costs of a deteriorating Salton Sea could approach $29 billion and may reach as much as $70 billion over the next 30 years if no action is taken. By having dedicated lands that are pre-approved for specific use as algae energy farms, and having a pilot demonstration facility built within one of these parks, the State of California will be prepared when the price of crude oil has climbed to the level where the significant investment required to build a commercial facility is less at risk from economic considerations.

Based on the research team’s knowledge of the state of research and commercial viability of algal biofuels, the CILMSF strongly believes the time is right for the State of California to actively expedite the commercialization of sustainable low carbon fuels from feedstocks like algae. These feedstocks can effectively address the State’s mandate to meet its obligations under AB32 and the LCFS, and make a significant contribution to California’s future energy needs while supporting robust economic growth, as well as environmental mitigation of the unrelated but very real problems associated with the Salton Sea. Additional targeted research and the establishment of energy parks will be the most effective way to do this – and the time to act is now.

65
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AB32</td>
<td>Assembly Bill 32</td>
</tr>
<tr>
<td>Cal-CAB</td>
<td>California Center for Algae Biotechnology</td>
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<tr>
<td>CEQA</td>
<td>California Environmental Quality Act</td>
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<tr>
<td>CILMSF</td>
<td>California Initiative for Large Molecule Sustainable Fuels</td>
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<tr>
<td>COSMOS</td>
<td>California State Summer School for Mathematics and Science</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeat</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>EDGE</td>
<td>Educating and Developing Workers for the Green Economy</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FAAS</td>
<td>Flame atomic absorption spectrometry</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>FF21</td>
<td>Food &amp; Fuel for the 21st Century</td>
</tr>
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<td>FMDV</td>
<td>Foot-and mouth-disease-virus</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography–mass spectrometry</td>
</tr>
<tr>
<td>GRAS</td>
<td>Generally regarded as safe</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<td>Greenhouse gases</td>
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<td>LMSF</td>
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<td>LCA</td>
<td>Life cycle analysis</td>
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<td>LCPUFA</td>
<td>Long chain polyunsaturated fatty acids</td>
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<td>LCFS</td>
<td>Low Carbon Fuel Standard</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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</tr>
<tr>
<td>MOOC</td>
<td>Massive Open Online Course</td>
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<td>NGO</td>
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<td>RFU</td>
<td>Relative fluorescent units</td>
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<td>PPTase</td>
<td>Phosphopantetheinyl transferase</td>
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<td>SANDAG</td>
<td>San Diego County of Governments</td>
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<td>SIO</td>
<td>Scripps Institution of Oceanography</td>
</tr>
<tr>
<td>SIT</td>
<td>Silicon transporter</td>
</tr>
<tr>
<td>TAC</td>
<td>Technical Advisory Committee</td>
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<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>UC</td>
<td>University of California</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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</tbody>
</table>
APPENDIX A: 
Student Outputs

Below are listed the 25 publications, 1 patent and 19 presentations with undergraduate or graduate student authors and presenters.

Patents:


Publications:


Presentations:

Barrera, Daniel J.; Rosenberg, Julian N.; Chiu, Joanna G.; Chang, Yung-Nien; Debatis, Michelle; Ngoi, Soo-Mun; Chang, John T.; Shoemaker, Charles B.; Oyler, George A.; Mayfield, Stephen P. Algal chloroplast produced camelid VHH antitoxins are capable of neutralizing botulinum neurotoxin. 2014 Food and Fuel for the 21st Century Symposium. 14-15 March 2014. Poster


Ferreira-Camargo, L., Tran, M., Tusakul, L., Beld, J., Burkart, M. D., Mayfield, S. P., Chemical and biological strategies for protein accumulation in Chlamydomonas reinhardtii chloroplasts, Cal-CAB Symposium, San Diego (March 2014), poster, award received


Taton, A., Amy Ma, You Chen, Federico Unglaub, Tyler Swinney, Edward King, Ron Cook, Nicole E. Wright, Susan S. Golden, and James W. Golden. 2013 "Improved genetic tools for cyanobacteria."
