Final Report

CONSORTIUM FOR ALGAL BIOFUEL COMMERCIALIZATION (CAB-COMM)

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CONSORTIUM FOR ALGAL BIOFUEL COMMERCIALIZATION

EXECUTIVE SUMMARY

The Consortium for Algal Biofuel Commercialization (CAB-Comm) was established by competitive award in 2010 to conduct research to enable commercial viability of alternative liquid fuels produced from algal biomass. CAB-Comm was funded to carry out basic research on three key aspects of the algal biofuels value chain: crop protection; nutrient utilization and recycling; and the development of genetic tools. Two commercial partners, Sapphire Energy and Life Technologies, initially participated as key collaborators and cost share partners.

In the final year of CAB-Comm funding, we also completed the worlds first EPA approved outdoor field tests of GMO algae, produced the world's first algae based surfboard, and working with commercial partners Heliae and Triton Health and Nutrition, completed development of strains for commercial production of protein co-products. We also continue our hands on education programs that have to date trained over two hundred research scientists and laboratory technicians for employment in the algal biofuels industry, and expanded our web based Massive Open Online course (MOOC) called Our Energy Future that has educated over 50,000 students world wide to the benefits of sustainable alternative fuels produced from algae.

Research highlights 2010 to 2015

Developed genetic tools to enable an algae industry: Perhaps our most important successes were achieved in developing genetic tools for cyanobacteria, green algae, and diatoms, as these tools have enabled the entire algal research community to be more productive. These tools were developed to support both the commercial and academic sectors to continue to drive commercial viability. To rapidly deploy these new tools, we made them available to everyone in the algal community through the Life Technologies catalog, where over 150 algae products are now listed and available for world-wide distribution. Additional tools that could not be made available through the LT catalog have been published and are available by material transfer agreement from UC San Diego, including the protein-targeting vectors that allow state of the art metabolic engineering in algae. We also incorporated synthetic biology approaches to develop next generation genetic control systems.

The genetic tool kit developed for cyanobacteria is fully supported by an advanced web-based interface that allows in silico design of genetic tools. We have used this system to create and test over 70 genetic parts and devices including: origins of replication, improved broad-host-range plasmids, homology regions for chromosome engineering, antibiotic markers, and functional devices for gene expression. For diatoms, a set of highly useful genetic manipulation tools were developed including: new promoters; new antibiotic resistance selectable markers; markers that enables selection without GMO classification; RNAi and antisense approaches for gene regulation; and an array of fluorescent proteins and targeting vectors for sending proteins to multiple cellular locations.

Devised crop protection strategies for cyanobacteria and algae: A critical factor for algal biomass production is combating pathogens (viruses, bacteria and fungi), predators (protozoa, planktonic crustaceans, rotifers and helminths), weeds (undesired algae and water plants), and high-density growth inhibitors produced by algae or associated bacteria. We examined all aspects of crop protection, from identifying the basic algal pathogenic microorganisms and predators to identifying strategies and molecules that allow for crop protection in algal production facilities. We characterized algal genetic resistance to chytrid fungi and identified a stress response

pathway; developed anti-viral technologies; developed strategies and methods for finding grazer resistant strains, and identified genes involved in resistance of cyanobacterium to grazing by an amoeba.

In addition, we also examined the impact that diverse communities of algae have on biomass accumulation. We examined how the diversity of algae polycultures impacts their interactions with grazers and fungal pathogens, and how multiple strains can result in greater productivity than monocultures. We found that Daphnia grazers were far more successful at invading monocultures of algae than communities containing 5-10 species. We also found that a single strain of a chytrid fungal pathogen could infect a number of algal species including chlorophytes, diatoms and cyanobacteria.

Enabled improved nutrient utilization and recycling: Nutrient supply and recycling is critical to a sustainable algal biofuels production system. Given that nitrogen is currently the most expensive of these nutrients, we examined whether nitrogen-fixing cyanobacteria (Nodularia) can be viable as outdoor production strains and can provide nitrogen to other microalgae through co-culturing. We also examined the interspecific tradeoffs among traits related to growth, biomass yield, and nutrient competitive abilities vs. lipid content and light competitive abilities. This analysis identified combinations of species that greatly "over-yield" or produce far more biomass together than any one of the component species alone. We will complete this work by asking if the highly productive and resilient algal communities identified in the lab show greater yield under more variable conditions in production type open ponds.

In collaboration with Sapphire Energy, we identified commercially relevant strains that utilize recycled nutrients after hydrothermal liquefaction and oil separation, the process used by Sapphire Energy to extract oil from wet algae. We also developed a nutrient, water, and carbon mass balance model to assess best-practices and technologies for nutrient recycling. Integration of this model with life cycle assessment (LCA) modeling also allows us to model the most effective recycling technologies from a system-wide perspective.

Conducted first EPA approved outdoor field testing of GMO algae: We conducted the world's first test of a genetically modified algae strain, *Scenedesmus dimorphous,* in outdoor field experiments. These tests were run in collaboration with Sapphire Energy under approval of the EPA TSCA Environmental Release Application (TERA, approved on 9/25/2013, R13-0003 through R13-0007) at the UC San Diego Biology Field Station algal growth facility in order to: examine the ability of the subject microorganism to disperse from cultivation ponds; assess the ability of the GM algae to invade and displace natural communities in local water bodies; and evaluate the translation of a GM phenotype (altered fatty acid profile) from the laboratory to an outdoor setting. We identified that the GM algae were able to disperse into algae traps located within 50 meters of the test ponds, but that these algae did not displace native species in test cultures using local waters. We also identified that the GM traits expressed in the test algae were stable throughout the three months of cultivation in open ponds.

Produced the world's first algae surfboard: In addition to this basic research we also engaged the commercial sector to develop and demonstrate the production of high value sustainable fossil fuel replacement products, specifically polyurethane made from algae oil. In collaboration with Arctic Foam, we developed polyurethane foams in which 100% of the polyols were derived from algae oil. The polyurethane made from the algae oil is both sustainable, as well as biodegradable, and test boards have demonstrated that the performance of algae polyurethane matches that of petroleum urethane. We plan a commercial launch of algae surfboards in 2016.

Created protein co-product strains for commercial partners: Finally, in collaboration with commercial partners Heliae and Triton Health and Nutrition we developed strain of green algae that express high value recombinant proteins as commercial co-products. These proteins have now undergone extensive cell based and animal testing and are reaching the final stages of product development. In the case of Triton Health and Nutrition, they have already entered into a co-development agreement with Nestle Pet Care to bring these products to market within a few years. Following regulatory approval and market analysis it is estimated that these products can enter the market within the next few years.

TABLE OF CONTENTS

CAB-Comm Final Report

CONSORTIUM FOR ALGAL BIOFUEL COMMERCIALIZATION

INTRODUCTION

The Consortium for Algal Biofuel Commercialization (CAB-Comm) was established by competitive award in 2010 to conduct research to enable commercial viability of alternative liquid fuels produced from algal biomass. CAB-Comm was funded to carry out basic research on three key aspects of the algal biofuels value chain: crop protection; nutrient utilization and recycling; and the development of genetic tools. Two commercial partners, Sapphire Energy and Life Technologies, have participated as key collaborators and cost share partners. The grant award period ran from 9/1/2010 to 8/30/2015. The total award support was $14,127,459, with $11,031,459 in federal funds and $3,096,000 in cost share.

Objectives

The main objective of CAB-Comm is to dramatically improve the viability of algae as a source of liquid fuels to meet US energy needs, by addressing several significant barriers to economic viability. To achieve this goal, CAB-Comm is taking a diverse set of approaches on three key aspects of the algal biofuels value chain: crop protection; nutrient utilization and recycling; and the development of genetic tools.

Crop Protection

A critical factor for algal feedstock production is combating pathogens (viruses, bacteria and fungi), predators (protozoa, planktonic crustaceans, rotifers and helminths), weeds (undesired algae and water plants), and high-density growth inhibitors produced by algae or associated bacteria. Formulating crop protection strategies for growing algae relies on knowledge and experience in plant pathology, microbial ecology, pest management, and many years of growing algae at several scales. Crop protection is the single most important challenge facing terrestrial agriculture, and a lack of crop protection is now being recognized as the chief limiting factor in algal biofuels production. Under CAB-Comm we undertook all aspects of crop protection, from identifying the basic pathogenic microorganisms to identifying strategies and molecules that allow for crop protection in algal production facilities. Specifically, our objectives were to:

1. Characterize algal genetic resistance to chytrid fungi;
2. Examine crop protection by secretion of extracellular products and their potential roles in suppressing growth of competing species;
3. Develop anti-viral technologies;
4. Identify and characterize quorum sensing molecules (QSMs) from algae that act as high-density growth inhibitors;
5. Produce antimicrobials for crop protection in eukaryotic algae; and
6. Develop strategies for finding or constructing grazer/competitor resistant strains.

Nutrient Utilization and Recycling

After de-watering, wastewater from the algae is still rich in nutrients. To decrease operational costs and environmental impact, all available nutrients need to be assessed for possible recycling and also for reducing downstream eutrophication. We have constructed highly controlled growth systems (chemostats and turbidostats)

that allow for reproducible physiological conditions. We use these to parameterize growth efficiencies (photons absorbed per unit carbon fixed). We have additionally used an algal growth chamber fitted with an integrating-sphere total-internal-reflection light metering system for recording in real time the total light energy absorbed during growth. This approach has been used to measure the solar to biomass conversion efficiency for algae from the combustion enthalpy of the dried biomass with exceptionally high precision. Under CAB-Comm, we have applied these methods to the following objectives:

1. Characterize the physiology of elite algae strains within the abiotic matrix that regulates growth and carbon partitioning;
2. Characterize carbon dioxide utilization in cyanobacteria at the molecular and cellular levels;
3. Examine biological nutrient supply & protection;
4. Develop and characterize a model pond; and
5. Create a model and analysis of nutrient recycling loops.

Genetic Tools

The development of genetic tools to enable crop protection and co-product production is critical the future success and economic viability of algal biofuels. Under CAB-Comm, we aimed to develop additional genetic tools, which could be leveraged by not only the activities of our corporate partners, but also by the greater academic and industry communities. Specifically, our objectives were to:

1. Develop additional selectable markers and crop protection tools for green algae and diatoms;
2. Develop new methods to control gene expression and cell viability in green algae;
3. Develop Cyanobacterial genetic tools;
4. Developing genetic tools and co-products for brown algae;
5. Develop methods for the rapid generation and expression of high affinity nanobodies to promote crop protection, facilitate harvesting, and express high-value co-products; and
6. Create a model and analysis of benefits of algae co-products.

Participants

These projects have been undertaken as collaboration between six academic institutions and two industrial partners: University of California, San Diego; Scripps Institution of Oceanography; University of Nebraska, Lincoln; Rutgers University; University of California, Davis; Johns Hopkins University; Sapphire Energy; and Life Technologies.

From the academic institutions, there were 20 faculty Principal Investigators participating as well as numerous postdoctoral fellows, PhD students, Masters students and undergraduate students. Participation from the PIs labs was extensive with a significant amount of training provided to postdoctoral fellows, PhD students, Masters students and undergraduate students.

CAB-Comm Faculty

UC San Diego	U Nebraska Lincoln	Rutgers University	UC Davis/LCA
Stephen Mayfield	Donald Weeks	Paul Falkowski	Alissa Kendall
Michael Burkart	James Van Etten	Charles Dismukes	Stefan Unnasch*
Susan Golden	George Oyler	Debashish Bhattacharya	
James Golden	Kenneth Nickerson*		
Steve Briggs	Heriberto Cerutti*		
Brian Palenik			
B. Greg Mitchell			
Mark Hildebrand			
Jonathan Shurin			
Bianca Brahamsha			

* PI either withdrew from project or did not pass Stage Gate review.

CAB-Comm Participant Breakdown 2011-2015

Participants	Subtotal
PIs	20
Other Professors	9
Scientists/Staff Researchers	15
Lab Technicians	4
Postdoctoral Fellows	35
PhD students	34
Masters students	8
Undergraduate students	99
HS students	5
Other Volunteers	2
Total Participants	*231*

RESEARCH PROGRESS

Crop Protection

A critical factor for algal biomass production is combating pathogens (viruses, bacteria and fungi), predators (protozoa, planktonic crustaceans, rotifers and helminths), weeds (undesired algae and water plants), and high-density growth inhibitors produced by algae or associated bacteria. Under our original scope, we examined all aspects of crop protection, from identifying the basic algal pathogenic microorganisms and predators to identifying strategies and molecules that allow for crop protection in algal production facilities. Specifically, we: 1) characterized algal genetic resistance to chytrid fungi and identified a stress response pathway; 2) developed anti-viral technologies; 3) developed strategies and methods for finding or constructing grazer resistant strains, established model systems for laboratory manipulation of predators, and identified 10 genes involved in resistance of a model cyanobacterium to grazing by an amoeba; and 4) examined the responses of algal polycultures to predation and found that predators are far more damaging to monocultures than they are communities consisting of 5-10 algal species. These important advances have laid the foundation for the elucidation and practical application of algal defense strategies and pathways.

To develop strategies for finding or constructing predator resistant strains, we first developed methods for the enrichment and isolation of protozoan grazers of both filamentous and unicellular cyanobacteria. We examined a variety of environments, including freshwater and brackish natural ponds, as well as experimental freshwater and marine outdoor production ponds. We used these grazers to establish model systems to uncover cyanobacterial resistance mechanisms and in doing so identified 10 genes involved in lipopolysaccharide (LPS) synthesis. Mutations in these genes confer resistance to grazing by an amoeba in a unicellular strain. We also identified a number of proteins that are upregulated in response to grazing in cyanobacteria. Because the mutations in LPS are not protective against all of the amoebae we isolated, we propose to expand our research to uncover additional mechanisms of resistance that may be more universal.

In addition to resistance genes, we also examined the impact that diverse communities of algae have on biomass loss from grazers. We examined how the diversity of algae polycultures impacts their interactions with grazers and fungal pathogens. We found that Daphnia grazers were far more successful at invading monocultures of algae than communities containing 5-10 species. We also found that a single strain of a chytrid fungal pathogen could infect a number of algal species including chlorophytes, diatoms and cyanobacteria.

Specific crop protection projects undertaken and their results and highlights are detailed below.

A.1 Characterization of algal signaling response to chytrid pathogen attack

Briggs, Mayfield

Chytrid fungi are a primitive and fast growing group of pathogens that have been identified in both the Sapphire pilot facility, and the UC San Diego algal growth facility, as significant pathogen of green algae and diatoms. Investigating these organisms will also provide a general model for understanding host-pathogen interactions in algae, and potentially in developing strategies for both genetic resistance as well as cropping practices that can reduce loss to fungal disease.

Under this task, we discovered that S. dimorphus responds to an analog, BTH, of the plant stress hormone, salicylic acid, in a manner similar to that of plants. Chytrid-induced disease development was delayed by BTH and accelerated by another plant stress hormone, meJA. These two hormones also act antagonistically during plant infection. Our results provide chemical modulators of chytrid infection in algae and suggest that some aspects of innate immunity are conserved between plants and algae.

A.2 Crop protection by secretion of extracellular products and their potential roles in suppressing growth of competing species

Van Etten, Bhattacharya

Overview: Improving feedstock is critical to facilitate the commercial utilization of algae, in particular in open pond systems where, due to the presence of competitors and pests, high algal growth rates and stress tolerance are beneficial. These considerations have led to the search for fast growing, stress resistant, lipid-producing algae. Target taxa would normally be subject to crop improvement by breeding (as done for crop plants), however this approach requires knowledge about the sexual cycle that is lacking for many algae. Therefore, absent access to sexual recombination to develop hybrids and public misgivings about cultivating genetically engineered algae in open ponds, an alternative approach to strain improvement is experimental evolution. This approach relies on the natural capacity of microbes to adapt rapidly to changing environment conditions and can lead to the generation of novel traits of interest. However, unlike culture perturbations that focus on specific pathways such as the carbon-concentrating mechanism or the response to sulfur deprivation, serial transfer of eukaryotes placed under selective regimes over hundreds of generations may open up a "Pandora's box" of genetic variation within populations with regard to DNA mutations, gene expression changes, and epigenetic changes that impact a variety of metabolic pathways. In this aim, we used two experimental evolution approaches to select for resistant strains using as a model the cell wall-deficient mutant *Chlamydomonas reinhardtii* CC-503 (cw92 mt+) that has a sequenced genome. Our work focused on adaptation and evolution of a novel phenotype that results from long-term selection for rapid growth to out-compete congeners and for salt tolerance as a crop protectant. The output from these experiments was measured on multiple fronts, including changes in cell size and growth rates, gene expression, and DNA sequence changes (i.e., SNPs/indels). The broad premise underlying our approach was that microbes have the ability to adapt rapidly to changing environmental conditions that can lead to the generation of novel traits of use for biofuel (and other) applications.

Research Highlights:

Selection for enhanced growth rate: After 283 serial transfers in liquid TAP medium under continuous light (ca. 1,880 generations; Fig. A.2.1) evolved (EL) *C. reinhardtii* populations had a doubling time that was ca. 35% lower than the progenitor population (PL). This relatively higher, evolved growth rate was achieved within 100 serial transfers (ca. 6 months). The elevated growth rate in TAP medium is most likely explained by more efficient uptake and metabolism of acetate, supported by the observation that PL and EL cells had significantly lower but equal

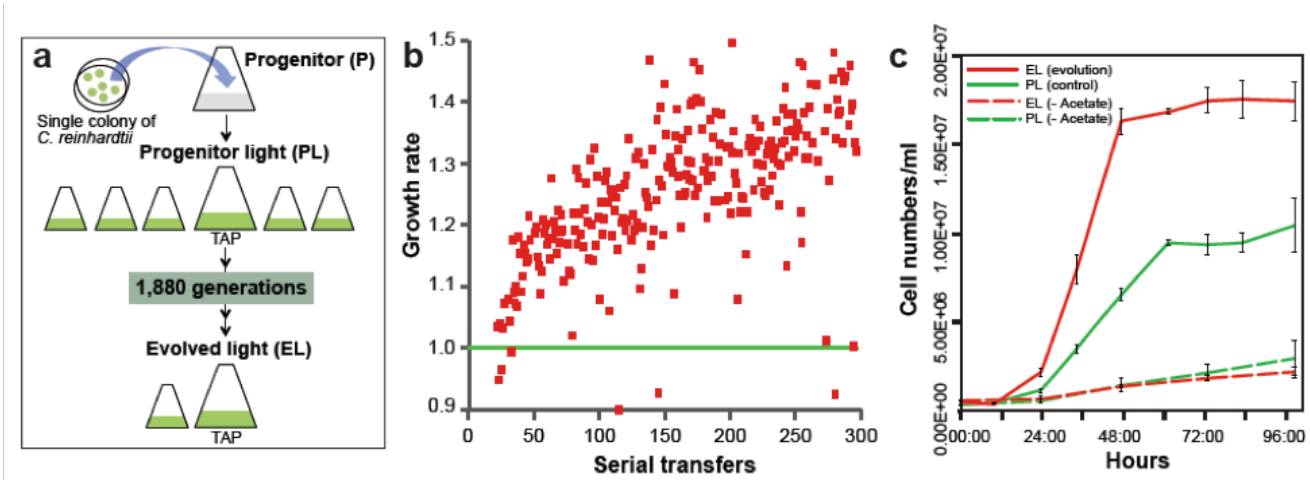


Figure A.2.1. Growth rate of an evolved *C. reinhardtii* population. (A) Design of the experimental approach used to generate a rapidly growing *C. reinhardtii* CC-503 cw92 mt+ population. (B) Results after 283 transfers (1,880 generations) of cells grown under continuous light in liquid TAP medium. At the end of the experiment, the evolved *C. reinhardtii* population grows ca. 35% faster than the progenitor strain. The progenitor growth rate is indicated with the solid green line. (C) Comparison of growth rates of the progenitor (PL, green line) and one population of the evolved (EL, red line) *C. reinhardtii* cells grown in TAP medium. The growth rate of these populations when raised in acetate-free (TAP) medium is shown with the dashed green line for PL and the dashed red line for EL. These curves are derived from three independent culture replicates.

growth rates in the absence of this organic carbon source. We estimated the DNA mutation rate to be 1.01x10-8 /base/generation for the EL population, which is 30-150 times higher than previous estimates made for this species ($3.23x10^{-10}$ and $6.76x10^{-11}$) but comparable to higher plants such as *A. thaliana* ($5.9x10^{-9}$). This result is not surprising if we take into account that our gene expression analyses (RNA-seq; see below) demonstrate that the DNA repair pathway was down regulated in the EL C. *reinhardtii* population (see below). This likely explains a higher mutation rate than has been previously estimated for this species. Among the 1,782 single base SNPs/indels, the mutations favored transitions G:C→A:T (30.1% and 22.0% for A:T→G:C) as has already been shown in previous studies and consistent with an elevated GC-content of 61.74% in the C. *reinhardtii* nuclear genome.

Analysis of global gene expression differences after 17 months under constant light and acetate as a carbon source demonstrates that gene expression in the EL population differs substantially from the PL population. Analysis of RNA-seq data from the EL population showed that, by far, the greatest number of significantly up regulated genes encoded ribosomal proteins. Concomitant with this emphasis on protein production in EL cells was the significant up regulation of genes involved in RNA-transport to facilitate gene expression and to support translation. These included the translation initiation factor subunits and two subunits of RNA polymerase. These results agree with the known correlation between higher ribosomal protein content and protein translation with increased cellular growth rates. The pathways significantly down regulated in the EL population included biosynthesis of secondary metabolites, DNA replication, and photosynthesis with the latter likely being a response to continuous light. For the DNA replication pathway, the mini-chromosome maintenance (MCM) complex was significantly down regulated. Several cell cycle genes (e.g., SCF, CycA, PCNA) were up regulated as would be expected in a rapidly dividing cell population.

Of particular interest was the impact of selection on lipid metabolism that is a key target for biofuel strain improvement. Our analysis shows that fatty acid biosynthesis (i.e., the precursors of storage lipids) is significantly down regulated in the EL population under the constraint of a high growth rate. Given this result, we tested whether fast growing EL cells could be induced to produce lipids by reducing nitrogen in the medium, as has been shown for many other algae. Staining of the PL and EL populations with the neutral lipid dye Bodipy 493/503 revealed the accumulation of lipids in both populations (Fig. A.2.2).

In summary, our results demonstrate that selection using a serial dilution regime can be used to substantially modify gene expression patterns in C. *reinhardtii*. In the case of the faster growing EL population, these cells can be manipulated to produce neutral lipids. The significant growth enhancement in C. *reinhardtii* was largely supported by improved acetate metabolism. This suggests that pathways of organic carbon usage present in many algae may provide useful targets for strain improvement. A large variety of DNA sequence and gene expression differences, the latter for fundamental process such as DNA replication and protein translation were uncovered in the PL-EL comparison. Our results clearly demonstrate the enormous capacity of algal genomes to adapt to changing conditions, a feature that can be exploited to advance basic and applied research in microbial eukaryotes (Perrineau et al. 2014a).

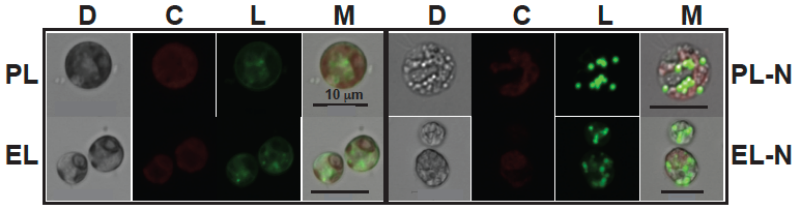


Figure A.2.2. Analysis of lipid production in the PL and EL populations of C. *reinhardtii* when grown under continuous light in liquid TAP medium. Shown are differential interference contrast images (D), chlorophyll autofluorescence (C), lipid bodies (Bodipy stain; L), and merged (M). Note that like the PL and EL cells show up regulation of lipid production after depletion of nitrogen (-N) in the medium.

Selection for salt tolerance: The second experimental evolution approach we employed was serial dilution of C.

reinhardtii cells under continuous light in liquid TAP medium containing 200 mM NaCl (see Perrineau et al. 2014b). This selection regime also resulted in significant changes in cell physiology. After 1,255 generations (189 serial transfers), the evolved salt (ES) population of cells grew at the same rate as progenitor cells (PL) raised in salt-free TAP medium. Comparison of triplicate PL and ES cultures grown for 4 days with and without 200 mM salt in the medium showed that both ES cultures grew at a faster rate than PL cells in salt-free medium and that PL cells grown with salt (PS) had a significantly lower growth rate. We again tested whether PS and ES cells could be induced to produce lipids by reducing nitrogen in the medium, as has been shown for many other algae. Staining of the PL, PS, and ES populations with Bodipy 493/503 revealed the accumulation of lipids in all populations under nitrogen depletion (Fig. A.2.3).

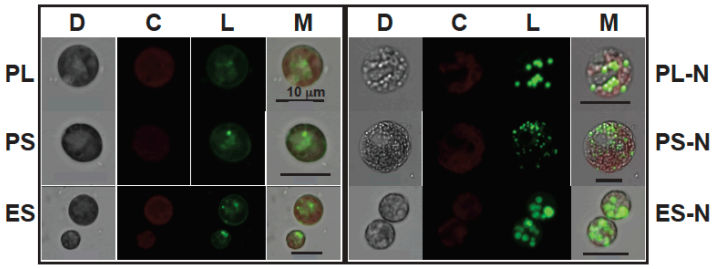


Figure A.2.3. Analysis of lipid production in the PL, PS, and ES populations of *C. reinhardtii* when grown under continuous light in liquid TAP medium. The images are labeled as shown in Fig. 2. Note that all cell populations show up-regulation of lipid production after depletion of nitrogen. (-N) in the medium with the ES cells showing the strongest response.

The results of this study into the salt stress response in C. *reinhardtii* provided several important insights into the adaptive potential of this, and likely other algae. First, we found that the short-term acclimation response after 48h incubation in 200 mM NaCl differs markedly from the long-term response after 1,255 generations. In the former case, PS cells exhibit many well-known responses such as a reduction in photosynthesis, up regulation of genes involved in the stress response, up regulation of glycerophospholipid signaling, and up regulation of the transcription and translation machinery. In contrast, the long-term acclimation response in ES cells showed down regulation of genes involved in the stress response, suggesting this population was well adapted to salt stress. The recovery of the progenitor growth rate in ES cells clearly shows however that this population is able to cope successfully with hyperosmolarity.

These results suggest that microalgae are able to adapt to abiotic stress without sex and meiosis and that this can happen over short time frames (i.e., 6 months for the recovery). A broader outcome of this study is that abiotic stress, engendered either by a rapidly changing climate or physical vicariance events that isolate population members in stressful environments may pose less issues than previously thought for gene-rich mixotrophic lineages such as C. *reinhardtii*. In summary, acclimating (short-term evolution) of algae to a high salt concentration in the medium offers a useful avenue for crop protection that can be applied to any biofuel target.

A.3 Develop anti-viral technologies

Van Etten

Overview: Among the known pests and pathogens predicted to limit algal biofuels/bioproducts production, viruses stand out as ubiquitous agents found in all aquatic environments. Viruses that infect algae are highly diverse and have RNA or DNA genomes, which are either single-stranded or double-stranded (ds), and icosahedral and filamentous particles. However, there are few systems that have been examined to the extent of the family *Phycodnaviridae*. Members of the *Phycodnaviridae* consist of a genetically diverse, but morphologically similar, group of large dsDNA-containing viruses (160 to 560 kb) that infect eukaryotic algae. These large viruses, which are being discovered with increasing frequency, are found in both terrestrial and marine waters throughout the world and play dynamic, albeit largely undocumented, roles in regulating algal communities, such as the termination of massive algal blooms commonly referred to as red and brown tides.

Research Highlights:

Our laboratory has studied one genera of the *Phycodnaviridae* for the last 35 years, the *Chloroviruses*. The chloroviruses we study infect three zoochlorellae that can be grown independently of their symbiotic partners in the laboratory, permitting plaque assay of the viruses, synchronous infection of their hosts and mass production of viruses for structure evaluations. These traits allow one to study the virus life cycles in detail.

Our goal of developing anti-viral technologies began by evaluating the key bottlenecks of chlorovirus infection; to a large extent we focused on the very early events during infection. Starting with the infecting agent, we determined the virus particle make-up by a comprehensive proteomic evaluation demonstrating that approximately 1/3 of the coding capacity (~400 protein encoding genes) of the virus is reflected in the virion composition. These proteins include the architectural factors that form the virion, but also many recognizable enzymatic functions, including proteins that regulate cellular functions. The structure of the prototype virion, PBCV-1, was refined (in collaboration with colleagues at Purdue University) to reveal an asymmetric icosahedron with a spike structure at a single unique vertex. The spike is involved in attachment to the host cell wall, the first point where resistance to viruses can occur. In fact, many of the virus-resistant algal mutants that we isolated are resistant to infection because the virus cannot attach to the alga, i.e., the host receptor was altered. Following successful attachment of PBCV-1 to the host, components of the virion are responsible for cell wall penetration by depolymerizing the wall matrix, and we have characterized a virion-associated protein with this property, referred to as vLysin, which may have utility in algal extraction for oils and other bioproducts.

In considering host-virus interactions and the consequences of infection, susceptible cells are either competent hosts for the viruses (often times the cell dies after virus replication and release) or the cell resists infection through cellular processes referred to as innate immunity. Using plants as a model, we examined the genome of one of the algal hosts for genes known to contribute to plant virus resistance. We also performed an RNA-Seq experiment to evaluate the temporal dynamics of the cellular mRNA populations during the early phase of virus infection, knowing that in our case study the virus eventually wins. Remarkably, this transcriptomic analysis revealed a significant flux in gene regulation at the level of mRNA abundance in the PBCV-1 host, *Chlorella variabilis*, *C. variabilis* exhibited innate immune responses to virus infection that are very similar to other metazoans. These data support our hypothesis that algae encode and utilize RNA silencing mechanisms to suppress virus replication. However, in the test case, the virus wins, the cell dies, and the innate immune activation appears to be overridden. Very recently, we have discovered another algal-chlorovirus system where the virus wins sometimes, but not always. This new host-virus system will allow us to address the question of what factors contribute to virus success, and what factors contribute to algal success when suppressing virus infection.

Our studies have led us to a new understanding of chlorovirus-algae interactions, yet much more research needs to be conducted to address the following questions about protecting biofuel producing algae from viruses. What is the range of algal hosts for these viruses? What is the natural biodiversity of the chloroviruses? When and where are chloroviruses most prevalent? What host nutrient factors contribute to virus activation or suppression?

A.4 Identify and characterize quorum sensing molecules (QSMs) from algae that act as high-density growth inhibitors.

Nickerson - Task Discontinued following 2011 Stage Gate review.

Overview: Often algae grow only to a certain density that is neither nutrient nor photon limited. It is hypothesized that algae, like bacteria, produce quorum-sensing molecules. If so, these molecules will reduce algal production. The Nickerson lab has studied cell density effects in several eukaryotic microbial systems including discovering the first eukaryotic QSM (farnesol) in the dimorphic fungus *Candida albicans*. They have also identified a QSM activity in the stalk-forming diatom *Acanthes longipes*. This QSM, which has not been chemically identified, triggers stalk formation, along with the adhesin, which is localized at the tip of the stalk. This task was aimed at expanding this research to

examine *Chlamydomonas, Nannochloropsis*, *Chlorella* NC64A, and *Chlorella vulgaris* C-169 for QSMs. Once compounds were identified, procedures were to be developed to prevent their appearance, degrade them, engineer resistance to them, and/or remove them by adsorbent materials.

A.5 Production of Antimicrobials for Crop Protection in Eukaryotic Algae

Burkart

Overview: Fatty acid synthases (FASs), non-ribosomal peptide synthases (NRPSs) and polyketide synthases (PKSs) are sophisticated assembly-line biosynthetic factories for the production of many bioactive compounds. These compounds have often antibiotic, immunosuppresive, cytostatic, anti-cancer or even probiotic activities. Most modern drugs are derived from these natural products and also many compounds used in agriculture find their origin in these synthases. These biological factories of compounds transform simple monomeric molecules like amino acids or acyl-CoAs into complex natural products utilizing a variety of enzymatic domains present in the synthase. A crucial part of these synthases is the carrier protein. This small protein carries intermediates in the biosynthetic process from enzyme to enzyme, from domain to domain. The intermediate is bound to the carrier protein via a 4'-phosphopantetheine arm, which is attached to a highly conserved serine residue. So called 4'-phosphopantetheinyl transferases transfer 4'-phosphopantetheine post-translationally from CoA to the carrier protein (Beld, Sonnenschein et al. 2014). Without this "arm" the carrier protein is inactive and thus also the synthase.

Our goal was to demonstrate that engineered eukaryotic green microalgae are capable and suitable to produce antibiotic (antibacterial/antifungal) substances via one of these synthases. These substances could also be used as valuable byproducts of biofuel production. Synthases are not common in eukaryotic organisms, to which green microalgae belong (Tiburzi, Visca et al. 2007). However, very recently an increasing number of synthases are found in eukaryotic species (Beld, Sonnenschein et al. 2014). Furthermore, green microalgae are at the crossroads between prokaryotic and eukaryotic organisms, harboring a bacteria-derived chloroplast. It should however be noted that these synthases are large proteins (1500 amino acids and larger) and so far such large proteins have never been successfully expressed in green microalgae (Specht, Miyake-Stoner et al. 2010). Without a doubt green microalgae are a novel and exciting new platform for heterologous protein expression but only ~30 proteins have been expressed to date, and all genetic tools are in its infancy.

As proof of concept, we decided upon the expression of the polyketide 6-methylsalicylic acid (6MSA) and the non-ribosomal peptide indigoidine, by the 6-methylsalicylic acid synthase (6MSAS) and the blue pigment synthase A (BpsA), respectively (Fig. 1). 6MSA is in nature produced by the *fungus Pennicilium patulum* (Beck, RIPKA et al. 1990) and it has been shown that the relatively small gene (5.3 kb) can be expressed in many heterologous hosts, including the bacteria *Escherichia coli* (Kealey, Liu et al. 1998), *Saccharomyces cerevisiae* (Kealey, Liu et al. 1998) and tobacco (Yalpani, Altier et al. 2001). Further, 6MSA has been shown to have antifungal activity. Also the non-ribosomal peptide indigoidine has been shown to have antibacterial activity (Cude, Mooney et al. 2012). This blue pigment is synthesized by the relatively small NRPS BpsA (3.9 kb), originally found in several *Streptomyces* species (Takahashi, Kumagai et al. 2007), and recently this synthase has also been expressed in mammalian cells (Müller, Ausländer et al. 2012).

Both these synthases require 4'-phosphopantetheinylation to be active. Thus, in parallel with our effort to engineer these two synthases into green microalgae, we also expressed a promiscuous PPTase from *B. subtilis* in the green microalgae C. *reinhardtii*, preparing the green microalgae for the introduction of a synthase.

A typical procedure for the metabolic engineering of the chloroplast of green microalgae consists of a several steps: 1. Cloning of desired gene into a C. *reinhardtii* chloroplast (or nuclear) plasmid, which contains homologous recombination regions and a antibiotic marker. 2. Coating of plasmid onto gold nanoparticles. 3. Ballistic transformation of wildtype C. *reinhardtii* using a Gene-Gun. 4. Colonies that show antibiotic resistance should contain plasmid/gene of interest but require colony PCR screening for homoplasmicity.

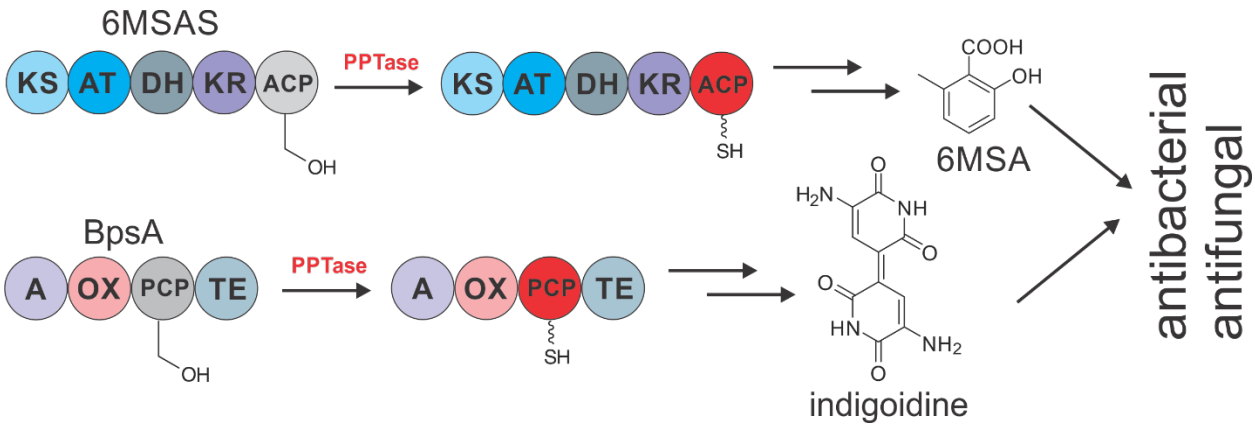


Figure A.5.1 – Overview polyketide and non-ribosomal peptide synthases 6MSAS and BpsA.

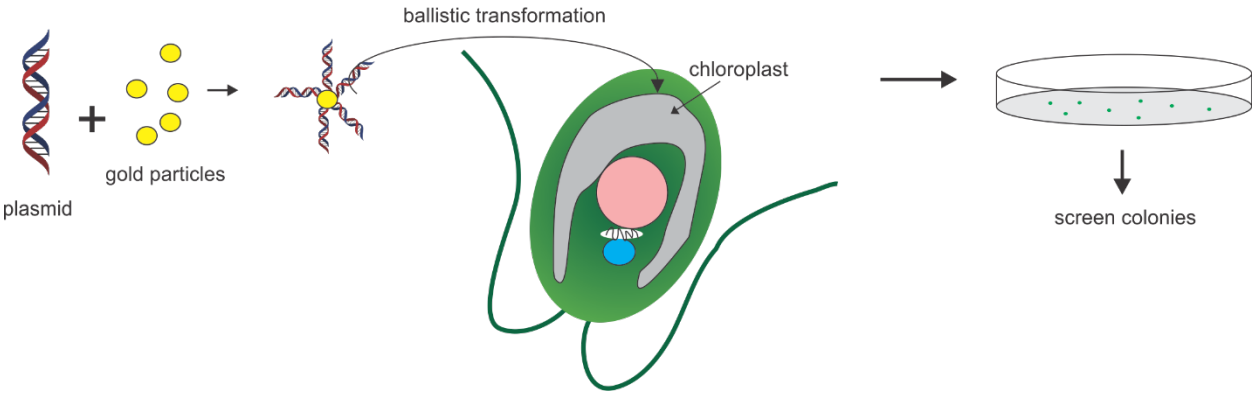


Figure A.5.2 – Typical chloroplast engineering of green microalgae C. reinhardtii. Plasmid DNA is coated on gold particles that are shot into wildtype strain C. reinhardtii. The plasmid contains an antibiotic marker that enables selection on antibiotic containing nutrient agar plates. Colonies are screened for the presence of the desired gene.

We also worked on the heterologous production of simple synthase-driven antimicrobials in C. *reinhardtii* and, in parallel, validating these compounds in biological assays for antifungal and antibacterial activity.

Research Highlights:

For industrial scale algal growth the use of crop protection measures is a necessity. For example, Sapphire Energy utilizes commercially available pesticides to protect outdoor raceways in their production facility in New Mexico. Here, we envisioned the production of antimicrobials in eukaryotic algae as a way to make the use of pesticides unnecessary. To do so, we set out to express, for the first time, large synthases that produce these antimicrobials in eukaryotic green microalgae. These synthases require post-translational modification with a 4'-phosphopantetheine arm, catalyzed by a PPTase

We successfully engineered a PPTase from *B. subtilis* in C. *reinhardtii*. While engineering *B. subtilis* PPTase Sfp into the chloroplast of C. *reinhardtii*, we found that C. *reinhardtii* contains two native PPTases in its genome (489 and 873). To study the effect of these PPTases on the green microalgae (and its ability to produce synthase-dependent

products), we tried to engineer these native enzymes into the algae and overexpress them. Interestingly, we were only able to express 489 but not 873. To our surprise, overexpression of the native PPTase 489 resulted in a large increase in fatty acid production. Studying this observation is part of ongoing research.

Next, we tried to engineer two synthases into C. *reinhardtii*. We chose the smallest PKS and NRPSs, which both produce antibiotic compounds. Engineering these large synthases into green eukaryotic algae appeared to be much harder than expected. The first synthase we tried to introduce into C. *reinhardtii* was 6MSAS and although PCR screening shows the presence of the synthase, Western-Blot analysis on the protein-level did not show any expressed synthase. A small peak in the GCMS analysis of the algae, suggest that the molecule is made. As part of ongoing research we are working on other vectors and better screening methods, to figure out why the molecule is not produced, on a RNA and protein level. We expected that the introduction of the synthase BpsA would result in blue-pigmented algae. However, also in this case we were unable to unambiguously show RNA or protein level of the synthase in engineered algae. We also worked on developing new ways to integrate the synthase (chloroplastic or nuclear) and have better control over the expression system. During the final year of this project, we were able to express terpene synthases and terpene cyclases in C. *reinhardtii*. We also demonstrated proof of concept that terpene synthase/cyclase expression in C. *reinhardtii* chloroplast can lead to production of new terpene small molecules.

A.6 Develop strategies for finding or constructing grazer/competitor resistant strains

Brahamsha, J. Golden, S. Golden, Palenik, Shurin

Overview: Algal species growing in open ponds are subject to predation by a variety of organisms. Grazing by members of the 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 grazer**s of both eukaryotic and prokaryotic algae 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. As summarized below, we met all of these goals.

Research Highlights:

We developed methods for the enrichment and isolation of protozoan grazers of both filamentous and unicellular cyanobacteria from a variety of environments, including freshwater and brackish ponds as well as experimental freshwater and marine outdoor ponds. Isolates included a ciliate (*Cyclidium* sp.) and diverse amoebae including members of the heteroloboseae and amoebozoae.

We established model systems consisting of filamentous (*Leptolyngbya* sp. BL0902 and *Anabaena* sp. PCC7120) as well as unicellular (*Synechococcus* sp. PCC7942 and *Synechocystis* sp. WHSyn) cyanobacteria and several clonal amoebae. We chose these cyanobacteria because they are all genetically tractable, the genome sequence for each is available, and they represent both very well-studied laboratory strains with sophisticated genetic manipulation resources (PCC7942, PCC7120), as well as recently isolated potential production (*Leptolyngbya* BL0902) and broadly halotolerant (WHSyn) strains. We have characterized feeding behavior in liquid as well as on solid substrates and have established pairs of cyanobacterium/amoeba for laboratory manipulation and genetic dissection of the interactions.

We used one of our model systems (*Synechococcus* sp. PCC7942 and amoeba HGG1) to isolate grazing-resistant mutants. We 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, we have expanded the catalog of mutations that confer resistance 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 and we are testing the hypothesis that 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.

In order to understand the cyanobacterial response to amoebal grazing, and to uncover signaling and defense pathways, we carried out a proteomic analysis of a time course of *Anabaena* sp. PCC7120 incubated with and without amoeba HGG1. We identified 184 proteins that were upregulated by at least 1.5-fold in replicate samples. To further analyze this dataset, we applied a P-value cut-off of <0.1 and have narrowed our list to 34 proteins. We have tested 22 of these genes for upregulation of mRNA by quantitative reverse transcriptase PCR. Of these genes, five showed consistent upregulation by *Anabaena* after grazing. Since the initial interaction between HGG1 and *Anabaena* occurs at the surface of the cells, we performed a targeted experiment to identify *Anabaena* outer membrane proteins that are upregulated after grazing. We performed outer membrane isolations of *Anabaena* incubated with and without HGG1 for 12 hours. We have identified 4 proteins that are present in the outer membrane preparations from *Anabaena* co-incubated with HGG1, and absent from preparations from *Anabaena* incubated alone. These datasets show promise and will be mined for additional *Anabaena* proteins upregulated during grazing.

We also performed experiments in field condition in 250 L tanks with algal communities differing in species richness (1, 2, 4, 6, 8 and 10 species). We identified a suite of invading grazers using microscopy. The most abundant grazers in the algal ponds were nauplii followed by cyclopoid copepods and *Hydrarachnida* sp. Besides the algae grazers, the ponds were also invaded by different members of hymenoptera, coleoptera and *Culicidae* and *Arachnida*. The minimum number of grazers was found in tanks with maximum algal species richness. Algal biomass production varied among diversity levels. The most productive community was a two species combination followed by four. Interestingly, the number of invading grazers in those algae tanks was comparatively low.

Identifying the genetic basis of resistance to predation has allowed for greatly improved crop productivity in all of agriculture. The studies conducted previously identified the first grazer (predator) resistance genes in cyanobacteria and showed (along with the fungal studies) that algal defense strategies to pest and pathogens is similar to defense strategies deployed in higher plants. We expanded our characterization of grazer-algae interactions to identify additional strategies and genes involved in these critical interactions.

Nutrient Utilization and Recycling

After de-watering, wastewater from the algae is still rich in nutrients. To decrease operational costs and environmental impact, all available nutrients need to be assessed for possible recycling and also for reducing downstream eutrophication. We have constructed highly controlled growth systems (chemostats and turbidostats) that allow for reproducible physiological conditions. We use these to parameterize growth efficiencies (photons absorbed per unit carbon fixed). We have additionally used an algal growth chamber fitted with an integrating-sphere total-internal-reflection light metering system for recording in real time the total light energy absorbed during growth. This approach has been used to measure the solar to biomass conversion efficiency for algae from the combustion enthalpy of the dried biomass with exceptionally high precision.

Given that nitrogen is currently the most expensive of algae critical nutrients, we examined whether nitrogen-fixing cyanobacteria (Nodularia) can be viable as outdoor production strains and can provide nitrogen to other microalgae through co-culturing. We also examined the interspecific tradeoffs among traits related to growth, biomass yield, and nutrient competitive abilities vs. lipid content and light competitive abilities. This analysis identified combinations of species that greatly "over-yield" or produce far more biomass together than any one of the component species alone. We will complete this work by asking if the highly productive and resilient algal communities identified in the lab show greater yield under more variable conditions in production type open ponds.

In collaboration with Sapphire Energy, we identified commercially relevant strains that utilize recycled nutrients after hydrothermal liquefaction and oil separation, the process used by Sapphire Energy to extract oil from wet algae. To decrease operational costs and environmental impact, these nutrients need to be recycled back into the algal ponds for continued rounds of growth. With Sapphire Energy, we identified improved commercial strains that efficiently utilize recycled nutrients following oil extraction including *Picochlorum* and *Haematococcus*. We will continue to characterize how species such as these acclimate or adapt to the use of raffinate (nutrient rich liquid fraction following oil extraction) to further improve nutrient recycling. We will incorporate physiological characterization of these elite algae to allow us to understand and model improved raffinate uptake and utilization.

A nutrient, water, and carbon mass balance model was created to assess best-practices and technologies for nutrient recycling. Integration of this model with life cycle assessment (LCA) modeling also allows us to model the most effective recycling technologies from a system-wide perspective. Two categories of nutrient and energy recovery technologies, anaerobic digestion and hydrothermal liquefaction, were examined to treat the algal biomass residual. We identified that recycling residuals for energy and nutrient recovery can reduce the carbon intensity of algal biodiesel by as much as 40% under some conditions, compared to exporting these residuals as co-products. These results are documented in three manuscripts.

B.1 Physiological characterization of elite algae strains within the abiotic matrix that regulates growth and carbon partitioning

Mitchell, Shurin

Overview: To meet the renewable energy needs from micro-algal mass culture, while avoiding competition for land and fresh water required for food production, commodity-scale mass culture must be pursued using salt-tolerant strains cultured on very large tracks of marginal lands that otherwise could not economically support viable food crop production. In support of a rational development pathway towards an economically viable model for algal bio-fuels from salt-tolerant algae, one must consider the inherent value of each of the major cellular constituents of the harvested algal biomass, i.e., the carbohydrates, proteins, lipids, and pigments, each as separate sources for products.

Research Highlights:

Understanding the dynamics of carbon allocation, e.g., carbon partitioning, among the cellular carbon pools for candidate algal strains is essential to understanding the potential magnitude of product yields in relation to varying environmental, i.e., abiotic conditions. Thus, our guiding methodology under this task was to vary nutrient, CO_2, pH, temperature and irradiance levels to assess the impact of these abiotic factors on algal growth rates, photosynthetic quantum yields, and cellular concentrations of lipid, protein, carbohydrate and pigments during both exponential and stationary growth phases. Our primary model organism has been *Thalassiosira pseudonana*, as the genome of this ubiquitous marine diatom has been fully sequenced and has the potential to accumulate large cellular concentrations of lipids. Additional experimental series were run with the marine haptophyte *Isochrysis galbana*, the chlororophyte *Dunaniella tertiolecta* and the diatom *Phaeodactylum tricornutum*. All of these strains are well-studied elite research strains and all are used commercially.

For *P. tricornutum,* growth was optimized when a continuous cultured regime was used, at or about oceanic salinities (i.e., 35 ppt), and within a pH range of 8.4-8.6 pH unit. For *Dunaliella teriolecta* maximum growth was achieved at a salinity of 10 ppt with reasonable growth between 20 - 35 ppt, but poor growth at 5 ppt and 65 ppt; optimal pH was between 8.2-8.6. For *I. galbana,* growth and biomass accumulation was best at salinity of 50 ppt, and optimal pH between 8 – 9. With *I. galbana*, varying light irradiances were tested, and the highest growth rate and light absorption per unit chlorophyll and C/Chl ratio were observed at the highest experimental irradiance level applied (744 uE/m^2/s). These results indicate lower overall pigment production at high light despite the observed increased growth rate, suggesting a need to grow at low light intensity if high cellular pigment concentration is desired.

For *T. pseudonana* growth rate was optimal between 18°C - 22°C and in diverse experiments, the sum of the four carbon pools studied (lipids, protein, carbohydrates and pigments) accounted for an average of 85% of total particulate organic carbon. See Figure B.1.1 for a summary of carbon partitioning for different treatments. Surprisingly, for the range of irradiances applied (80, 250, and 420 uE/m^2/s), and the various levels of CO_2 supplementation tested, the growth rates observed during mid-log phase were not significantly different (within the uncertainty of the data), but growth rates between treatments did vary from 1.2 – 1.6 day^{-1}, and cellular allocation within the major carbon pools was significantly affected by the abiotic regulation. Hence, even for similar growth rates, abiotic control on physiology dramatically affects the carbon partitioning. The percentage of cellular carbon allocated to lipid storage reached a maximum of 45% during stress induced stationary phase but was significantly lower during log growth. While total fatty acids increased during stationary phase and at higher irradiances, the high value polyunsaturated fatty acid (PUFA) fraction decreased. For fuel production there is a desire for high yields of monosaturated fatty acids, minimal polyunsaturated acids, and controlled proportions of saturated acids. C18:1 and C16:1 are generally considered the most suitable fatty acids in terms of oxidative stability and cold weather behavior of fuels. Applying the data from this study, *T. pseudonana* should be cultured at saturating irradiance and CO_2 supplementation for the purpose of fuel lipid feed stock. Polyunsaturated fatty acids should be avoided for fuel production due to their contribution to autoxidation of biodiesel. However, given the importance of the polyunsaturated fatty acids and especially the omega-3 PUFAs for nutraceutical products in the balanced economic model suggested, low irradiance and minimal CO_2 should be utilized to optimize this product fraction.

We found that partitioning of carbon among lipids, proteins and carbohydrates varied as a function of culture conditions and growth phase, with a number of important implications for optimizing growing and harvesting strategies.

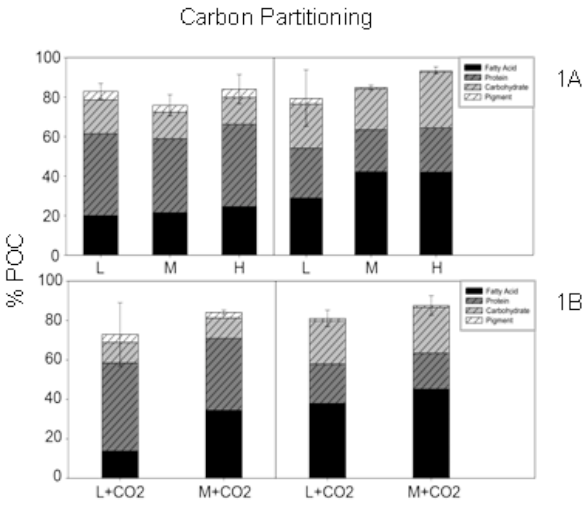


Figure B.1.1 - Carbon fractionation for Thalassiosira pseudonana as a function irradiance and CO2 expressed as a percentage of total particulate carbon (POC) during mid-log phase (left) and stationary phase (right). A) Cultures treated with irradiances low: 83±11, medium: 237±11 and high: 420±11 □mol photon · m□2 · s□1 bubbled with ambient air. B) Cultures treated with irradiances low: 83±11 and medium: 237±11 bubbled with 2% CO2. Error bars represent the standard deviation of triplicate cultures.

Respiration(R) compromises can result in a significant loss of photosynthetically fixed carbon yet knowledge of the balance between photosynthesis and respiration is very poorly known due to diverse methodological and physiological experimental challenges. In this study we acclimated *Phaeodactylum tricornutum* at 143 µmol quanta m^{-2} s^{-1} of 24°C, then exposed the culture to a wide range of intensity and darkness to investigate the relationship between respiration in the dark and photosynthesis for varying light intensity. We divided the respiration rate into two components: Maintenance cost (r_m) that enables the survival of a living cell, and biosynthetic cost (r_{bio}) that supports the growth. We consider r_m a constant for a cell that has been photo-acclimated to a specific irradiance, and r_{bio} is therefore the difference between R(measured) and r_m (extrapolated). Results from the experiment showed the dynamics relationship among the four parameters that are critical for cell growth. Interestingly, we found the ratio of respiration to gross photosynthesis declined from 4.91 to 0.20 in response to the instantaneous irradiance increment from 5 to 200 µmol quanta m^{-2} s^{-1}, and stabilized at 0.20 from 200 to 500 µmol quanta m^{-2} s^{-1}, which is different from previous literature suggesting a constant ratio. This has important implications for mass culture where a significant fraction of the crop is out of the well-lit first few centimeters of the pond, and hence will experience significant respiratory loss of the crop.

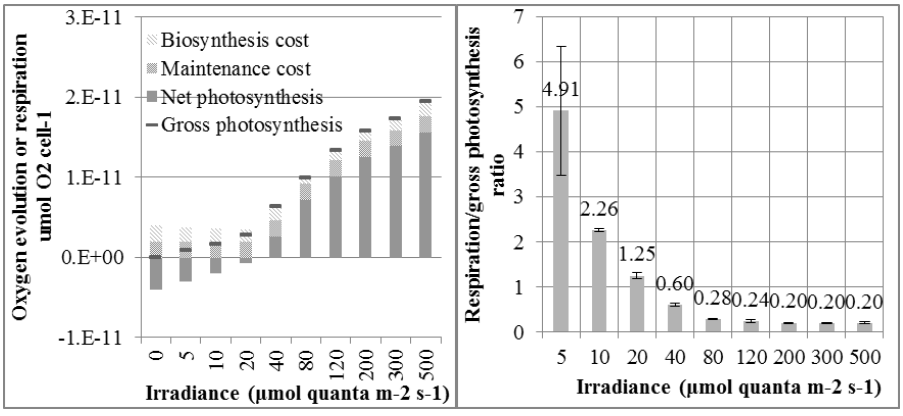


Figure B.1.2 - (Left) Cell specific oxygen evolution/consumption rates allocated into sub-components: net photosynthesis, maintenance cost, and biosynthesis cost, as function of instantaneous irradiance. (Right) Ratios of respiration to gross photosynthesis as a function of instaneous irradiance. Error bars shows the standard deviation, n=2.

B.2 Characterization of carbon dioxide utilization in cyanobacteria at the molecular and cellular levels

Dismukes

Overview: We have constructed and experimentally tested the most complete genomic-scale computational model of photoautotrophic growth by a cyanobacterium, based on 771 genes and 723 metabolites of *Synechococcus sp.* PCC 7002. In contrast to conventional genomic models (FBA), which use a stoichiometry-fixed biomass objective function (BOF), our model incorporates a variable biomass objective function (*v*BOF) in which stoichiometries of major biopolymer products are allowed to vary according to light intensity (accounting for differential fluxes through the photosystems). *v*BOF was constrained by measurements of biomass composition under different light conditions. The model provides rigorous agreement to experimentally measured growth rates, inorganic carbon uptake rates and the carbohydrate/protein content as a function of light intensity during photoautotrophic growth. The incorporation of *v*BOF adds greater metabolic flexibility for simulation of more realistic compositional changes in response to light stresses, thus providing more reliable prediction of growth rate and carbon partitioning. We developed a further advanced model (*t*FBA), which incorporates constraints based on experimentally measured mRNA expression data (transcriptome) under different nutrient conditions. *t*FBA allows qualitative prediction of repartitioning of cellular protein and carbohydrate pools under +/- nitrogen stress.

Research Highlights:

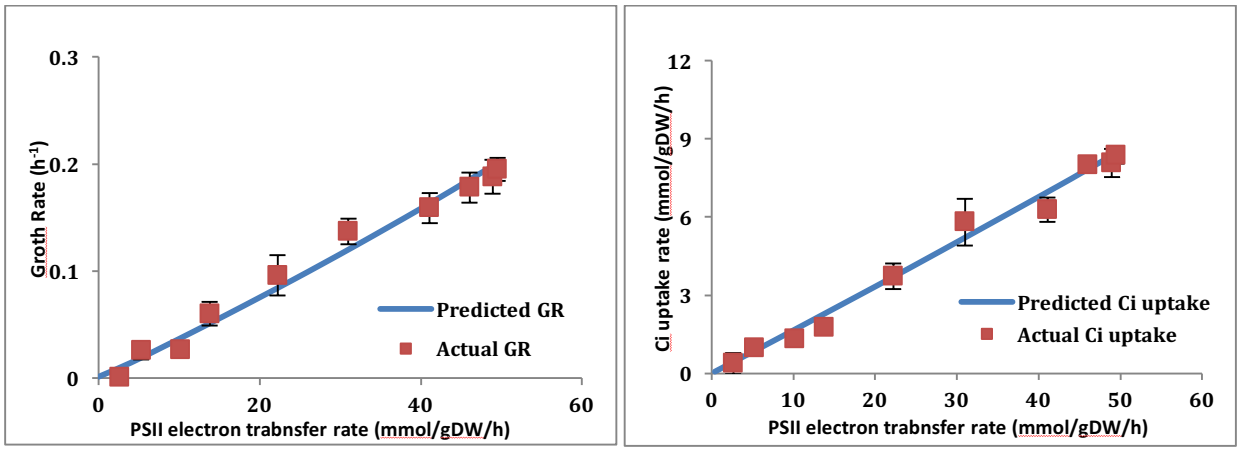
The overall goal of this research was to improve our understanding of CO_2 utilization by cyanobacteria during photosynthesis by developing an accurate and flexible computational model of its metabolism. The cyanobacterium *Synechococcus* sp. PCC 7002 was selected because it has a complete genome sequence with many annotated gene functions, well established transformation tools, and a relatively fast photoautotrophic growth rate of 0.20 d^{-1} [1,2]. This organism is also used for testing of hypotheses used for the development of production strains in the biofuels industry.

The power of computational modeling is that it allows a holistic understanding of how cell integrates metabolic chemistry to produce growth, without doing time-consuming, expensive experiments. We developed a genomic-scale model of metabolism in *Synechococcus* 7002 to predict photoautotrophic growth and biomass composition.

		***i*Syp611** **(Hamilton et al., 2012)**	***i*Syp708** **(Vu et al., 2013)**	***i*Syp771** **(Qian et al., in prep)**
Genes		611	708	771
Reactions (Metabolic and Transport)	**GPR**	517	568	682
	NGPR	35	34	87
Exchange Reactions		37	44	31
Metabolites		554	581	723
Experimentally measured BOF		NO	YES	YES

Table B.2.1. A comparison of input parameters for computational model *i*Syp771 to the two existing models, *i*Syp611 and *i*Syp708, for cyanobacterium *Synechococcus* 7002. GPR: gene & protein for the reaction are known in *Synechococcus* 7002; NGPR: gene & protein for the reaction are not known in *Synechococcus* 7002.

This genomic-scale model, *i*Syp771, features the most comprehensive reconstruction to date of genes and gene products (Table B.2.1) and utilizes an experimentally measured variable biomass objective function (vBOF). The model was validated against experimentally measured biomass growth rate and inorganic carbon uptake rate (Figure B.2.1) and carbohydrate/protein content as a function of light intensity. This model significantly improves the accuracy of predictions of carbon fluxes during photosynthesis and, accounts, for the first time, the repartitioning of carbon between biopolymer productions observed with varying light intensity.



A) B)

Figure B.2.1. Prediction by *i*Syp771 of the growth and inorganic carbon (Ci) uptake rates at different PSII electron flux rates. The predicted (A) growth rate and (B) C_i uptake rate (line) of *Synechococcus* 7002 on A^+ medium as a function of PSII electron transfer rate is shown alongside experimental measurements of growth rate and C_i uptake rate (squares). The results are the mean values of 3 biological replicates.

B.3 Biological nutrient supply & protection

J. Golden, S. Golden, Palenik

Overview: Nutrient supply for algal growth is critical to a sustainable biofuels production system. As currently the most expensive nutrient is nitrogen, our projects largely focused on nitrogen supply. We investigated using nitrogen-fixing cyanobacteria as potential production strains including the possibility of providing nitrogen to other microalgae through co-culturing or transgenic approaches that would reduce algal competition. We also investigated the use of hydrothermal liquefaction waste as a nitrogen and total nutrient source.

Research Highlights:

We devised a strategy of using a nitrogen fixing cyanobacterium to supply nitrogen to co-cultured phytoplankton. The advantage is that fixed nitrogen does not need to be externally supplied as a nutrient and the co-cultured phytoplankton may provide a better lipid profile. We were able to show that the nitrogen-fixing heterocystous cyanobacterium *Nodularia* is halotolerant and grows robustly in greenhouse and outdoor miniponds with relatively little crop contamination. Moreover, it can be co-cultured with diatoms so that the final lipid profile represents both species.

In a novel transgenic approach, we designed a strategy in which a nitrogen-fixing cyanobacterium *Anabaena* PCC7120 will synthesize and export a non-native compound that has a high nitrogen to carbon ratio, and a specific target strain will utilize that compound, which will not be available to non-target species. As proof of concept, we engineered *Anabaena* PCC 7120 to produce octopine, and attempted to engineer *Synechococcus*

elongatus PCC 7942 to utilize octopine. A provisional patent application based on this project and titled "TARGETED DELIVERY OF NUTRIENTS TO RECIPIENT ORGANISMS" has been submitted through UCSD.

Another strategy for nutrient supply is to develop an engineered system where an alga is grown, harvested, and processed using high temperature liquefaction (HTL). After green crude production, the waste is then utilized to re-grow the original alga. Not all algae will grow on HTL waste as we learned. We screened diverse algal strains for growth on amino acids, protein, and HTL waste. We evaluated the performance of these strains in terms of growth rate and final biomass yield. We were able to show that *Haematococcus, Picochlorum*, and a few other species are capable of robust growth on HTL waste, that itself was generated from *Haematococcus* biomass. We thus demonstrated the feasibility HTL waste recycling.

Interestingly, the use of nitrogen fixing cyanobacteria may naturally help with crop protection as these are known producers of toxins that may inhibit grazing. The use of HTL waste may also help in crop protection as we found it is somewhat toxic to algae and presumably other organisms, so HTL waste-using algae would have a strong competitive advantage. Thus it seems that “crop protection” and nutrient utilization have subtle links. Additional research on crop protection is found in task A.6.

B.4 Development and Characterization of a Model Pond

Shurin, Brahamsha, Briggs, J. Golden, S. Golden, Palenik, Mitchell

Microalgal growth models were derived for *Scenedesmus dimosphus* and *Scenedemus* sp-1, two species of interest to our industrial collaborators at Sapphire Energy. We based the model on quantum yield, e.g., moles of carbon fixed through photosynthesis per mole of photons absorbed, as a function of solar irradiance for outdoor ponds exposed to full sunlight. Model inputs are: light intensity, chl-a (cellular chlorophyll a content), carbon and biomass light absorption coefficient (to determine quantum yield), specific growth rate, and algal biomass produced on average per day (productivity) throughout a typical batch growth cycle. The model showed high fidelity in predicting the growth rates of algae as daily sunlight dose varied over a factor of 2 between cloudy and clear days.

Additionally, as part of this task, pond samples were taken from a Sapphire Energy Las Cruces pond that was fertilized, treated for fungal pathogens, and harvested to maintain biomass over one year. Composition of the eukaryotic and prokaryotic communities was characterized by sequencing of the 16S and 18S ribosomal DNA region of the genome from environmental samples. Our goal was to determine the relationships among the diversity of algae, bacteria and other organisms, the production of biomass and its variability over time, and seasonal fluctuations in the environment. Our study determined that biomass productivity was positively correlated with the diversity of eukaryotic algae found in the pond, negatively correlated with the diversity of bacteria, and declined in association with outbreaks of planktonic fungi. We also found lower temporal variation in biomass production during periods of higher algal diversity. Our work supports the importance of algal diversity for maintaining the productivity and resilience of algal biomass in an outdoor, industrial setting over an entire annual seasonal cycle. Management interventions aimed at optimizing algal diversity and species composition are therefore likely to be effective as part of integrated strategies to build productive and robust algal biomass production systems.

B.5 Modeling and analysis of nutrient recycling loops

Kendall

Overview: Nutrient demands in algae biofuel production systems may constitute as much as 12% to 26% of life cycle energy requirement and 22% of CO_2-equivalent (CO_2e) emissions (Lardon et al., 2009; Stephenson et al., 2010). Understanding and optimizing the nutrient flow and balance within the algal oil production system can improve the environmental and economic sustainability of producing algal biofuels. The research conducted in Task

B5 quantifies the nutrient demand and flow during the life cycle of a simulated algal biofuel production system, with integrated energy and nutrient recovery technologies. The study also tracks the flows of water, energy, and carbon to provide a broader picture of mass flows through the system, to identify co-benefits of nutrient recovery, and to serve as the foundation for a detailed life cycle assessment (LCA) model of algal biodiesel production. In Phase 2 of this project we intended to incorporate new information on nutrient recycling based on validation from laboratory studies (see B.1.4 and B.1.5). Laboratory results were not incorporated into the model due to challenges for laboratory pond experiments; however, significant advancements were made in simulating nutrient recycling loops during Phase 2 by incorporating a new conversion pathway that reflects Sapphire Energy's production system; whole-algae hydrothermal liquefaction (HTL). This process yields a biocrude product suitable for upgrading to renewable diesel (in contrast to the lipid extraction pathway modeled in Phase 1 which yields algal oil suitable for biodiesel production), and changes the nutrient, energy, and carbon cycling opportunities.

Research Highlights: This research provides a system-level mass balance of nutrients, carbon and water for algal biofuel production systems. The mass balance model evaluates the energy savings brought by nutrient, carbon, energy and water recycling within the system and provides the underlying framework for a detailed and complete LCA with the capability of assessing the best technology choices and uses of co-products from the system from the standpoint of life cycle energy and greenhouse gas (GHG) emissions.

In Phase 1 of this research, which considered algal biodiesel production based on lipid extraction followed by transesterification, the system level mass balance showed that anaerobic digestion of residual biomass after a wet oil extraction process was the most effective technology for nutrient recycling, and had the added benefit of energy recovery through the production of biogas from the anaerobic digester. Other promising technologies were examined, such as hydrothermal treatments of residuals, but proved to be less effective when considered within the algae oil production system and when technology uncertainty was accounted for. For example, hydrothermal treatments did not provide as much usable energy to the algal system as anaerobic digestion despite producing more gross energy, much of it was in the form of heat, which far exceeded the demand for heat in the algal oil production system. Findings show that when the preferred technologies are used, lipid extraction followed by anaerobic digestion of residuals, algal biodiesel has a life cycle energy requirement of 1.06 – 1.08 MJ/MJ biodiesel, and a life cycle GHG intensity of 73 – 85 g CO_2e/MJ (Yuan et al. 2014, Zhang and Kendall in preparation). These results show the energy return on investment (EROI) is not favorable for algal biodiesel, and the life cycle GHG intensity is only a modest improvement over petroleum diesel (approximately 95 g CO_2e/MJ).

Because this modeling relied on data from existing studies and engineering calculations, uncertainty assessment was required. The sensitivity analysis revealed that CH_4 yield from anaerobic digestion was the most sensitive parameter; a ±15% change in yield of CH_4 could result in a ±35% change in primary energy requirement and ±32% change in GHG emissions. Anaerobic digester performance varies widely based on design and operation practices, this finding indicates that more research is required to optimize algal residual biomass digestion and maximize the energetic and GHG emissions performance of algal oil and algal biodiesel production systems. These results are detailed in two peer reviewed publications; Yuan et al. 2014 and Zhang et al. 2014.

In Phase 2 of this research, whole-algae hydrothermal liquefaction (HTL) resulting in renewable biodiesel was examined and showed significantly improved robustness in carbon intensity results based on scenario analysis. This is illustrated in Figure B.5, where the renewable diesel outcomes are significantly more consistent than biodiesel outcomes.

The large variability in results is, in part, explained by differences in process technologies and operating conditions that affect the type and quantity of co-product generated, and thus the value of co-products. In addition, the method used to conduct co-product allocation also affects the credit assigned to the biodiesel produced. Testing of co-product allocation methods (economic allocation, mass allocation, or displacement) showed that for biodiesel co-product decisions have a significant effect on the performance of the resulting fuel. For HTL pathways that

produce renewable diesel, only economic allocation resulted in a significant difference from all other scenarios, and even then the variability was much smaller than the biodiesel scenarios.

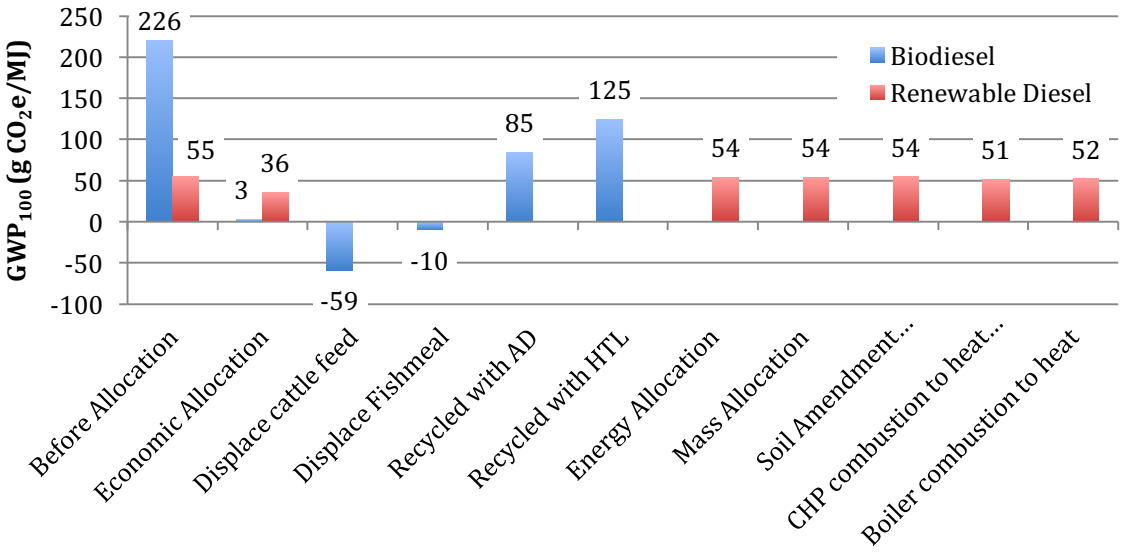


Figure B.5 Carbon intensity per MJ of biofuel for different production pathways and different co-product utilization and allocation methods. Biodiesel refers to lipid extraction pathways, and renewable diesel refers to whole-algae HTL pathways. For reference the carbon intensity of petroleum diesel is estimated at about 95 g CO2e/MJ (Zhang and Kendall, in preparation).

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Genetic Tools

Perhaps our most important successes were achieved in developing genetic tools for cyanobacteria, green algae, and diatoms, as these tools have enabled the entire algal research community to be more productive. The development of genetic tools to enable crop protection and co-product production is critical for the future success and economic viability of algal biofuels.

We made significant headway in developing genetic tools for green algae, cyanobacteria, and diatoms, and these tools have been made available to the entire algae community through Life Technologies or material transfer agreements. These tools have enabled the activities of our corporate partners, as well as the greater academic and industry algal communities. These tools will be enhanced and expanded under continued support and rapidly deployed into the commercial and academic sectors to continue to drive commercial viability. To rapidly deploy these new tools, we made them available to everyone in the algal community through the Life Technologies catalog, where over 150 algae products are now listed and available for rapid world-wide distribution. Additional tools that could not be made available through the LT catalog have been published and are available by material transfer agreement from UC San Diego, including the protein-targeting vectors shown in Figure C.1 below. We have also generated a set of synthetic promoters that are capable of driving high nuclear expression that will enable synthetic engineering in green algae. All of these tools will be made available to the algal community via distribution through Life Technologies or by MTA from UC San Diego.

The genetic tool kit developed for cyanobacteria is fully supported by an advanced web-based interface that allows in silico design of genetic tools. We have used this system to create and test over 70 genetic parts and devices including: origins of replication, improved broad-host-range plasmids, homology regions for chromosome engineering, antibiotic markers, and functional devices for gene expression. The genetic tools developed in this project have been used for several basic research applications including efforts to understand the cyanobacterial host-predator interactions. As a proof-of-principle metabolic engineering application, the system was also successfully used to engineer the production of polyunsaturated fatty acids into several different cyanobacterial species.

For diatoms, a set of highly useful genetic manipulation tools were developed including: 1) new promoters; 2) new antibiotic resistance selectable markers; 3) markers that enables selection without GMO classification; 4) RNAi and antisense approaches for gene regulation; and 5) an array of fluorescent proteins and targeting vectors for sending proteins to multiple cellular locations. Specific genetic tool projects undertaken and their results and highlights are detailed below.

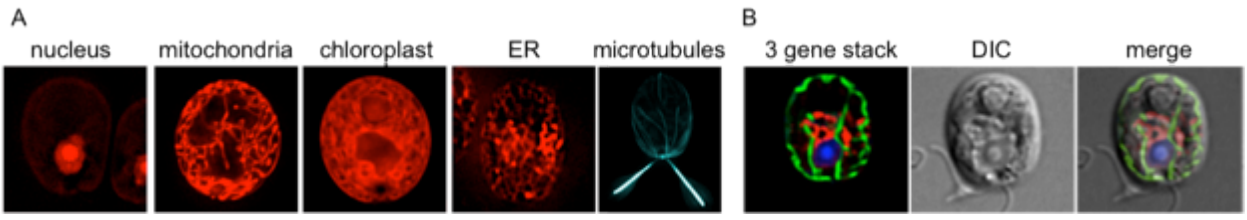

Figure C.1. Gene stacking and protein targeting. A. Nuclear encoded fluorescent reporter proteins were targeted to specific subcellular compartments using protein targeting domains. B. Gene stacking was used to generate a transgenic strain with Venus targeted to the mitochondria (green) mCherry directed to the Endoplasmic Reticulum (ER, red) and mCerulean localized to the nucleus (blue).

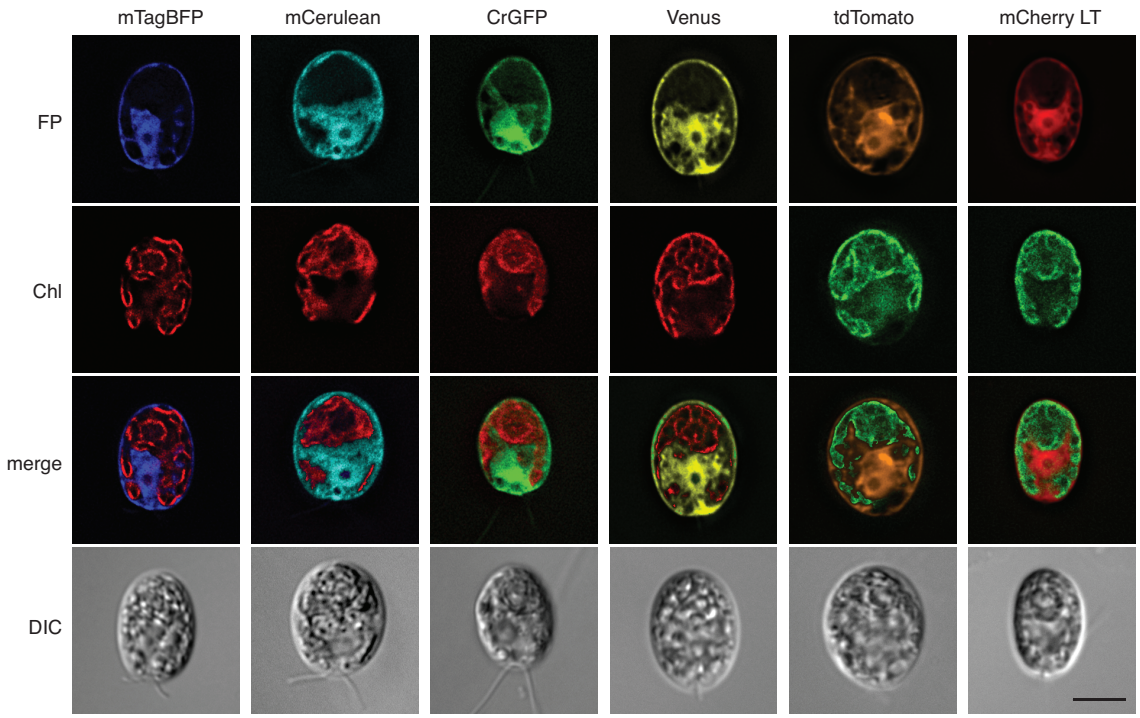

Figure C.1.1. Live cell fluorescence microscopy of the FP-expressing strains. Live cells were plated on agar pads and subjected to deconvolution fluorescence microscopy. Images were collected using the optimal filters for each FP. Chlorophyll auto-fluorescence images were collected using the best alternative filter set available. Differential interference contrast (DIC) images are also shown. Chl, chloroplast autofluorescence. Scale bar, 5 µm.

C.1 Develop additional selectable markers and crop protection tools for green algae and diatoms

Mayfield, Weeks, Falkowski

Overview: Microalgae have recently attracted attention as potential low-cost platform for the production of a broad range of commercial products including biofuels, nutraceuticals, therapeutics, industrial chemicals and animal feeds; and genome engineering will enable and enhance algae-produced bio-products. However, while much has been written about the potential of transgenic microalgae, little of that potential has yet to be commercialized. A major obstacle to generating useful transgenic algae strains has been the lack of molecular tools and overall poor expression of heterologous genes from the nuclear genome of many microalgae species, at least partially due to rapid gene silencing. Furthermore, a set of validated vectors for targeting transgene products to specific subcellular locations do not exist, nor does the vector to allow the expression of multiple nuclear-encoded genes within a single cell. Our objective in the task was to address these critical areas – transgene silencing, protein targeting, and gene stacking. But first, we developed essential molecular genetic tools to aid in our understanding of algal biology and the development of algal biotechnology.

Our project successfully created numerous tools for the high throughput characterization of algae. To build on this project, we demonstrated the utility of these newly developed tools through use of high-throughput genetic screens and fluorescent proteins.

Research Highlights:

The Mayfield Lab's first objective for this task was to develop and validate a set of fluorescent and enzymatic reporters for the use in the green alga *Chlamydomonas reinhardtii*. Fluorescent protein (FP) technology has revolutionized many fields in the life sciences, including molecular biology, cell biology, biomedicine and biotechnology. Indeed, the 2008 Nobel Prize in Chemistry was awarded for discovery and development of the Aequorea victoria green fluorescent protein, avGFP. Today, FP technology is being used in an ever-expanding list of applications and thus has become an essential tool for biological research. However, FPs had not been widely developed for microalgal research.

To overcome silencing of nuclear-encoded transgenes, we developed a nuclear transformation vector that transcriptionally fuses the transgene-of-interest to the selection marker, ble. A viral self-cleaving sequence (2A) was cloned between the selection marker and the transgene so that an unfused recombinant protein product accumulates. This strategy leads to up to 100-fold better recombinant protein expression and our vector has been made available to the research community through Life Technologies (*Chlamydomonas* Protein Expression Kit, A24244).

We codon-optimized, synthesized, cloned, and transformed 8 FPs: mTagBFP, mCerulean, mEmerald GFP, Venus, tdTomato, Cerianthus OFP, mKO2, and mCherry. All were well-expressed from the nuclear genome of C. *reinhardtii*. Each FP was validated by flow cytometry, live-cell microscopy (Figure C.1.1), western blotting, and fluorescence microplate reader analysis. In addition, we demonstrated successful FP protein-tagging by fusing FPs to endogenous proteins (i.e. alpha-tubulin and histone H2B). Live-cell microscopy was used to visualize protein localization and monitor function. Our results were recently published in The Plant Journal. We also synthesized and validated two enzymatic reporters: *Gaussia* luciferase and β-glucuronidase.

Next, we constructed and validated a set of protein targeting vectors (Figure C.1.2A). Targeting vectors enable recombinant protein localization to precise subcellular locations and are crucial for advanced genetic and metabolic engineering. We developed vectors to target proteins to the nucleus, endoplasmic reticulum (ER), mitochondria, chloroplast, and for secretion (Rasala et al., Plos One, in revision). We also developed a multi-cistron vector that enabled transgene stacking following a single transformation event. For example, nucleus-targeted mCerulean was co-expressed with ER-targeted mCherry (Figure C.1.2B).

We then developed a mating strategy to stack up to four transgenes within a single cell. All recombinant proteins were well-expressed and targeted to four different subcellular locations (Figure C.1.3).

Finally, all high-expressing fluorescent strains were obtained through fluorescent activated cell sorting, we which have demonstrated is efficient in pulling out high fluorescent protein expressing algal cells (Figure C.1.11).

For diatoms, new antibiotic resistance selectable markers were developed and tested for selection on appropriate herbicides.

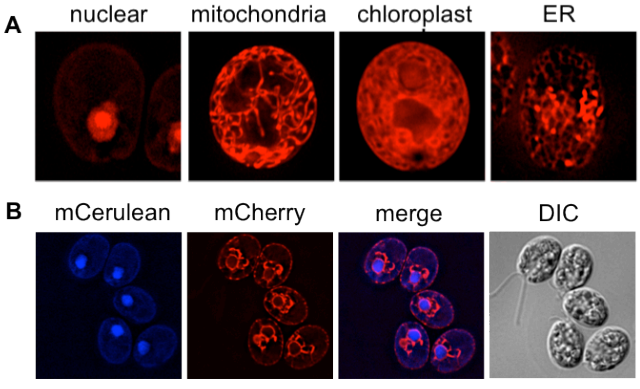

Figure C.1.2. Protein targeting and gene stacking vectors.

(A) Nuclear genome transformation vectors were constructed to targeted proteins of interest to the nucleus, mitochondria, chloroplast, and ER.

(B) A multi-cistronic vector was designed and validated to co-express two transgenes. mCerulean was targeted to the nucleus and mCherry was sent to the ER.

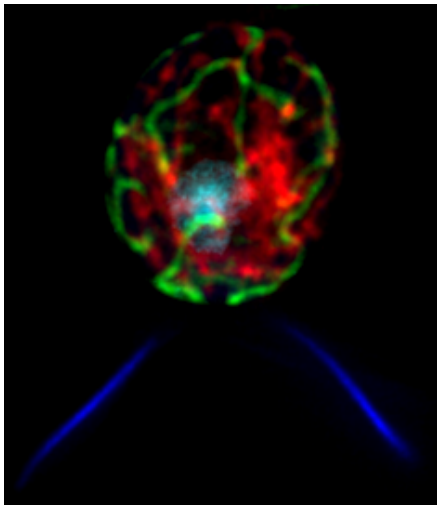

Figure C.1.3. Gene stacking through mating. Mating was used as an alternate strategy to stack up to four transgene with a single cell. This clone expresses mTagBFP-tagged alpha-tubulin (blue), mitochondria-targeted Venus (green), ER-targeted mCherry (red), and nuclear-mCerulean (cyan).

C.2 Develop advanced tools for genetic and metabolic engineering of green algae.

Mayfield, Oyler, Van Etten

Overview: A major obstacle to generating useful transgenic algae strains has been the lack of molecular tools and overall poor expression of heterologous genes from the nuclear genome of many microalgae species, at least partially due to rapid gene silencing. Viral promoters, which tend to be more robust than endogenous promoters, have been used in other eukaryotic expression hosts to boost transgene expression. One of the objectives of this task was to test a set of viral promoters in C. *reinhardtii* for their ability to drive expression of a reporter fusion construct: ble-GFP. Ble confers resistance to the antibiotic zeocin, and GFP is a fluorescent reporter whose expression is detected using a fluorescence microplate reader.

Our results for testing of promoter elements led to identification of functional but not enhanced promoter elements. As previously we have had success in combining functional elements we will explore these elements further in the coming year.

Research Highlights: Two vectors were designed and built to complete this task (Figure C.2.1). The first vector, pBR11, was designed to clone viral sequences upstream of the reporter fusion construct, *ble-GFP*, and thus tests the ability of the chosen viral elements to function as **promoters** in C. *reinhardtii*. *Ble* is a gene that confers resistance to the antibiotic zeocin. GFP is a fluorescent protein that can be detected in C. *reinhardtii* by flow cytometry. The second vector, pBR10, was designed to clone viral elements upstream of an endogenous C. *reinhardtii* promoter, *rbcs2*, driving the expression of *ble-GFP*. pBR10, therefore, tests the ability of the chosen viral elements to act as **enhancer elements**.

The chosen viral elements are of two classes. The first class were viral sequences that are commonly used in other species for transgene over-expression: AMT promoter from Chlorella NC1A virus, SV40 promoter from simian virus 40, CMV promoter from cytomegalovirus, and CaMV 35S promoter from cauliflower mosaic virus (Table C.2.1). The second class of viral sequences were all cloned from Paramecium bursaria Chlorella virus 1 (PBCV-1; Table C.2.2). Ten sequences were chosen based on gene expression data from the Van Etten laboratory. Sequences were cloned up to 500 bp upstream of the highly expressed open reading frames: A158L, A214L, A256L, A289L, A308L, A312L, A404L, A441L, A625R, and A1260L. All 14 viral sequences were subcloned into the pBR11 promoter vector and pBR10 enhancer vector.

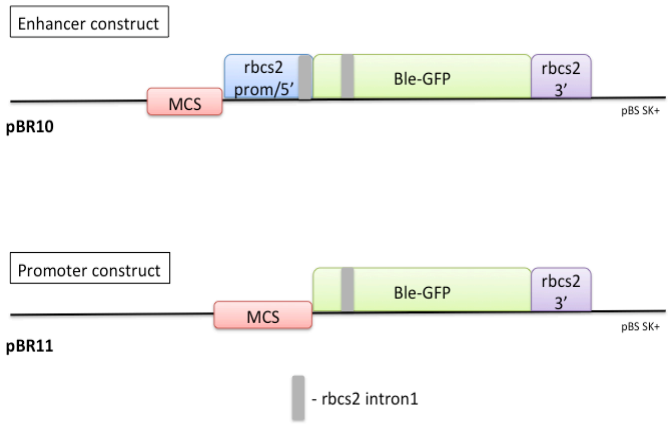

Figure C.2.1. Vector maps of pBR10 (enhancer construct) and pBR11 (promoter construct). The selected viral sequences were cloned into the multiple cloning site (MCS) for each vector. pBR10 contains a minimal endogenous rbcs2 promoter driving the expression of the reporter construct ble-GFP. The viral sequences were cloned upstream of the rbcs2 promoter and were assay for the ability to enhance the expression of ble-GFP from the rbcs2 promoter. The viral sequences were also cloned directly upstream of ble-GFP in the pBR11 construct and therefore tested for the ability to act as promoters in *C. reinhardtii*.

Table C.2.1. Viral promoters selected for *C. reinhardtii* studies based on successes in other organisms

Viral Promoter	Host virus	Commonly used in:
CMV IE1 promoter	Human cytomegalovirus	Mammalian cells. Commonly found in commercially available vectors from mammalian overexpression.
SV40 early promoter	Simian virus 40	Mammalian cells. Commonly found in commercially available vectors from mammalian overexpression.
AMT promoter	Chlorella virus NC1A	Higher plants, bacteria
CaMV 35S promoter/ TMV leader fusion	Cauliflower mosaic virus Tobacco mosaic virus	Higher plants. Commonly found in commercially available vectors from plant cell overexpression.

Table C.2.2. PBCV-1 sequences selected based on viral gene expression studies from the laboratory of James Van Etten.

ORF	Time of expression	Function
A158L	10 min	unknown
A625R	20 min	unknown
A312L	60 min	unknown
A289L	60 min	Signaling
A256L	60 min	unknown
A214L	60 min	unknown
A441L	60 min	unknown
A308L	60 min	unknown
A1260R	60 min	Cell wall degradation
A404R	120 min	unknown

The 14 viral sequences cloned into pBR10 and pBR11 were tested for promoter and enhancer function in C. *reinhardtii*. Briefly, cells were transformed with the 28 constructs, along with control vectors, and selected on TAP/ 15 ug/ml zeocin plates. For the pBR11 promoter constructs, only viral elements that display promoter activity in C. *reinhardtii* will support *ble-GFP* expression and therefore form colonies on the selection plates. For the pBR10 enhancer constructs, the number of colonies yielded were documented and compared to the empty vector, which

contains just the minimal *rbcs2* promoter. Bonafide enhancer elements should lead to increased number of colonies on the selection plates. Enhancer activity was further characterized by analyzing GFP accumulation by flow cytometry.

Table C.2.3 summarizes the results for this objective. The AMT promoter from NC1A did not yield any zeocin-resistant clones, and therefore does not function as a robust promoter in C. *reinhardtii*. Further, when the sequence was cloned upstream of the endogenous rbcs2 promoter, fewer colonies were obtained compared to just the rbcs2 promoter alone. This data suggests that not only does AMT not act as an enhancer, but that it reduces or inhibits transcription from the rbcs2 promoter. Similar results were obtained with CMV and SV40 (Table C.2.3). Neither CMV nor SV40 yielded significant zeocin-resistant clones when cloned in the promoter position. And both also led to reduced colonies when cloned in the enhancer position compared to the rbcs2 promoter alone. However, the CaMV promoter did support *ble-GFP* expression, yielding significant zeocin-resistant colonies when cloned in the promoter position, and led to slightly more colonies compared to the rbcs2 promoter alone when cloned in the enhancer construct. Flow cytometry analysis of the CaMV sequence suggests that CaMV supports similar levels of GFP accumulation to the rbcs2 promoter (data not shown).

Table C.2.3 also summarizes the results obtained from the second class of viral sequences, selected elements cloned from PBCV1 that are located upstream of highly expressed ORFs. Of the ten tested, none were able to function as promoter elements in C. *reinhardtii*. Furthermore, six sequences led to significantly reduced colony formation when cloned into the enhancer construct compared to the rbcs2 promoter alone. The other four had either no positive effect or a slight suppression effect on rbcs2.

In summary, of the 14 viral promoters tested, only one – CaMV – functioned in C. *reinhardtii*. These results are surprising, and suggest that C. *reinhardtii* may possess a strict set of rules for promoter function.

Table C.2.3. Summary of the performance of viral promoters/sequences in C. reinhardtii.

Viral sequence	Promoter function in Chlamydomonas?	Effect on endogenous rbcs2 promoter
Common viral promoters		
CMV IE1	No	Suppression
SV40 early promoter	No	Neutral
NC1A AMT	No	Suppression
CaMV 35S/ TMV leader	**Yes**	**Neutral/slight enhancement**
PBCV-1 sequences		
A158L	No	Suppression
A625R	No	Suppression
A312L	No	Neutral/slight suppression
A289L	No	Neutral/slight suppression
A256L	No	Neutral/slight suppression
A214L	No	Neutral/slight suppression
A441L	No	Suppression
A308L	No	Suppression
A1260R	No	Suppression
A404R	No	Suppression

C.3 Develop Cyanobacterial genetic tools

S. Golden, J. Golden, Brahamsha, Palenik

Objectives: Our overall objectives were to develop broad-host-range genetic tools for synthetic biology approaches in diverse cyanobacterial strains. Our specific aims were to produce new or improved replicating and integration vectors, along with new or improved selectable markers, promoters, and reporter genes, and to test the function of these devices in several cyanobacterial strains, including the newly identified potential production strains *Leptolyngbya* sp. BL0902 and *Synechocystis* sp. WHSyn.

Research Outcomes:

Inspired by the need for improved genetic tools to exploit cyanobacteria for the production of renewable bioproducts, we used state-of-the-art synthetic biology approaches to construct host-vector systems for knockouts, knockins, and regulated heterologous expression of genes and pathways in a broad range of cyanobacteria. We developed a versatile platform that includes the following features. (i) 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. (ii) A growing library of devices categorized in 3 major groups: (a) replication and chromosomal integration; (b) antibiotic markers; (c) expression cassettes, reporter cassettes, and promoter-reporter modules. (iii) A web service, the CYANO-VECTORs assembly portal, which was built to organize the various modules, facilitate the *in silico* construction of shuttle vectors, and share our toolkit with the scientific community. This work also resulted in the construction 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 elongatus* PCC7942, *Synechocystis* sp. WHSyn, *Synechocystis* sp. PCC6803, *Anabaena* sp. PCC7120, and *Leptolyngbya* sp. BL0902.

Background and Significance:

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 (3, 7), 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 (2), the presence of different restriction/modification systems (1), the presence of interacting differentiating cell types in some species (4), and their circadian rhythms (5). 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 (6) (i) by defining and developing standards and (ii) by the characterization of parts and devices. In the synthetic

biology lexicon, 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. We have focused on producing broad-host-range parts and devices for synthetic biology research and development.

C*onclusions:* To enable advanced synthetic biology methods for metabolic engineering and strain improvement in both model organisms and production strains of cyanobacteria, we have developed an integrated and expandable platform 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, we 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 and this platform is easily expandable by addition of new biological devices carrying the proper GC-adaptor overlapping sequences.

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C.4 Developing genetic tools and co-products for brown algae

Hildebrand

O*verview*: Diatoms are excellent candidate organisms for biofuels production because of their intrinsic growth and lipid accumulation characteristics1. Fundamental genetic manipulation tools have been developed for some species of diatoms, but not for model or production strains for biofuels. The objectives of this task were to develop a set of genetic manipulation and selection tools for the model species *Thalassiosira pseudonana*, and to transition the tools into the potential production strain *Cyclotella cryptica*, to enable a sophisticated level of genetic manipulation, controllable protein expression, and controlled intracellular targeting of proteins. The overall goal was to develop genetic manipulation approaches that would put diatoms on a par with other model organisms.

Highlights of the research: One desirable capability for genetic manipulation is to be able to control expression of mRNA to different levels. We identified four different expression control elements (promoters) that enabled such control (Fig. 1). In addition, we characterized the SIT1 silicon transporter promoter, which allowed highly inducible expression during silicon starvation, and little expression in the presence of silicon. Use of these promoters allows tailored and inducible expression of genes placed under their control.

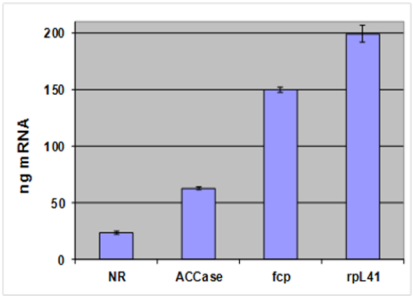

Fig. 1. Control of gene expression in *T. pseudonana.* mRNA accumulation levels driven by expression control elements from four different genes, nitrate reductase (NR), acetyl-CoA Carboxylase (ACCase), fucoxanthin-chlorophyll binding protein (fcp) and ribosomal protein L41 (rpL41). Data are qRT-PCR measurements of RNA isolated from exponentially-growing cultures.

The SIT1 promoter has proven especially useful in protein expression studies because it represses expression in the presence of silicon during growth, and induces expression under silicon starvation and lack of growth. This enables expression of proteins that are detrimental to the cell. Using this promoter and applying other improvements resulted in expression of a test protein in *T. pseudonana* to 1.2% of total soluble protein (TSP), and in *Cyclotella cryptica* to 1.8% TSP.

Sophisticated metabolic engineering to improve production characteristics could involve manipulation of multiple genes. This will require sequential insertion of genes into a strain, using multiple selectable markers. We developed two new antibiotic resistance markers for *T. pseudonana* in this project. Codon optimization of the Sh ble gene enabled zeocin resistance. Collaborative work with the Weeks lab enabled the development of a codon optimized glyphosate acetyltransferase that engendered resistance to glyphosate. This was the first demonstration that a herbicide resistance gene could be used for selection in a diatom.

Highly effective transcript knockdown approaches using RNAi and antisense were developed on this project. Initially tested by knocking down silicon transporter genes, the approaches have been applied to several genes with similar effectiveness (e.g. 2). With typical RNAi or antisense constructs, extensive screening of transformants is required to isolate strains with good knockdown characteristics, typically two transformants out of 50. Consistently with our constructs, out of 6 transformants screened, 3-4 have excellent knockdown capability.

All of the vectors developed for genetic manipulation used the Gateway™ system, which allows rapid generation of constructs, and easy swapping of components.

One set of Gateway™ vectors were tagged expression vectors for one step cloning with a variety of fluorescent reporters, including CFP, eGFP, mWasabi, bflo1, eYFP, RFP, and TagRFP. All of these reporters worked in *T. pseudonana* without codon optimization. These constructs were used to target expressed proteins to various cellular locations, which aided in elucidation and manipulation of metabolic processes. We have successfully targeted fluorescent proteins to the cytoplasm, plasma membrane, chloroplast, chloroplast membrane, pyrenoid, mitochondria, peroxisome, endoplasmic reticulum (ER), chloroplast ER, as well as a previously uncharacterized cellular compartment. Examples are shown in Fig. 2. In addition, transgenic lines containing two fluorescent

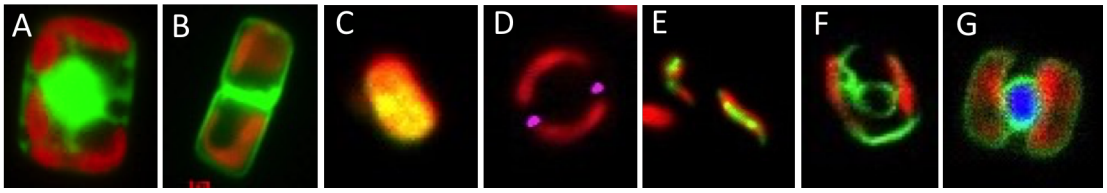

Fig 2. Fluorescent proteins expressed at various locations by virtue of specific signal peptides. A) cytoplasm; B) plasma membrane; C) chloroplast; D) mitochondria; E) pyrenoid; F) endoplasmic reticulum (ER); G) chloroplast ER).

markers (GFP and RFP) were generated to more precisely clarify intracellular location between closely associated compartments.

A novel and highly efficient mutagenesis and selection approach was developed that enabled isolation of large numbers of high lipid accumulating mutants with no adverse effects on growth 3. Typical mutagenesis and selection approaches result in 1:10,000 isolated mutants, the new procedure enriches for mutants prior to plating such that 36% of screened mutants had substantially improved lipid accumulation ability. This is a 3,600x improvement. Because the approach involved iterative selection in liquid, competitive growth was a selective pressure, and all mutants grew just as well as wild-type. Plate screening does not include a requirement for competitive growth, and mutants with detrimental secondary mutations are common, reducing the potential applications of mutants. Because a large number of mutants can be isolated, multiple genotypes and phenotypes may be represented, which will greatly facilitate genome resequencing approaches to identify the mutation locus responsible.

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Manandhar-Shrestha,K, Hildebrand, M. Development of flow cytometric procedures for the highly efficient isolation of improved lipid accumulation mutants in a Chlorella–like microalga. J. Appl. Phycology. DOI 10.1007/s10811-013-0021-8.

Mark Hildebrand and Kalpana Manandhar-Shrestha. U.S. Provisional Application Serial No. _61/836,848, SELECTION OF MICROORANISMS. 2013.

C.5 Develop methods for the rapid generation and expression of high affinity nanobodies to promote crop protection, facilitate harvesting, and express high-value co-products.

Oyler, Mayfield

Overview: The ability to produce high affinity nanobodies recognizing green algae surface antigens was successfully demonstrated and resulted in one patent application and two publications to date. Even greater success was obtained in the production of high-value co-product by expression of anti-botulinum toxin nanobodies in *Chlamydomonas* chloroplast. The functionality of the algae expressed nanobody and ability to remain stable and active in oral feeding have been demonstrated and resulting in a publication and patent application. Preliminary feeding trials of the anti-botulinum toxin nanobody expressing *Chlamydomonas* demonstrated protection of mice from oral botulinum toxin. Further development of this system may offer potential for a novel therapy for human infant botulism as well as proof of concept for algae expressed nanobodies to treat diseass of the intestinal tract both in animals and humans caused by toxins or pathogenic bacteria. Further development of nanobodies as high value co-products expressing anti-campylobacter, anti-Shigatoxin, and anti-E. coli ETEC pathogenic nanobodies. These nanobodies have been cloned in to both *Chlorella vulgaris* chloroplast targeting vectors and *Chlamydomonas* chloroplast as well as *Chlorella vulgaris* and *Chlamydomonas* nuclear genome expression systems was completed to allow the development of *Chlamydomonas* expression of nanobodies as functional feed additives in chickens and livestock to improve food safety and reduce animal feed antibiotic use. Expression of the nanobodies in relevant levels has been demonstrated in both the *Chlamydomonas* chloroplast and the nuclear vectors. While there have been several promising transformants of the *Chlorella vulgaris* chloroplast and nuclear systems protein expression of the nanobodies is awaiting confirmation. This effort has provided high value transformed algae for further testing in humans and animals of nanobodies specifically: 1. Anti-botulinum, 2.

anti-campylobacter, 3. Anti-Shiga toxin, and 4. Anti-E. coli ETEC nanobodies in *Chlamydomonas* chloroplast and nuclear systems for advancement to animal and eventually human texting. Further proof of concept of this novel way of expressing and delivering high value products in algae has successfully been provide by this research.

Research Highlights:

We have expressed camelid VHH domains in C. *reinhardtii* chloroplast, which function as antitoxins against the botulinum neurotoxin (BoNT). These single-chain variable binding domains were expressed as either 2 different monomers (C2 or H7) or a heterodimer (H7-fs-B5). In neutralization assays using primary rat neurons, all were capable of neutralizing BoNT, and the heterodimer was able to neutralize at near equimolar concentrations with the toxin. Furthermore, we were able to demonstrate that the antitoxin was stable inside the gastrointestinal tract when delivered in antitoxin-producing microalgae fed to mice.

Moving forward, since we have evidence that the antitoxins are functional and are stable inside the gut, we will perform in vivo BoNT challenge experiments in mice to see if algae-produced antitoxins can serve as a therapeutic treatment for botulism. This will involve administering a lethal dose of BoNT to mice orally, followed by administration of antitoxin-producing microalgae to investigate therapeutic value in a real world scenario. The concept of algae expression of nanobodies as functional food components is being extended to expression of anti-Campylobacter nanobodies to improve food safety.

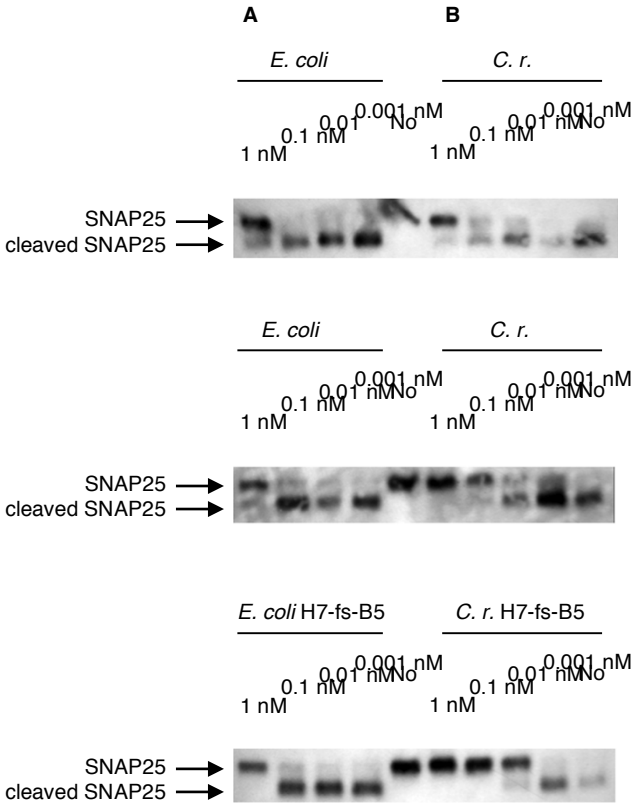

Figure 5. *C. reinhardtii* chloroplast produced V_HH domains neutralize BoNT/A. (A) V_HH domains produced from *E. coli* were capable of preventing BoNT/A from cleaving its target SNAP-25 when co-administered with toxin to rat primary neuron cells. A western blot of SNAP-25 integrity is shown for harvested primary neuron cell cultures treated with each V_HH. Domains C2 and H7 were capable of preventing cleavage, although not entirely, at 1 nM anti-toxin. The heterodimer B5-fs-H7 was able to completely neutralize BoNT/A at 1 nM, and had some anti-toxin activity at 100 pM. The lane indicating "no toxin" contained cells incubated without an anti-toxin and serves as a negative control. (B) V_HH domains produced from *C. reinhardtii* chloroplasts also demonstrated anti-toxin activity. Domain C2 was capable of preventing SNAP-25 cleavage at 10 nM. Domain H7 offered complete neutralization at 1 nM and partial neutralization at 100 pM. The heterodimer B5-fs-H7 had potent anti-toxin activity, completely neutralizing BoNT/A at 100 pM and mostly neutralizing BoNT/A at 10 pM.

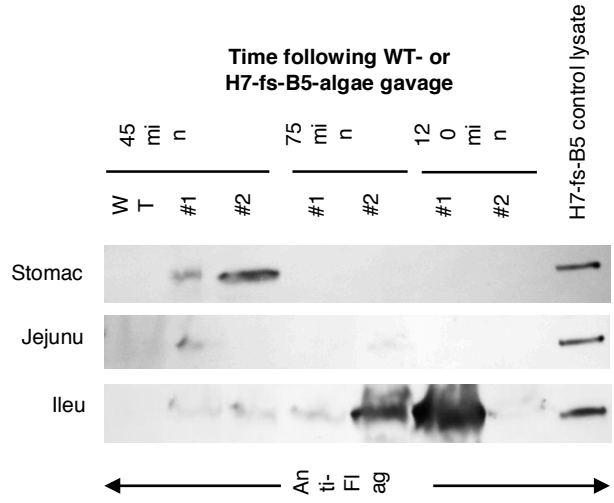

Figure 6. Orally administered microalgae producing H7-fs-B5 can deliver intact antitoxin to mouse stomach and small intestine. Whole cell fresh algae was administered to mice by oral gavage, and sections of the gastrointestinal tract were examined 45, 75 or 120 minutes later. Two mice (#1 and #2) were examined per time point for antitoxin (H7-fs-B5) by blotting for Flag-tag of H7-fs-B5, and one mouse given wild type algae (WT) was examined after 45 minutes. The last lane on the right is control lysate derived from the the same preparation of antitoxin-producing algae orally administered to mice. Antitoxin was detected in the stomach at 45 minutes post-feeding, but moved to jejunum and ileum by 75 minutes and was primarily found in the ileum (or passed into colon) by 120 minutes.

C.6 Enable homologous recombination in the *Chlamydomonas reinhardtii* nuclear genome

Mayfield

Overview: The inability to efficiently achieve homologous recombination in the nuclear genome has hampered complex metabolic engineering as well as a reverse genetics approach to elucidate nuclear gene function. Previous reports of homologous recombination relied on endogenous gene targeting, often with low reproducibility and ambiguous readouts for recombination events. Our objectives were to develop a high-fidelity, high-throughput system to reliably measure the homologous recombination rate; use this system to quantitate a reproducible basal recombination rate; and then test for incremental increases in homologous recombination efficiency using a suite of approaches. Increasing the HR rate of C. *reinhardtii* could have profound impacts on all aspects of genome engineering in green algae, and as such, we will continue to develop these technologies as described.

Research Highlights:

The recombination detection system we developed consists of two polycistronic plasmids, each of which contains a non-functional half of the hygromycin resistance gene with an artificial intron inserted in the middle of the gene to provide additional homology (Figure C.6.1). The first plasmid is incorporated into an algal nuclear genome at random by non-homologous end-joining. Then, the second plasmid is introduced into this recipient strain, and only a homologous recombination event will result in hygromycin resistance.

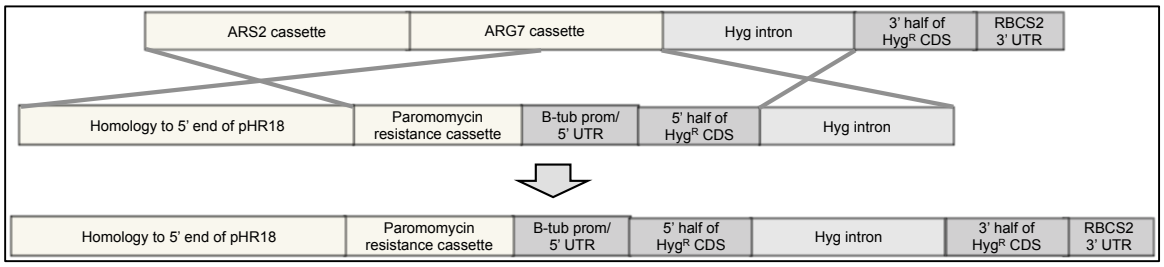

Figure C.6.1. The recipient strain creation vector, shown at top, is first introduced into a naïve strain. Subsequently, the complementing vector is introduced; only a recombination event in the homology provided by the artificial hygromycin intron will confer hygromycin resistance. A double recombination event will also disrupt the ARG7 gene, leading to arginine auxotrophy.

This system provides highly reproducible measures of homologous recombination efficiency using two readouts: first, an intact paromomycin resistance in the second plasmid allows measurement of overall transformation efficiency; secondly, hygromycin resistance indicates recombination events. The intron in the middle of the hygromycin resistance gene allows imperfect recombination events to also be observed, such as insertions and deletions in the crossover region. We can screen for recombinants in a high-throughput fashion, testing hundreds of thousands of transformants in a single experiment for growth on hygromycin (Figure C.6.2). This system and the results from the first four strategies for increasing HR have been assembled in a manuscript which has been accepted pending revision at Algal Research.

Using this system, we have determined the basal homologous recombination rate in strains grown in constant light to be around 6 x 10^{-5}; this rate holds even when additional variables strongly affect the overall transformation efficiency. We tested several approaches for increasing homologous recombination frequency, including two that had previously been reported in the literature. Contrary to one previous report, we did not find that using particle bombardment significantly increased recombination compared to

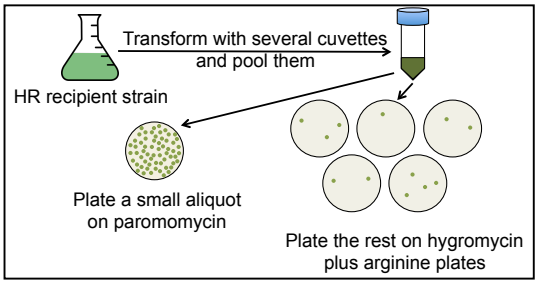

Figure C.6.2. Schematic of the high-throughput screening protocol to detect rare recombination events.

electroporation. However, in agreement with a report indicating that single-stranded DNA is a more effective substrate for homologous recombination, we found approximately a ten-fold increase in recombination efficiency using a plasmid in which one strand had been digested. Using diurnally cycled cultures, we were also able to achieve over 8-fold higher recombination during the middle of the light cycle. Finally, incubating the DNA with a suite of DNA-binding proteins prior to transformation increased homologous recombination by approximately 2- to 5-fold. These are very promising results, and indicate that perhaps combinations of multiple approaches may have a synergistic effect to drastically increase homologous recombination efficiency.

In addition to the initial set of strategies, new methods remain under investigation for increases to HR rate. Due to its success in a diverse set of other organisms, the CRISPR/Cas system is being developed for use in C. *reinhardtii*. We have encountered difficulties with both the expression of the Cas9 protein in vivo as well as introducing purified Cas9 protein through electroporation. Because of the incredible utility of this system, development remains a priority. Also in development are a set of protein knockout strains that are deficient for various components of non-homologous end-joining repair. This repair mechanism is the predominant means by which C. *reinhardtii* repair DNA, in lieu of homologous recombination. It has been shown in *P. pastoris* that knocking out proteins associated with NHEJ can upregulate HR. The knockout strains are under investigation using our HR detection system. These next-generation strategies pose the best chance at dramatically altering the HR efficiency to develop molecular tools for C. *reinhardtii*.

C.7 Use over-expression of algal transcription factors to improve traits including crop protection, growth and co-product production

Mayfield

Overview: While expressed and environmentally regulated genes have been identified in C. *reinhardtii* (Cr), the transcription factors (TF) that result in changes of the genetic program are unknown. To date, we cloned over 100 native transcription factors (TFs) from the 340 total TFs founding C. *reinhardtii*. We developed a yeast one-hybrid assay to characterize the binding activity of a CrTF library to some of the most highly expressed endogenous C. *reinhardtii* promoters (Figure C.7.1). We have over-expressed several of these TFs, and are presently characterizing the transcriptional activation of native C. *reinhardtii* genes in response to this over-expression.

To fully take advantage of these tools, we also need to develop genetic tools for conditional gene expression in C. *reinhardtii*. Continued analysis of the array of CrTF-promoter combinations will eventually inform promoter design to improve transcription factor binding and invaluably enable functional characterization of little-known CrTFs. As TFs undoubtedly play a major rule in algal traits the results from this characterization will enable future construction of synthetic networks for trait improvements.

Research Highlights:

The yeast one-hybrid (Y1H) assay is a well-established, high-throughput assay to test a protein's ability to bind to a DNA sequence. For this study, putative CrTFs have been fused to the yeast GAL4 transcriptional activation domain. Promoter elements were cloned upstream of a yeast minimal promoter element and the reporter gene *Gaussia luciferase*. Each vector is transformed into separate haploid *Saccharomyces cerevisiae* cells and in this way the CrTF library can be crossed against the opposite mating type of any strain harboring a DNA bait of interest. The ease of outcrossing the library allows for many combinations of CrTFs and promoters to be tested in a 96-well plate format. Conveniently, luciferase activity is measured in whole yeast cells using the substrate coelenterazine. Our data has shown that CrTFs can be produced within *S. cerevisiae* cells with most of the 93 CrTFs having high enough accumulation to be detected by western blotting.

Our experiments have described several CrTF and promoter binding combinations. Twenty-five different Cr promoters have been assayed for CrTF binding and a total of 15 binding hits were identified from 10 different CrTFs. Figure 7.1 shows an example for results from CrTF Y1H assay. Of particular interest several of the

interacting CrTFs are regulated by CO2 and Nitrogen. Two heterologous promoters from *A. thaliana* were also screened and one CrTF was found to bind one of these regions. Interestingly the *A. thaliana* TFs orthologous to the interacting CrTF also showed binding to the *A. thaliana* promoter indicating potential conservation of binding sites between *A. thaliana* and Cr TFs.

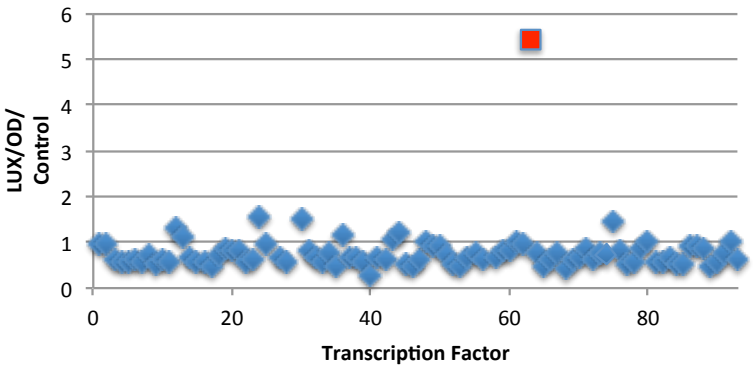

Figure C.7.1: Example results from a Y1H on a bait fragment for the promoter from a gene product that is induced under low light/low CO2. The data point highlighted in red indicates a positive result for CrTF binding, in this case a luminescent signal more than 5X the un-fused GAL4AD control was detected.

The TF library was then inserted into a C. *reinhardtii* constitutive promoter to determine its potential in a synthetic expression system. Constitutive expression of many of the individual TFs was debilitating. One transcription factor, T64, which is of a typical helix-loop-helix structure, was investigated by RNA-seq analysis because of its efficacy in promoter-binding. The overexpression of this transcription factor affected the transcripts of many endogenous nuclear genes, some negatively, some positively. Bioinformatic analysis indicates that T64 is implicated in activation of light harvesting complex II components, which will have dramatic affects on transcripts cell-wide. This type of whole-transcript analysis is required for our eventual goal of developing a synthetic transcription factor/promoter system for production of recombinant proteins.

The results of our transcription factor studies have been assembled in a manuscript and are ready for submission under the working title "Towards a synthetic expression system in green algae: Characterization of *Chlamydomonas reinhardtii* nuclear transcription factors and identification of targeted promoters".

C.8 Modeling and analysis of benefits of algae co-products

Kendall

Overview: As has been observed for many biofuel production systems, the utilization of co-products can have significant effects on the energetic and environmental performance of fuels; moreover, the method of co-product allocation and assumptions about how co-products will be used are critical to determining their value [e.g. (Cherubini et al. 2009, Kendall and Chang 2009)]. Task C.8 examines the benefits of algae co-products by assessing their best use in terms of improving the energy return on investment (EROI) and reducing life cycle greenhouse gas (GHG) emissions for the primary products of concern – algal biodiesel or algal renewable diesel. This research also explores the trade-offs between reducing co-products from the system by utilizing them internally for nutrient and energy recovery, or exporting them from the system, and trade-offs among conversion processes (lipid extraction for algal oil, or hydrothermal liquefaction for biocrude). These conversion processes yield different outcomes for the mix and quantity of co-products per unit of bioenergy produced.

Research Highlights: Phase one of this project focused on algal biodiesel produced through lipid extraction pathways. For lipid extraction pathways, the primary co-product is residual biomass or *algal cake* remaining after the lipid extraction process. The cake contains nearly all of the nitrogen and phosphorous assimilated by algal cells, along with a significant amount of carbohydrate. Thus, the primary co-product is also the most promising target for nutrient, energy and carbon recycling; entwining nutrient recycling and co-products utilization strategies.

The primary technologies assessed include anaerobic digestion (AD), hydrothermal liquefaction (HTL), and hydrothermal gasification (HTG); others were considered but eliminated from detailed analysis. AD was found to perform best under a number of scenarios and assumptions (Zhang et al. 2014).

Co-product allocation methods were tested and found to have little effect on outcomes under optimal process design. This is, in part, because the largest potential co-product (residual biomass) is recommended to be recycled for nutrient and energy recovery within the production system, which requires no allocation, and because the two co-products used outside the system; solid digestates used as soil amendments, and glycerin from the transesterification process of algal oil to biodiesel yielded similar total credits, nearly 15 g CO_2e/MJ biodiesel, regardless of allocation method. When displacement methods are used glycerin contributes about half of the credit, while in the economic allocation it contributes less than 7% (Yuan 2014).

In Phase 2 of this research whole-algae HTL was added as a conversion pathway in the LCA model. This led to entirely new results (as illustrated in figure B5 of this report). In fact, the most surprising insight from this research is the trade-off between a high-risk, high-reward pathway (lipid extraction with algal cake exported as livestock feed under favorable economic conditions and displacement assumptions), or a robust pathway (HTL for biocrude production) that delivers renewable diesel at a favorable, but not outstanding carbon intensity (around 50 g CO_2e/MJ) across a variety of scenarios. Given that the high-risk, high-reward approach hinges on market conditions for algal cake feed and a methodological decision about co-product displacement calculations, whole-algae HTL is likely the preferable pathway.

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Yuan J (2014) Mass Balance Modeling and Life Cycle Assessment of Microalgae-derived Biodiesel Production. Dissertation, University of California, Davis, CA.

Zhang Y, Kendall A, Yuan J (2014) “A comparison of on-site nutrient and energy recycling technologies in algal oil production.” Resources, Conservation and Recycling, 88: 13–20. doi:10.1016/j.resconrec.2014.04.011

C.9 Develop new methods to control gene expression and cell viability in green algae

Weeks

Overview: For successful production of renewable biofuels and other high-value products from green algae, it is important to be able to alter metabolic pathways leading to lipids or to other desired molecules. Discovery and testing of gene promoters and other molecular tools that allow increased or decreased expression of a particular enzyme or enzymes in a metabolic pathway is one approach that has proven highly successful in the biotechnology industry using a variety of living organisms. Our goals in this high-risk/high-payoff set of projects was to develop a set of new molecular tools that could allow us to control gene expression in the model green alga, *Chlamydomonas reinhardtii*, and in other microalgae of commercial significance as methods for their genetic transformation become available.

Research Highlights:

We have successfully tested a number of conditional promoters in *Chlamydomonas* and have been able to demonstrate, as expected, that they provide a practical means for controlling expression of genes of interest when

these genes are introduced into cells by electroporation. One very recent example of this success was our ability to control the expression of a potentially toxic protein with the chimeric 70S heat shock/Rubisco small subunit gene promoter combination whose activity can be rapidly and strongly induced when cells are shifted from 25° C to 42° C. Because this promoter has very low activity at 25° C, we were able to use it to drive the expression of the toxic gene coupled through the FMDV 2A coding region (developed as part of the CAB-COMM project) to the paromomycin resistance gene and select transformed cells resistant to paromomycin. Subsequent exposure to a brief heat shock treatment at 42° C allowed transient, high-level production of the toxic protein without causing cell death. This result was particularly important to the project aimed at gene editing in algal cells described in the following paragraph.

Two new methods for targeted gene knockout and gene editing by homologous recombination have recently become available. These are the TALEN and CRISPR/Cas9 systems that are designed to target specific genes and cause double-stranded DNA breaks. When such breaks are joined using nonhomologous end-joining DNA repair, there are often insertion or deletion of nucleotides that can cause the target gene to be inactivated. If a segment of DNA with strong homology to the target sequence is present at the time of the double stranded DNA break, there is an opportunity for gene replacement though homologous gene recombination. The Weeks laboratory has tested both the TALEN and CRISPR systems in both higher plants and algae. While the TALEN system was highly successful in producing rice plants with resistance to a major plant disease, bacterial blight [Li et al., Nature Biotechnology (2012) 30:390-392] and the CRISPR/Cas9 system is highly efficient in causing gene modification in Arabidopsis, tobacco, rice and sorghum plants [Jiang et al., Nucleic Acids Research (2013) 41:e188], both systems are toxic when expressed continuously in *Chlamydomonas* cells. Thus, the new combined FMDV 2A system and conditional 70s heat shock/Rubisco promoter system described above offers an exceptional opportunity to gain transient expression of the TALEN and CRISPR/Cas9 systems in *Chlamydomonas* - an effort presently underway. This same combined system for conditional gene expression also provides the opportunity to gain adequate control of the expression of the PhiC31 integrase system that we have explored as a means to insert genes into preselected spots in the genome of *Chlamydomonas* that can provide highly predictable levels of expression of transgenes at low, medium or high levels of expression.

Finally, we have submitted for publication an article in which we describe success in using a modification of the TALEN system to gain activation of a targeted gene, in this case, the arylsulfatase (ARS) gene of *Chlamydomonas*. In this research conducted in collaboration with the Spalding laboratory at Iowa State University, we have used an synthetic TALEN targeting the promoter region of the ARS gene coupled with the natural C-terminal activation domain of the TAL effector to cause artificial activation of the ARS gene. This discovery likely will lead to our ability to conditionally express such activator genes (or, alternatively, repressor genes) to help control the synthesis of enzymes needed at specific times to control the flow of intermediates through pathways needed to control the quantity and quality of lipids and oils useful in algal biofuel production.

C.10 Develop additional selectable markers and crop protection tools for diatoms

Falkowski

Overview: Our goal was to develop new selectable markers and crop protection features for diatoms that could greatly improve the potential organisms as a robust feedstock for biofuel production. Our strategy was to develop strains that are more resistant than the wild type diatom *Phaeodactylum tricornutum* while producing enough lipids and biomass as required from a biofuel feedstock strain.

Research Highlights:

We tried to obtain a glyphosate resistant *Phaeodactylum tricornutum* strain, by using constructs coding for a glyphosate acetyltransferase (GAT), and two 5-enolpyruvylshikimate-3-phosphate synthases from Agrobacterium CP4 (EPSPS) (provided by Donald Weeks, University of Nebraska, Lincoln). GAT adds acetyl groups to

glyphosate, resulting in a non-toxic product, and the two EPSPS are glyphosate-insensitive. We used microparticle bombardment to transform *Phaeodactylum tricornutum* with constructs that were optimized for *Chlamydomonas reinhardtii*, and without its own plasmid harboring a *Phaeodactylum tricornutum*-optimized glyphosate acetyltransferase gene (GATopt).

We completed several transformations and obtained 12 strains that are glyphosate resistant. Unfortunately, we couldn't find fast growing/high lipid strains, thus no farther characterization of their physiology lipid content was done. Upon reviewing our screening results, we conclude that we couldn't obtain a transformant that was sufficiently fast growing to make it worthwhile to pursue.

Cost-share Task: Field Testing of GMO Algae

Sapphire Energy, Mayfield, Shurin

At the end of 2013, we conducted the world's first test of a genetically modified algae strain, *Scenedesmus dimorphous,* in outdoor field experiments. These tests were run in collaboration with Sapphire Energy under approval of the EPA TSCA Environmental Release Application (TERA, approved on 9/25/2013, R13-0003 through R13-0007). These tests were run at the UC San Diego Biology Field Station algal growth facility in order to: 1) examine the ability of the subject microorganism to disperse from cultivation ponds; 2) assess the ability of the GM algae to invade and displace natural communities in local water bodies; and 3) evaluate the translation of a GM phenotype (GFP expression) from the laboratory to an outdoor setting. We identified that the GM algae were able to disperse into algae traps located within 50 meters of the test ponds, but that these algae did not displace native species in test cultures using local waters. We also identified that the GM trait, green fluorescent protein, expressed in the test algae throughout the 8 weeks of cultivation in open ponds. These results showed that GM traits are likely to be stable when introduced into commercial settings, and that GM algae, although able to disperse into natural environments, do not appear to have any selective advantage over native species and do not appear to displace native species in these environments. A manuscript has been prepared and submitted for publication to PlosOne, and is currently in review. Additional GM trials will be planned to address both environmental and physiological aspects of GM algae introduced into commercially relevant settings.

Algae Surfboard Task: Creating algae-based polyols for polyurethane production

Mayfield, Burkart, Pomeroy with commercial partners, Arctic Foam and Solazyme

In addition to this basic research, we also engaged the commercial sector to develop and demonstrate the production of high value sustainable fossil fuel replacement products, specifically polyurethane made from algae oil. In collaboration with Arctic Foam, we developed polyurethane foams in which 100% of the polyols were derived from algae oil. The polyurethane made from the algae oil is both sustainable, as well as biodegradable, and test boards have demonstrated that the performance of algae polyurethane matches that of petroleum urethane. We plan a commercial launch of algae surfboards in 2016.

INDUSTRY PARTNERSHIPS

Industry partnerships were a core part of the CAB-Comm program. Industry partnership not only provided cost-share via cash contribution and provision of in-kind supplies, but also through dedicating personnel time and access to commercial facilities to work on truly collaborative projects, which have had a significant impact on making technology and research findings from this consortium available to the greater algae research community. These collaborations have also provided rare and invaluable training opportunities for Cal-CAB graduate students and postdoctoral researchers. CAB-Comm has, over the past five years, spurred the creation of three new companies: Triton Health and Nutrition, Verdant Therapeutics, and a yet to be named company focusing on the partnership with Arctic Foam.

Life Technologies

Life Technologies was the key commercial partner for the genetic tools component of the grant. In addition to supplying significant financial support to enable the construction of alga land cyanobacteria tools, they were, and remain, a key partner for the distribution of the genetic tools created under this grant. Over 150 specific tools are presently available through the Life Technologies Catalog for genetic transformation, metabolic engineering, and recombinant protein expression for both C. *reinhardtii* and cyanobacteria. Through Life Technologies, our genetic tools are available to the entire algal community; academic, industrial, or National Lab, anywhere in the world.

Sapphire Energy

Sapphire Energy was the primary commercial partner for both crop protection and nutrient recycling aspects of this award. A significant portion of the crop protection work was carried out on pests and pathogens identified by Sapphire as problems for their large-scale production in New Mexico. Additionally, another of the major highlights of the CAB-Comm grant, the first outdoor GMO algae field trial, was made possible through the close collaboration between UC San Diego and Sapphire Energy. In addition to cost share, Sapphire provided a variety resources for examining nutrient utilization and recycling using the residual from their hydrothermal liquefaction treatment of algae for green crude production.

Triton Algae Innovations

Triton Algae Innovations became a partner in 2013, when we began to examine recombinant proteins as potential co-products for economic production of algal biofuels. Although not a cost share partner, Triton Algae Innovation provided significant resources to CAB-Comm as we helped to develop their production strains, which today are entering into animal trials on their way to becoming products in 2016.

Arctic Foam and Solazyme

Like Triton, Arctic Foam and Solazyme were not cost share partners on the CAB-Comm grant, but were important commercial partners during the last year of the project. Specifically, Solazyme provided the algae oil that was used by UC San Diego chemists to turn into polyols. These algae-based polyols were then used by Arctic Foam to produce the world's first algae surfboards. We have now perfected the formulation for algae-based polyurethane and expect to enter into commercial production of algae-based polyurethane surfboard blanks with Arctic Foam in 2016.

OUTREACH

Outreach has been a core part of CAB-Comm, as the training and recruitment of talented individuals to the algae industry will be crucial as the industry starts to grow. In addition to supporting the annual Food & Fuel for the 21st Century Symposium and the Cal-CAB Summer Bioenergy Research Program, CAB-Comm has provided the opportunity for 30 Postdoctoral Fellows, 29 PhD students, 6 Masters students, 71Undergraduate students, 4 High School students, and 2 EDGE Biofuels Certificate students to receive training as volunteers and staff working on CAB-Comm research projects across all five research institutions. The CAB-Comm field station facilities also became a core part of the EDGE Biofuels Certificate Biomass Production Lab course, which taught over 130 students from 2011 through 2013. Other CAB-Comm research is also directly integrated into the EDGE Biofuels Certificate lecture and lab courses, which are often taught by CAB-Comm members, getting knowledge and skills from CAB-Comm directly into the hands of students and algae industry professionals.

Food & Fuel for the 21st Century Symposia

CAB-Comm has supported the SD-CAB/FF21 Annual Symposium, which has been an important meeting for researchers, industry professionals, and students from around the country to come together to discuss the latest research in photosynthetic biotechnology for the production of biofuels and bio-products. In 2012, the symposium was held on May 11-13 in the Frederic de Hoffman Auditorium at the Salk Institute for Biological Studies. The theme of the event was "Sustainable Production of Food and Fuel for the 21st Century" and drew 257 attendees. There were 16 poster session presenters and 31 speakers including keynote talks from Rob Horsch of the Gates Foundation and Chris Cassidy of the U.S. Department of Agriculture. In 2013, the symposium was held on April 19-20 in the Hojel Auditorium at the Institute of the Americas, UC San Diego with the theme "Expanding the Opportunities." 202 attendees came to hear 23 speakers, including keynote speaker Lawrence Johnson of the Salim Group, UC San Diego Chancellor Pradeep Khosla, and California State Senator Ben Hueso, and to discuss research with 18 poster session presenters. A number of talks from both of these meetings were from CAB-Comm investigators who were presenting research findings from the grant. Symposia agenda for both meetings is attached in **Appendix D**.

Summer Bioenergy Research Program

In 2012, 2013 and 2014, CAB-Comm has supported the Cal-CAB Summer Bioenergy Research Program, which gives undergraduate students the opportunity to gain hands-on experience in CAB-Comm labs. The Summer Bioenergy Program aims to: 1) Provide students with the skills to become research scholars; 2) Stimulate students' serious consideration of graduate study; and 3) Increase learning and networking opportunities for students committed to pursuing a professional or academic research career in bioenergy.

Each participant was matched with a member of CAB-Comm's faculty and worked in one of UCSD or SIO's state-of-the-art research facilities. Participants worked with both a faculty member and a lab mentor to craft a research project. In addition to lab work, students were given the opportunity to participate in science field trips to the UCSD Biology Field Station and to Sapphire Energy, to learn about the scale up and commercialization side of algae biotechnology, and where their research fits in. The program culminates with a research symposium each year where students presented talks on their summer project for faculty, lab mentors, and other student interns.

CAB-Comm supported 28 undergraduates during this time, helping many of them to gain employment in the algae and biotechnology industries. Many others have also gone on to be accepted into graduate programs.

Cal-CAB Summer Bioenergy Research Program Projects
2014
Garri Arzumanyan, Lab: Stephen Mayfield Project: Integration of Cas9 Gene into *Chlamydomonas Reinhardtii* with NHEJ knockouts
Eric Barber, Lab: Jonathan Shurin Project: Effect of Chytrid Fungi on algal productivity
Rosalie Ellis, Lab: Stephen Mayfield Project: Confirming PSII Core Protein Knockout
Elizabeth Hann, Lab: Bianca Brahamsha Project: Is the Resistance Mechanism in *Synechococcus* Mutant 7D3 Universal to all Amoeba Grazers?
Karl Hong, Lab: Brian Palenik Project: Salt and Raffinate tolerances of *Synechocystis* Strains
Regina Izquierdo, Lab: Stephen Mayfield Project: Immunotoxin Production within *Chlamydomonas reinhardtii* Chloroplasts
Matt Paddock, Lab: S. Golden Project: Evaluation of genetic circuits composed of transcriptional repressors controlled by riboswitches to down regulate gene expression in cyanobacteria
Vivian Pham, Lab: J. Golden Project: Evaluation of genetic circuits composed of transcriptional repressors controlled by riboswitches to down regulate gene expression in cyanobacteria
Wilson Wong, Lab: Michael Burkart Project: Large Scale Lipid Extraction of Algae Biomass and Subsequent Polyol Synthesis
2013
Christina Aguila, Lab: Greg Mitchell Project: Comparison of pond conditions to lab conditions with respect to salinity
Nate Gulizia, Lab: Bianca Brahamsha Project: Analysis of interactions between cyanobacteria and amoebae
Prema Karunanithi, Lab: Stephen Mayfield Project: Overcoming Gene Silencing and Positional Effect using mCherry-Venus Plasmid Constructs
Eddie Lin, Lab: Biology Field Station Project: Improving & Maintaining Robust Algae Growth through Ultraviolet Sanitized Media
Yen Hong Lu, Lab: Briggs Lab Project: Selection of *Scenedesmus dimorphus* mutant strains resistant to jasmonic acid
Lindsey Pieper Lab: Jim Golden Project: EPA Production In Photosynthetic Cyanobacteria
Yuan Pu, Lab: Michael Burkart Project: Inserting Features into *Chlamydomonas*
Alice Tung, Lab: Brian Palenik Project: A study of growth under various cultivation systems and selection of a potential biofuel diatom
Steven Villareal, Lab: Jonathan Shurin Project: Effect of Chytrid Fungi and Elevated CO2 on Algal Productivity
Xue (Scarlett) Zou Lab: Susan Golden Project: Looking at the subcellular localization of clock proteins in Cyanobacteria
2012
Rodrigo Abelin Tackaert, Lab: Greg Mitchell Project: Growth Rate evaluation of *D. tertiolecta* using AlgaStat automated photobioreactor
Amir Begovic, Lab: Brian Palenik Project: Coculture of *Anabaenopsis* and *Scendesmus* in tapwater/seawater media
Ron Cook, Lab: Jim Golden Project: Improvements of a Modular Vector System for Cyanobacteria
Brian Fan, Lab: Michael Burkart Project: Developing a nuclear transformation protocol for the nrt2 gene in *Scenedesmus dimorphus*
Emily Fu, Lab: Steven Briggs Project: Algae produced malaria transmission blocking vaccines
Austin Hallgren, Lab: Stephen Mayfield Project: Relationship between the Cell Wall, Growth and FAME content
Josh Kenchel, Lab: Susan Golden Project: Protozoan Grazer Resistance in *Synechococcus elongatus*
Matthew Krause, Lab: Stephen Mayfield Project: Growth and Media Studies
Natalie Ortiz, Lab: Jim Golden Project: The role of a novel antisense transcript in phycobilisome degradation in *Anabaena* sp strain PCC 7120

OUTPUTS

The outputs from CAB-Comm are extensive and go beyond making the research findings accessible in scientific publications and presentations to enabling commercial development of not only the PI's own technology via patents, but also providing access to genetic tools to the algae industry to enable further commercial development. To date, there have been 117 publications, 12 patent applications and 316 presentations from CAB-Comm research. Many of the genetic tools created have also been made available via websites and the Life Technologies catalog. In addition, two different models around LCA have been created.

Patents

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Falkowski, PG., Dinamarca, J., Levitan, O., "Overexpression of Dgat2D gene in *Phaeodactylum tricornutum*". U.S. provisional Application Serial No. 61/894,197.

Mayfield SP, Barrera D, Oyler G, "Using algae to deliver bioactive proteins to modify gut flora". Docket No. SD2014-222.

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Hildebrand, M., Manandhar-Shresta, K., "Efficient Method for Selecting Microalgae using Flow Cytometric Sorting". US Provisional Application Serial no. 61/836,848.

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S.S. Golden, B. Brahamsha, R. Simkovsky, E. Daniels, B. Palenik, J.W. Golden "Cyanobacterial Strains Resistant to Grazers and Capable of Autoflocculation." Provisional patent application UCSD064.001PR.

S.S. Golden, J.W. Golden, A. Daulo "Targeted Delivery of Nutrients to Recipient Organisms." Provisional patent application UCSD062.001PR.

Trentacoste EM, Shrestha RP, Hildebrand M, Gerwick WH, "Method for Increasing Algal Biofuel Production". United States UC Case: 2012-190-0.

Oyler GA, Rosenberg JN, Weeks DP, "Single chain antibodies for photosynthetic microorganisms and method of use". Provisional patent application LLC. PCT/US2012/032662.

Products

Algae Expression & Engineering Kits: Genetic tools from CAB-Comm made available to the algae community via the Life Technologies catalog.
https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-expression/algal-protein-expression.html

Websites/Tools

CYANO-VECTOR portal: http://golden.ucsd.edu/CyanoVECTOR/

LCA Model: Microsoft Excel-based model that combines a facility mass balance calculation with life cycle assessment with a user-friendly interface (see Appendix B.5.4).

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Presentations

Crop Protection

P Seitzer, M Facciotti, F Ma, A Jeanniard, JL Van Etten. "Gene gang analysis of genus Chlorovirus: Surprising conservation of gene synteny."

Beld, J, Michaud J; Pu, Y, Schoepp N, Burkart MD. "Chemistry approaches to Algal Biotechnology: from natural product isolation to metabolic engineering." Food and Fuel for the 21st Century Symposium poster presentation on March 12-13, 2015, La Jolla, CA.

Simkovsky, Ryan; Nagar, Elad; Parnasa, Rami; Benjamin; Wang, Jingtong; Kench, Joshua; Schwarz, Rakefet; and Golden, Susan. "Molecular mechanisms for regulating biofilm formation in cyanobacteria: potential for crop protection and harvesting" Food and Fuel for the 21st Century Symposium poster presentation on March 12-13, 2015, La Jolla, CA.

Beld, J , Finzel, K and M D Burkart. "Revealing flexibility in fatty acid biosynthesis using acyl-acyl carrier protein synthetases" ASM New Orleans.

Burkat, MD. "Green algae cultivation and engineering of its fatty acid synthase" 249th ACS National Meeting & Exposition, Denver, CO.

Golden, S. "How Cyanobacteria Tell Time." Seminar, Plant Research Lab, Michican State Univ., East Lansing, MI, March 2, 2015.

Simkovsky, R. "Molecular mechanisms for regulating biofilm formation in cyanobacteria: potential for crop protection and harvesting." Algal Biomass Organization's Summit in Washington, DC. September 2015, oral presentation.

Ma, AT, EF Daniels, N Gulizia, B Brahamsha. "Examination of Cyanobacterial-Amoebal Interactions" American Society of Microbiology General Meeting, New Orleans, Louisiana, May 2015, poster presentation.

Ma, Amy. "Bacterial Interactions with Amoebae" Department of Biological Sciences, Ohio University, Athens, Ohio, May 2015, invited seminar.

Ma, Amy. "Bacterial Interactions with Amoebae" Department of Biology, University of Kentucky, Lexington, Kentucky, February 2015, invited seminar.

Beyter, Doruk, Tang, Pei-zhong, Becker, Scott, Hoang, Tony, Peterson, Todd, Beiligin, Damla, Lim, Yan Wei, Haerizadeh, Farzad, Shurin Johnathan, Bafna Vineet, McBride Robert. "Ecology of Open Algae Ponds for the Production of Biofuels." Food and Fuel for the 21st Century Symposium poster presentation on March 12-13, 2015, La Jolla, CA.

Brahamsha, Bianca. "Molecular approaches for protection of cyanobacterial crops" 2014 Food & Fuel for the 21st Century Symposium, UC San Diego, March 14-15, 2014, oral presentation.

Simkovsky, Ryan; Effner, Emily; Iglesias-Sanchez, Maria Jose; Tang, Karen; Kenchel, Joshua; Bearmar, Nicholas; Golden, Susan. "Lipopolysaccharide Mutants Reveal Novel Targets for Cyanobacterial Grazer Resistance and Tolerance to LPS Deletion" 2014 Food & Fuel for the 21st Century Symposium, UC San Diego, March 14-15, 2014, poster presentation.

Beld, J. "Interrogating the fatty acid synthase in bacteria and algae" NOBCCHe, San Diego, USA (March 2014), http://ucsd.academia.edu/NOBCChEUCSD, oral presentation.

Beld, J Cang, H, Burkart, M D. "Visualizing the chain-flipping mechanism in fatty acid biosynthesis" GRC Bioorganic, Proctor Academy (June 2014), Poster presentation.

Shurin, J. "Experimental risk assessment for dispersal, invasion, and impact on wild algae of genetically-modified *Scenedesmus dimorphus*" Algae Biomass Organization Summit Meeting; Sept. 29-Oct 2nd, San Diego, CA. Presentation 2014.

Simkovsky, R . "Pathways to Grazer Resistance in Cyanobacteria." Algae Biomass Organization Summit Meeting; Sept. 29-Oct 2nd, San Diego, CA. Oral presentation.

Simkovsky, Ryan; Nagar, Elad; Parnasa, Rami; Rubin, Benjamin; Wang, Jingtong; Kenchel, Joshua; Schwarz, Rakefet; and Golden, Susan. "Exploring alternative stages of lipopolysaccharide production for crop protection in the cyanobacterium *S. elongatus*." Cal-CAB Student and Postdoc Symposium in La Jolla, CA. January 2014, oral presentation.

Bianca Brahamsha. "Grazing and the Cyanobacterial Cell Surface." Oral presentation at the San Diego Microbiology Group Meeting of March 20, 2013.

Ma, AT. "Predator-prey Interactions Between Cyanobacteria and Amoebae" Oral presentation at the SD-CAB student and Postdoc Symposium, March 15, 2013.

S Wehrkamp-Richter. "Proteomics Analysis of *Scenedesmus* infection with Fungus" Oral presentation at the SD-CAB/Sapphire Collaborators Meeting, La Jolla, CA. March 2013.

Bhattacharya D*, Gross J, Price DC, Chan CX . "The Cyanophora genome: analysis of gene and small RNA content in a pivotal algal lineage." 10th International Phycological Congress (http://ipc10.intphycsoc.org/), August 9, 2013. *speaker.

Bhattacharya D*, Perrineau MM, Gross J, Price DC, Zelzion U . "Biofuel crop improvement using experimental evolution." 10th International Phycological Congress (http://ipc10.intphycsoc.org/), August 5, 2013. *speaker.

D D Dunigan, J L Van Etten. "PBCV-1 infection of Chlorella variabilis: A transcriptomic analysis of the immediate-early innate immune response; " 2013 Symposium on Food and Fuel for the 21st Century, held in La Jolla, California, USA; April 2013, oral presentation.

Dunigan, D D. "The immediate-early events of Chlorovirus infections" Invited seminar at Purdue University, West Lafayette, Indiana, USA: May 2013 .

Amy Ma, EDaniels, B Knight, J Golden, B Brahamsha. "Characterizing interactions between *Anabaena* sp. PCC7120 and Amoeba HGG1" Poster presentation at the Food and fuel for the 21st Century Symposium. April 19, 2013.

Amy Ma, EDaniels, B Knight, J Golden, B Brahamsha. "Characterizing interactions between *Anabaena* sp. PCC7120 and *Amoeba* HGG1" Poster presentation at the Annual Meeting of the San Diego Microbiology Group. May, 11, 2013.

Ryan Simkovsky. "Protecting cyanobacteria from amoebal predators " Oral presentation at the San Diego Microbiology Group Meeting of April 17, 2013.

Joris Beld, Jen Michaud, Nathan Schoepp, Eva Sonnenschein, Michael Burkart: . "Towards a sustainable future – Algae research in the Burkart lab" poster presentation at the Food and Fuel for the 21st Century, April 19-20, 2013.

Eva Sonnenschein, Michael Burkart: . "Activation of Secondary Metabolite Pathways in Microalgae, " General Meeting of the American Society for Microbiology, May 18-21, 2013.

Van Etten, JL. "The genetic potential of the chloroviruses is huge." 2013 Symposium on Food and Fuel for the 21st Century, held in La Jolla, California, USA; April 2013, oral presentation.

Duncan, G (2013) Co-authors: D Dunigan, J Gurnon, JL Van Etten. "Antigenic mutants of the Chlorovirus PBCV-1: Sequence analysis of the gene a064r" Annual Meeting of The American Society for Virology, held at State College, Pennsylvania; July 2013, poster.

Agarkova, IV Co-authors: B Hertel, X Zhang, D D Dunigan, M G Rossmann, G Thiel, J L Van Etten. "Chlorovirus attachment to the host cell wall is reversible" Annual Meeting of The American Society for Virology, held at State College, Pennsylvania; July 2013, oral presentation.

Bhattacharya D. " Experimental evolution of microalgae and its application to biofuels." International Congress of Protistology (ICOP; http://www.icoprotist.com), July 31, 2013, Vancouver, Canada. *speaker.

Bhattacharya D, Gross J, Price DC, Chan C. "The Cyanophora genome: analysis of gene and small RNA content in a pivotal algal lineage." 10th International Phycological Congress (http://ipc10.intphycsoc.org/), August 9, 2013.

Bhattacharya D, Perrineau MM, Gross J, Price DC, Zelzion U. "Biofuel crop improvement using experimental evolution." 10th International Phycological Congress (http://ipc10.intphycsoc.org/), August 5, 2013.

Foflonker F1, Palenik B, Price DC, Bhattacharya D . "Genome sequence of a biofuel candidate alga provides insights into stress adaptation." 10th International Phycological Congress (http://ipc10.intphycsoc.org/), August 9, 2013.

D D Dunigan, A Jeanniard, G Blanc, J Gurnon, I Agarkova, G A Duncan, J-M Claverie, J L Van Etten. "A genomic analysis of giant viruses in the genus Chlorovirus" Annual Meeting of The American Society for Virology, held at State College, Pennsylvania, USA; July 2013, oral presentation.

Amy Ma, E Daniels, J Golden, B Brahamsha. "Proteomic Response of *Anabaena* sp. PCC7120 to grazing by natural amoebal isolate HGG1." Poster presentation at the 11th Workshop on Cyanobacteria, Washington University, St. Louis, Aug. 7-11, 2013.

Ryan Simkovsky, Maria José Iglesias-Sánchez, Karen Tang, Joshua Kenchel, Nicholas Bearmar, and Susan Golden. "Rough Phenotype Screen Reveals New Candidate Genes Conferring Resistance to Grazing in a Model Cyanobacterium-Amoeba System" Poster presentation at the 11th Workshop on Cyanobacteria, St. Louis, MO, Aug. 7 – Aug 11, 2013.

Ryan Simkovsky, Maria José Iglesias-Sánchez, Karen Tang, Joshua Kenchel, Nicholas Bearmar, and Susan Golden. "Rough Phenotype Screen Reveals New Candidate Genes Conferring Resistance to Grazing in a Model Cyanobacterium-Amoeba System" Poster presentation at the UCSD Postdoctoral Research Symposium, La Jolla, CA, Sept 13, 2013.

Dunigan, D D. "Rise of the giant viruses: A view from the midrealm" Invited seminar at University of California-Davis, Davis, California, USA: October 2013.

Dunigan, D D. "A view of giant viruses from the midrealm of the chloroviruses" Invited seminar at Oklahoma State University, Stillwater, Oklahoma, USA: October 2013.

Dunigan, DD. "Oral presentation" Aquatic Virus Workshop 7, 2014.

Dunigan, DD. "Algae-virus interactions: A transcriptomic Evaluation of the inate immune response of *Chlorella Variabillis* to PBCV-1 infection" Food and Fuel for the 21st Century-Expanding the Opportunities. April 2013, oral presentation.

De Castro, C, D Garozzo, R Lanzetta, A Molinaro, M Parrilli, L Sturiale, J Gurnon, JL Van Etten, M Tonetti. "A molecular modeling view into the complex N-linked oligosaccharide from A430L envelope protein isolated from Paramecium bursaria chlorella virus PBCV-1." Gordon Conference on Glycobiology, 2013.

De Castro, C, D Garozzon, L Sturiale, A Molinaro, F Piacente, J Gurnon, JL Van Etten, M Tonetti . "Structure characterization of the N-linked glycans associated with the major capsid protein of the giant DNA virus PBCV-1." Gordon Conference on Glycobiology, 2013.

Dunigan, DD, Van Etten, JL, Jeanniard, A. "Exploration of Chloroviruses through genomics" Algae Biomass Summit , 2013.

S Briggs. "Reconstruction of Protein Networks from an Atlas of Proteotypes" FF21 Symposium La Jolla, CA. April 2013, oral presentation.

F Nohilly. "Algae chytrid interactions" SD-CAB Student and Post-doc symposium, La Jolla, CA. April 2013, oral presentation.

S Wehrkamp-Richter. "Proteomics Analysis of *Scenedesmus* infection with Fungus" SD-CAB/Sapphire Collaborations Meeting, La Jolla, CA. March 2013, oral presentation.

F Nohilly. "Conservation of plant stress hormone responses in the unicellular alga, *Scenedesmus dimorphus*" Food and Fuel for the 21st Century Symposium. April 19, 2013, poster.

Ma, Amy. "Development of Cyanobacteria for Biotechnological Applications" Department of Biological Science Seminar, California State University, Fullerton, October, 2013, invited seminar.

Van Etten . "Unusual life-style of giant algal viruses." Invited seminar at the University of California – Riverside. Riverside, CA, Feb 2012.

Dunigan, DD. "Chloroviruses in North America, " U. S. Geological Survey/Nebraska Water Science Center, Lincoln, NE, Jan 2012.

Van Etten. "Unusual life-style of giant algal viruses." Invited seminar at Centro de Investigación Científica y de Educación Superior de Ensenada. Ensenada, Mexico, Mar 2012.

Van Etten. "Chlorella viruses: possible infectious agents of humans and experimental animals." Invited talk at Algal Biofuels Symposium 2012 held in La Jolla, CA, May 2012.

Duncan, G Co-authors: David Dunigan, James Gurnon, James Van Etten. "Genomic analysis of 11 antigenic mutants of the Chlorovirus PBCV -1; " Annual Meeting of the Nebraska Academy of Sciences held in Lincoln, NE, oral presentation, Apr 2012.

Blatti: Jillian L Blatti, Michael D Burkart. "Releasing stored solar energy within pond scum: Biodiesel from algal lipids, " 243rd American Chemical Society National Meeting, San Diego, CA, 29 Mar 2012.

Burkart: . "Modular synthase engineering in algae, " oral presentation at the Food and Fuel for the 21st Century, La Jolla, CA, April 22-23 2012.

Burkart: . "Role of protein-protein interactions in algal fatty acid synthase engineering, " 243rd American Chemical Society National Meeting, San Diego, CA, March 28, 2012.

Dunigan: Co-authors: Claire Mueller, David Dunigan, James Gurnon, James Van Etten. "An evaluation of Nebraska River Systems for chloroviruses; " 2012 Flyswat, held in Nebraska City, Nebraska, Poster Presentation, Mar 2012.

D D Dunigan, J L Van Etten. "Algal – virus interactions: A proteomic approach to host range specficity; " Algal Biofuels Symposium 2012, held in La Jolla, California, Oral presentation, May 2012.

Amy Ma, Emy Daniels, Javier Paz Yepes, Jim Golden, and Bianca Brahamsha . "Proteomic analysis of *Anabaena* sp. strain PCC7120 after co-culture with wild amoebal isolate HGG1" Poster presentation at the Food and Fuel for the 21st Century, La Jolla, Ca USA, April 22-23, 2012.

Ryan Simkovsky, Emy Daniels, Karen Tang, Stacey Hyunh, Susan Golden, and Bianca Brahamsha. "Impairment of O-antigen production confers resistance to grazing in a model cyanobacterium-amoeba predator-prey system." Poster presentation at the Food and Fuel for the 21st Century, La Jolla, CA, USA, April 22-23, 2012.

Ryan Simkovsky, Emy Daniels, Karen Tang, Stacey Hyunh, Susan Golden and Bianca Brahamsha: . "O-antigen Impairment Confers Resistance to Grazing in a Model Cyanobacterium-Amoeba System" poster presentation at the San Diego Microbiology Meeting, La Jolla, CA, USA, June 9, 2012.

Shurin. "Engineering algal communities for biofuel productivity and resilience" Invited Symposium on Biodiversity and Ecosystem Functioning at the Association for the Sciences of Limnology and Oceanography Annual Meeting, Otsu, Japan, July 10, 2012.

Simkovsky. "Grazer-resistant and auto-flocculating mutants of the cyanobacterium *Synechococcus elongatus* PCC 7942;" Oral presentation at the SD-CAB Student and Postdoc Symposium, La Jolla, CA, USA, April 20 2012.

Van Etten. " Chlorella viruses have a sweet tooth" Invited seminar at the University of Genova, Genova, Italy, July 2012.

Van Etten. "Chlorella viruses have a sweet tooth." Invited seminar at the University of Naples, Naples, Italy, July 2012.

Van Etten. "Giant viruses change the perception of viruses." Invited talk at the American Society of Microbiology annual meeting, June 2012.

Van Etten. "Chlorella viruses: possible infectious agents of humans and experimental animals." Invited talk at the Second International Conference on Viruses of Microbes held in Brussels, Belgium, July 2012.

S Golden. "Impairment of O-antigen production confers resistance to grazing in a model cyanobacterium-amoeba predator-prey system," 14th International Symposium on Phototrophic Prokaryotes, Porto, Portugal, 5 - 10 August 2012.

Van Etten. "Unusual life-style of giant algal viruses." Invited seminar at Nebraska Wesleyan University. Lincoln, NE. Oct. 2012.

Van Etten. "Chloroviruses: giant algal viruses." Invited talk at the annual meeting of the Mediterranee Infestion held in Gordes, France. Oct. 2012.

Dunigan, DD, JL Van Etten. "PepScanning the really big viruses: Immuno-reactivity of patients with mental disorders to NCLDV proteins" Annual Meeting of the Stanley Medical Research Institute, held in Baltimore, Maryland, December 2012, Oral presentation.

Dunigan. "A two year comparison of chloroviruses in Nebraska river systems." Plant Virus Ecology Network Workshop 5, held in Lawrence, Kansas, USA, September 2012.

Gross J. "Systemic genomics of *Chlamdomonas* for understanding salt tolerance mechanisms in biofuel algae" Annual Meeting, Consortium for Algal Bioguels Commericialization, San Diego, CA May 13.

Gross, J, Wajid, S, Price, D, Chan, C X, and Bhattacharya, D. "sRNAs of *Cyanophora paradoxa* and *Chlamydomonas reinhardtii*, a system genomics approach to understand algal evolution." Phycological Society of America Annual Meeting, Chareston SC, June 20-23, 2012.

Van Etten, JL. "A fascinating journey with giant chlorella viruses" Invited seminar at Nebraska Wesleyan University. Lincoln, NE. Oct, 2012.

Van Etten, JL, L Jones-Brando, E Severance, M Webster, S Kim, J Gurnon, DD Dunigan, F Dickerson, RYolken. "Chlorella viruses: potential infectious agents of humans and experimental animals. Viruses of Microbes 2012" Viruses of Microbes. 2012.

Blatti. "Releasing stored solar energy ithin pond scum: Biodiesel from algal lipids" 243rd American Chemical Society National Meeting, San Diego, CA, March 28,2012, oral presentation.

Z Shen. "Chytrid pathogenesis programs the algal host proteotype" SD-CAB & FF21 Symposium, La Jolla, CA, May 2012, oral presentation.

F Nohilly. "Chytrid pathogensis programs the algal host proteotype" SD-CAB & FF21 Symposium, La Jolla, CA. May 2012, poster.

F Nohilly. "Chytrid pathogensis programs the algal host proteotype" International Conference on Algal Biomass, Biofuels, and Bioproducts. San Diego, CA. June 2012, poster.

F Nohilly. "Chytrid pathogensis programs the algal host proteotype" UCSD Biological Sciences Research Sympsoium, La Jolla, CA. June 2012, poster.

F Nohilly. "Chytrid pathogensis programs the algal host proteotype" California Energy Commission Roadmap Meeting, La Jolla, CA. October 2012, poster.

F Nohilly. "Chytrid pathogensis programs the algal host proteotype" French BioBeach Symposium, La Jolla, CA. November 2012, poster.

J Golden. "July seminars on Cyanobacterial Biotechnology" China at the Hydrobiology Institute and Central Normal University (Wuhan), Qingdao Institute of Bioenergy and Bioprocess Technology (Qingdao), and the Shanghai Institute of Plant Physiology and Ecology (Shanghai), talk.

Sonnenschein and Burkart. "Metabolic engineering of microalgae towards the production of secondary metabolites" Food and Fuel for the 21st Century, April 22-23, 2012, La Jolla, CA, poster.

Sonnenschein and Burkart. "Metabolic engineering of microalgae towards the production of secondary metabolites" 15th International Conference on the Cell & Molecular Biology of *Chlamydomonas*, June 5-10, 2012 Postdamn, Germany, poster.

Sonnenschein and Burkart. "Metabolic engineering of microalgae towards the production of secondary metabolites" CEC Large Molecule Sustainable Fuels Roadmap Meeting, October 18, 2012, poster.

Dunigan, DD Co-authors: Dunigan, DD, and Van Etten, JL. "Chlorovirus major capsid proteins." Invited talk at Future crop protection: viruses of eukaryotic algae. Algal Biofuels Symposium in La Jolla, CA, 2011.

Van Etten. "Unusual life-style of giant algal viruses." Invited seminar at the University of Mediterranean, Marseille, France, 2011.

D D Dunigan, L C Lane, G L Lewis, G A Duncan, G M Yanai-Balser, J C Vitek, R L Cerny, J L Van Etten. "Chlorovirus major capsid proteins." Flyswat, held in Nebraska City, Nebraska, March 2011.

Van Etten. "Unusual life-style of giant algal viruses." Invited seminar at Temple University, Philadelphia, PA. April 2011.

D D Dunigan, J L Van Etten. "Future crop protection; viruses of eukaryotic algae." Algal Biofuels Symposium 2011, held in La Jolla, California.

Claire Mueller, David Dunigan, James Gurnon, James Van Etten. "An evaluation of Nebraska River Systems for chloroviruses; " 11th Annual Symposium in Virology, held in Lincoln, Nebraska, September 2011, poster.

Shurin. "Species invasion and large-scale algae cultivation." Ecological Society of America, special symposium on Microbial Diversity, Aug 10, 2011.

Jones-Brando, L, E Severance, M Webster, SKim, J Gurnon, D D Dunigan, F Dickerson, J L Van Etten, R Yolken. "Crossing kingdoms: association of phycodnaviruses with human psychiatric disorders." Annual Meeting of The American Society for Virology, held at Minneapolis, Minnesota, USA, July 2011.

Burkart. "University of Maryland Center for Environmental Science (College Park, Maryland; October 2011)" University of Maryland Center for Environmental Science, College Park, Maryland, Oct 2011.

Dunigan, DD, Duncan, GA, Lane, LC, Zhang, X, Rossmann, M, Van Etten, JL. " The evolving view of chlorovirus structure; " Aquatic Virus Workshop 6, held in Texel, The Netherlands, October 2011, oral presentation.

Mueller, CM, Dunigan, DD, Gurnon, J R, Van Etten, JL. "An evaluation of Nebraska Streams for Chloroviruses; " Aquatic Virus Workshop 6, held in Texel, The Netherlands, October 2011, oral presentation.

Van Etten. "Chlorella viruses continue to surprise." Aquatic Virus Workshop #6. Texel, Netherlands, Oct 2011.

Van Etten. "Unusual life-style of giant algal viruses." Invited seminar at Johns Hopkins University, Baltimore, MD, Dec 2011.

Van Etten. "Unusual life-style of giant algal viruses." Invited seminar at the University of Minnesota, Minneapolis, MN, Dec 2011.

Van Etten. "Algal viruses – the good and the bad." Invited talk at a Food and Fuel meeting at the state capital in Lincoln, NE., Oct 2011.

Dunigan, DD. "Exploring giant viral genomes: Why so many genes? " The James Hutton Institute, Dundee, Scotland, United Kingdom, invited talk, Oct 2011.

Dunigan, DD, and JL Van Etten. "Chlorovirus safari." Annual Meeting of the Stanley Medical Research Institute, held in Baltimore, Maryland, USA, December 2011, oral presentation.

Claire Mueller, David Dunigan, James Gurnon, James Van Etten. "An evaluation of Nebraska River Systems for Chloroviruses." 11th Annual Symposium in Virology, held in Lincoln Nebraska, September 2011, poster presentation.

Simkovsky, R; Hyunh, S; and Golden, S. "Social behaviors and macrostructure development in the cyanobacteria *Synechococcus elongatus* PCC 7942." ESF-Bielefeld-CeBiTec Conference on Microorganisms for Biofuel Production from Sunlight, Bielefeld, Germany, Sep. 2011, poster presentation.

Rowe, JM, JR Gurnon, LC Lane, and JL Van Etten. "Analysis of PBCV-1 resistance in *Chlorella* NC64A." Viruses of Microbes, Paris, France, 2010, poster.

Rowe, J M, J R Gurnon, L C Lane, I V Agarkova, and J L Van Etten. "Isolation and characterization of PBCV-1-resistant *Chlorella* NC64A." International Society for Microbial Ecology, 13th Symposium, 2010.

Van Etten. "Unusual life-style of giant algal viruses." Invited seminar at the University of Missouri – Kansas City, Mar 2010.

Van Etten. "Early events associated with infection by chlorella virus PBCV-1." Invited keynote talk at the First International Conference on Viruses of Microbes held in Paris, France, 2010.

Van Etten. "Unusual life-style of giant algal viruses." Invited seminar at the University of Milano, Milano, Italy, 2010.

Nutrient Utilization and Recycling

Palenik, Brian. "Algal strains for recycling nutrients from hydrothermal liquefaction waste" Food and Fuel for the 21st Century Symposium oral presentation on March 12, 2015, La Jolla, CA.

Shurin, Jon. "Experimental risk assessment for dispersal, invitation, and impact of genetically modified biofuel algae" Food and Fuel for the 21st Century Symposium oral presentation on March 12, 2015, La Jolla, CA.

Bafna, V. "Ecology of Open Algae Ponds for the Production of Biofuels." Food and Fuel for the 21st Century Symposium oral presentation on March 13, 2015, La Jolla, CA.

Beyter, D. "Diversity, Productivity and Stability of an Industrial Microbial Ecosystem." Algae Biomass Organization Summit Invited Talk, Sept. 29-Oct 2nd (2015) Washington, DC.

Palenik, Brian. "Algal Biofuels Research." Talk, Russian delegation from Vladivostock (San Diego Sister City Association) April 29 2015, La Jolla CA.

Mendoza W, Schieber, B, Mendola, D, Du, N, Weiss, E, Mitchell, BG. "Irradiance-dependent biomass growth model for *Scenedesmus dimorphus* cultivated in outdoor ponds." 5th International Conference on Algae Biofuels, Biomass and Bioproducts; 8 June 2015, San Diego, CA. Poster presentation.

"Mendoza, W, Weiss, E, Du, N, Mendola, D, Mitchell, BG. ""Modeling *Scenedesmus dimorphus* Growth, Carbon Partitioning and Optimized Harvest

Schedules for an Outdoor Raceway System."" Poster presented at ABO 2015, Washington DC."

Du, N, Allen, A, Mitchell BG. "Membrane inlet mass spectrometry (MIMS) integrated to photobioreactor for measurement of in vivo carbon uptake in algal cultures." Poster presented at ABO 2015,Washington DC.

D Mendola, I Woertz, J Benemann, R Kent, W S Rickman, B G Mitchell. "Conceptual Design & Preliminary Engineering for Capture & Reuse of CO2 & NOx for Algae Production from Stationary Engine Flue-Gas." NAABB 2015, San Diego, CA.

Mitchell, Greg. "Carbon partitioning into lipids, protein, carbohydrates, and pigments induced by irradiance, CO2 and N-limitation in the marine diatom *Thalassiosira pseudonana*" 2014 Food & Fuel for the 21st Century Symposium, UC San Diego, March 14-15, 2014, oral presentation.

Zhang, Yizhen; Kendall, Alissa; and Yuan, Juhong . "A comparison of on-site nutrient and energy recycling technologies in algal oil production" 2014 Food & Fuel for the 21st Century Symposium, UC San Diego, March 14-15, poster.

Patricia Abelin, Alyssa Velloze, Frank Shang, Laura T Carney, Wilson G Mendoza and B Greg Mitchell. "Application and validation of TPTZ method for measuring total carbohydrate content in microalgae culture." Algae Biomass Organization Summit Meeting; Sept. 29-Oct 2nd, San Diego, CA. Poster Presentation.

Margarita Godoy, Elliot Weiss, Patricia Abelin, Wilson G Mendoza, and B Greg Mitchell. "Photo-physiology of the Haptophyte marine alga *Isochrysis galbana* under a light gradient in culture: carbon partitioning into cellular constituents." Algae Biomass Organization Summit Meeting; Sept. 29-Oct 2nd, San Diego, CA. Poster Presentation.

Niu Du, Daniel Yee, Egil Sakshaug, Maria Vernet, Osmund Holm-Hansen, Satoru Taguchi, B Greg Mitchell. "Modeling of Light dependent growth and respiration for the marine diatom *Thalassiosira pseudonana*." Algae Biomass Organization Summit Meeting; Sept. 29-Oct 2nd, San Diego, CA. Poster Presentation.Awarded third prize for the Young Algae Research Awards, presented to winners for research conducted in two subject areas: biology and engineering, for outstanding research in algae biology.

Wilson G Mendoza, Patricia Abelin, Dominick Mendola, Du Niu, Elliot Weiss, Brian Schieber and Greg Mitchell. "Irradiance-dependent biomass growth model of *Scenedesmus* sp. in outdoor ponds. " Algae Biomass Organization Summit Meeting; Sept. 29-Oct 2nd, San Diego, CA. Poster Presentation. Awarded first prize for the Young Algae Research Awards, presented to winners for research conducted in two subject areas: biology and engineering, for outstanding research in algae biology.

Palenik, Brian. "Characterization of the Use of Wastewater from Hydrothermal Liquefaction as a Nitrogen Source by the Potential Algal Biofuel Strain *Picochlorum* sp." Algae Biomass Organization Summit Meeting; Sept. 29-Oct 2nd, San Diego, CA. Presentation, 2014.

Alyssa Velloze, Elliot Weiss, Niu Du, Patricia Abelin, B Greg Mitchell. "Variation in cellular growth and pigment packaging in response to growth irradiance for *Dunaliella tertiolecta* and *Thalassiosira pseudonana*." Algae Biomass Organization Summit Meeting; Sept. 29-Oct 2nd, San Diego, CA. Poster Presentation.

Zhang, Y, Kendall, A, Yuan, J. "A comparison of on-site nutrient and energy recycling technologies in algal oil production." Poster presented at ISSST2014. May 19-21: Oakland, CA.

Zhang, Y. "Nutrient and Energy recycling in Algal biodiesel production with different technologies." Jan. 28, 2014. NextSTEPS Seminar. Institute of Transportation Studies, Davis, CA.

W Lambert, (MS Student Palenik Lab). "Greenhouse and Pond Cultivation of Nitrogen-fixing Cyanobacteria Co-cultured with Diatoms" SD-CAB Student and Postdoc Symposium - March 15, 2013.

Specht, EA. "Characterizing chloroplast gene regulatory elements to construct optimized synthetic regulatory regions." Talk, Quantitative Biology Winter Conference. Waikiki, Hawaii. February 18, 2013.

Shuyi Wang, Postdoctoral Scholar ". "Microbial Dynamics in Model Algal Biofuels Ponds" Oral presentation at the SD-CAB student and Postdoc Symposium, March 15, 2013.

Lambert W, Palenik B. ""Greenhouse and pond cultivation of nitrogen-fixing cyanobacteria co-cultured with diatoms."" Poster presentation at the Food and fuel for the 21st Century Symposium. April 19, 2013.

Shuyi Wang, Sophia Giang, W Lambert, and Brian Palenik. "Microalgal assemblages in a poikilohaline pond and isolates transition to biofuels pond growth." Poster presentation at the Food and fuel for the 21st Century Symposium. April 19, 2013.

Shurin JB. "The ecology of algae biofuels." Universidad Pedagologico y Tecnologico de Colombia in Tunja, Colombia, May 24, 2013.

Shuyi Wang. "Microalgal assemblages in a poikilohaline pond and isolates transition to biofuels pond growth." Talk. Xiamen University, China. April 2013.

Wang, S Lambert, W, Giang, S, Goericke, R, and Palenik, B. "Microalgal Strains for Algal Biofuels Production in Outdoor Ponds" Algae Biomass Summit, Orlando, Florida (September 30-October 3, 2013).

Lambert, Billy. Public master's thesis defense. SIO, 2013.

Mendola, Dominick. "Micro-Algae Biofuels & Bio-Products Research & Pilot Commercial-Scale Projects Worldwide." Ningbo University, Ningbo, China, April, 2013, Oral presentation.

Dismukes, C. "Metabolic Switching During Carbohydrate Catabolism: Redox Energy Dynamics Revealed in Real-time" SEBS Microbiology Symposium, Feb 2012.

Danielli Matias Dantas, Frank Shang, S Shaleh, P Abelin, E Weiss, D Mendola, B G Mitchell. " Effect of Light and Temperature on Pigment, Lipid, Protein and Carbohydrate Content in the Marine Diatom *Thalassiosira pseudonana*." NAABB, June 10, 2012, San Diego, CA.

Jonathan Shurin, B G Mitchell. "Effect of irradiance and CO2 on the utilization of carbon in the marine diatom *Thalassiosira pseudonana*." SD-CAB Symposium, May 13, 2012, La Jolla, CA.

Sonnenschein and Burkart. "Metabolic engineering of microalgae towards the production of secondary metabolites" SD-CAB student and postdoc symposium - March 16, 2012, La Jolla, CA, USA.

Daulo, A Golden, S. "Engineering cyanobacteria to provide nutrients for algal biofuel strains" Oral presentation at the SD-CAB Student and Postdoc Symposium, La Jolla, CA, USA, April 10, 2012.

Kumaraswamy, G Kenchappa. "Metabolic engineering of *Synechococcus* sp. PCC 7002 for improved utilization of fixed carbon. " Food and Fuel for 21st Century, May 11-13 2012, La Jolla, CA.

Lambert. "Co-culturing nitrogen fixing cyanobacteria and diatoms in nitrogen deplete media for biofuels production" Oral presentation at the SD-CAB Student and Postdoc Symposium, January, 2012, La Jolla, CA, USA.

Lambert. "Co-culturing nitrogen fixing cyanobacteria and diatoms in nitrogen deplete media for biofuels production" Poster presentation at the Food and Fuel for the 21st Century, April 22-23, 2012, La Jolla, CA, USA.

B G Mitchell. "Modeling gross production, net production, and respiration of photosynthetic microalgae." NAABB Conference on Algal Biomass, Biofuels and Bioproducts, June 10-13 2012, San Diego, CA.

B G Mitchell. "Modeling growth of photosynthetic microalgae and carbon partitioning between lipid, protein and carbohydrate." 31st IUBS General Assembly and Conferences on Biological Sciences and BioIndustry, July 5, 2012, Suzhou, Jiangsu Province, China.

Shurin. "Algal traits and diversity as components of productivity and stability for biofuels." Oral presentation at the Food and Fuel for the 21st Century, April 22-23, 2012, La Jolla, CA, USA.

Sonnenschein, Eva. "Sonnenschein and Burkart: Metabolic engineering of microalgae towards the production of secondary metabolites, " Poster presentation at the 15th International Conference on the Cell & Molecular Biology of *Chlamydomonas*, June 5-10, 2012, Potsdam, Germany.

Sonnenschein and Burkart. " Metabolic engineering of microalgae towards the production of secondary metabolites, " Poster presentation at the Food and Fuel for the 21st Century, April 22-23, 2012, La Jolla, CA, USA.

Wang, Shuyi. "Microalgal use of diverse nitrogen sources for growth" Oral presentation at the SD-CAB Student and Postdoc Symposium, January 20, 2012, La Jolla, CA, USA.

Wang, Shuyi. "Microalgal use of diverse nitrogen sources for growth" Poster presentation at the Food and fuel for the 21st Century, April 22-23, La Jolla, CA, USA.

G Greg Mitchell (presenter), Du Niu, Daniel Yee. "Model of Growth, Respiration, Light Absorption and Photosynthetic Quantum Yield of Microalgae" Algae Biomass Summit meeting, Denver, CO. 26 September, 2012.

Mitchell, Greg. Program Committee Member and Session moderator; Algae Biomass Summit meeting, Denver, CO., 23-27 September, 2012.

Sonnenschein and Burkart. "Metabolic engineering of microalgae towards the production of secondary metabolites," Poster presentation at the CEC Large Molecule Sustainable Fuels Roadmap Meeting - October 18, 2012, La Jolla, CA, USA.

J Golden. "July seminars on Cyanobacterial Biotechnology" China at the Hydrobiology Institute and Central Normal University (Wuhan), Qingdao Institute of Bioenergy and Bioprocess Technology (Qingdao), and the Shanghai Institute of Plant Physiology and Ecology (Shanghai), 2012.

Shurin, Jon. "Ecology of algae biofuel" Departmental Seminar, University of Kansas. March 6, 2012.

Shurin, Jon. "Ecology of algae biofuel" Departmental Seminar, Missouri State University, March 9, 2012.

Shurin, Jon. "Ecology of algae biofuel" Associations for the Sciences of Limnology and Oceanography, special symposium on Biodiversity, July 10, 2012.

Shurin, Jon. "Ecology of algae biofuel" Sapphire Energy, Special Seminar, August 21, 2012.

BG Mitchell, Danielli Matias Dantas, Frank Shang, Sitti Raehanah Muhamad Shaleh, Patricia Abelin, Niu Du, Elliot Weiss, Dominick Mendola. "Effect of Light and Temperature on Photosynthetic Quantum Yield, Pigment, Lipid, Protein, and Carbohydrate Contenct in the Marine Diatom *Thalassiosira pseudonana* " National Alliance for Advanced Biofuels and Bioproducts, 2nd International Conference, June 10-13 2012, San Diego, CA, poster presentation.

B G Mitchell, Niu Du, Daniel Yee. "Photosynthetic Quantum Yield of Algae" Algae Biomass Organization Summit Meeting Sep. 24-27, 2012. Denver, CO, oral presentation.

Patricia Abelin, Laura T Carney, Frank Shang, and B Greg Mitchell. "Determination of water soluble carbohydrate for microalgae cultures using TPTZ method and application to the study of cellular carbon partitioning." Algae Biomass Organization Summit Meeting Sep. 24-27, 2012. Denver, CO, Poster presentation.

Yuan, J, & Kendall, A. "Life Cycle Assessments of Second and Third Generation Biofuels: A Review of Feedstocks, Processes and Environmental Implications." Growing the Bioeconomy Conference. Oct. 2-5, 2012. Bannf, Canada.

Du, N. "Life-cycle assessment of Algal Biofuel: Why do we biologists care?" SD-CAB Student and Postdoc Symposium, February 17, 2012, La Jolla, CA, Oral presentation.

Shang, F. "Carbon Partitioning in the Diatom *Thalassiosira pseudonana*." SD-CAB Student and Postdoc Symposium, May 13, 2012, La Jolla, CA, Oral Presentation.

Weiss, E. "Photophysiological acclimation of *Thalassiosira pseudonana*." SD-CAB Student and Postdoc Symposium, December 7, 2012, La Jolla, CA, Oral presentation.

Du, N. "A Light Dependent Model for the Growth Rate of *Thalassiosira pseudonana*." SD-CAB Student and Postdoc Symposium, December 7, 2012, La Jolla, CA, Oral presentation.

Falkowski P. "The Global Carbon Cycles" Oral presentation at American Society of Plant Physiology Meeting, June 2011, Minneapolis, MN, USA.

Dominick Mendola. "Energy from Algae" Aquatic Biomass Conversion for the Coachella Valley, Coachella Valley Energy Summit, May 12, 2011.

Dominick Mendola. "The Future for Algae Crops in the Imperial Valley" Imperial Valley Renewable Energy Expo, March 17, 2011.

B G Mitchell. "Growth Rates and Yields of Oil, Protein and Carbohydrate for Microalgae as Regulated by Growth Conditions, " Algae World Summit, May 23, 2011, Del Mar, CA.

B Greg Mitchell. "Photosynthetic Quantum Efficiency Measurement and Modeling: How and Why?" Algal Biofuels Symposium, April 30, 2011, La Jolla, CA.

B G Mitchell. "Opportunities for Improving Environmental Quality and Enhancing Natural Resource Base Provided by Algal Biofuels" NAS-NRC Study, March 17, 2011, San Diego, CA.

B G Mitchell. "Photosynthetic Quantum Efficiency Measurement and Modeling: How and Why?" ABO Summit, October 25, 2011, Minneapolis, MN.

Du Niu, Ian Woertz, James Rhodes, Dominick Mendola, Greg Mitchell, Tryg Lundquist, John Benemann. "Critical review on algal biofuel LCA articles' parameters and results, " ABO Summit, October 25, 2011, Minneapolis, MN.

Shurin, Jon. NRC Committee on Sustainable Development of Algal Biofuel, UC Irvine, CA; Sept 2011.

Amir Neori, Patricia Abelin, Sitti Raehanah M Shaleh, Dominick Mendola, B G Mitchell. "Spray Irrigation Culture of Macroalgae in CO2 Enriched Atmosphere using Recirculated Seawater, " ABO Summit, October 25, 2011, Minneapolis, MN.

Shurin, Jon. "Ecology of algae biofuel" Ecological Society of America, special symposium on Microbial Diversity, Aug 10, 2011.

Daulo, A, Godlen, S. "Engineering cyanobacteria to provide nutrients for algal biofuel strains" 2011 UC San Diego Division of Biological Sciences/SALK Institute Annual Retreat, Lake Arrowhead, CA.

B G Mitchell. "Are (Micro/Macro) Algae a Viable Option for Biofuel? " G'Day USA Clean Tech Conference, January 12, 2010, San Diego, CA.

B G Mitchell. "Prediction of the Consequences of the Obvious, " SD-CAB Symposium, April 23, 2010.

B G Mitchell. "Algal Biofuels: Because A Pig Won't Eat a Lump Of Coal" BIOCOM, September 15, 2010 San Diego, CA.

B G Mitchell. "Consortium for Algal Biofuels Commercialization (CAB-Comm)" DOE Webinar, September 8, 2010.

B G Mitchell. "Imperial Valley, Salton Sea and Water: The Alage Alternative" California Independent Voter Project, November 15, 2010, Maui, Hawaii.

Frank Shang, Laura Carney, Patricia Abelin, Rex Brookhart, B G Mitchell. "Effect of Temperature and Light Variation of on Fatty Acids, Pigment and Carbohydrate Yields for Marine Diatom, *Thalassiosira pseudonana*," ABO Summit, September 9, 2010, Denver, CO.

Genetic Tools

Anderson, M. "Building a synthetic transcription system in algae" Food and Fuel for the 21st Century Symposium oral presentation on March 13, 2015, La Jolla, CA.

Ferreira-Camargo, Livia; Tran, Millderl Tusakul, Lydia; Mayfield, Stephan. "Increase of protein accumulation in C*hlamydomonas reinhardtii* chlorolats: DsbA" Food and Fuel for the 21st Century Symposium poster presentation on March 12-13, 2015, La Jolla, CA.

Ostrand, J. "Breeding and FACS selection for increased recmbinant protein accumulation in C. *reinhardtii*." Food and Fuel for the 21st Century Symposium poster presentation on March 12-13, 2015, La Jolla, CA.

Ruben, B. "Exploring Cyanobacteria's Essential Genes." Food and Fuel for the 21st Century Symposium oral presentation on March 13, 2015, La Jolla, CA.

Scranton M. "Synthetic Promoters capable of driving robust nuclear gene expression in C*hlamydomonas reinhardtii*" Food and Fuel for the 21st Century Symposium oral presentation on March 13, 2015, La Jolla, CA.

Taton, Arnaud. "Synthetic Biology of Cyanobacteria: Further Development of the Genetic Toolbox." CAL-CAB Student and Postdoct Symposium oral presentation, 16 Jan 2015 La Jolla.

Golden, J. "Broad-Host-Range Genetic Tools for Cyanobacteria." Talk. ASM-2015 115th General Meeting; May 30-June 2 2015, New Orleans LA.

Golden, S. "Day, night, and time: how a cyanobacterium knows when to do what." Talk. American Society for Microbiology annual meeting; 31 May 2015, New Orleans, LA.

Mayfield, SP. "Photosynthetic bio-manufacturing in green algae." 5th International Conference on Algae Biofuels, Biomass and Bioproducts; 8 June 2015, San Diego, CA. Invited speaker.

Molino J. "Signal Peptides development for Algae." Food and Fuel for the 21st Century Symposium poster presentation on March 12-13, 2015, La Jolla, CA.

Taton, Arnaud. "Synthetic Biology of Cyanobacteria: Further Development of the Genetic Toolbox." Oral presentation at the Cal-CAB Student and Postdoc Symposium; Jan 16, 2015 La Jolla CA.

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

Arnaud Taton, Amy Ma, You Chen, Federico Unglaub, Tyler Swinney, Edward King, Ron Cook, Nicole E Wright, Susan S Golden, and James W Golden. "Improved genetic tools for cyanobacteria" 2014.

Oyler, George. "Algae expression of Nanobodies for Oral Enteric Theray" 2014 Food & Fuel for the 21st Century Symposium, UC San Diego, March 14-15, 2014., oral presentation.

Golden, Jim. "Cyanobacterial Strains, Genetic Tools, and Crop Protection" 2014 Food & Fuel for the 21st Century Symposium, UC San Diego, March 14-15, 2014., oral presentation.

Trentacoste, Emily. "The two sides of algae biofuels: building both molecular and political toolboxes" 2014 Food & Fuel for the 21st Century Symposium, UC San Diego, March 14-15, 2014, oral presentation.

Specht, Liz. "Tools for algal nuclear engineering: homologous recombination" 2014 Food & Fuel for the 21st Century Symposium, UC San Diego, March 14-15, 2014, oral presentation.

Abbriano, Raffaela; Smith, Sarah; Hildebrand, Mark. "An Integrative Approach to Investigate the Regulation of Carbon Partitioning in the Marine Diatom *Thalassiosira pseudonana*" 2014 Food & Fuel for the 21st Century Symposium, UC San Diego, March 14-15, 2014, poster.

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 & Fuel for the 21st Century Symposium, UC San Diego, March 14-15, 2014, poster.

Chen, You; Taton, Arnaud; Ma, Amy T; Pieper, Lindsey; Allen, Eric E; Golden Susan S; Golden, James W. "Engineering biosynthesis of long-chain polyunsaturated fatty acids in cyanobacteria" 2014 Food & Fuel for the 21st Century Symposium, UC San Diego, March 14-15, 2014, poster.

Ferreira-Camargo, Livia; Tran, Miller; Tusakul, L; Beld, Joris; Burkart, Michael; Mayfield, Stephen P. "Chemical and biological strategies for protein accumulation in C*hlamydomonas reinhardtii* chloroplasts " 2014 Food & Fuel for the 21st Century Symposium, UC San Diego, March 14-15, 2014, poster.

Georgianna, D Ryan; Muff Travis J; Carruthers, David N; Taylor, Bryn; Mayfield, Stephen P. "Development of Functional Synthetic Promoters for C. *reinhardtii*" 2014 Food & Fuel for the 21st Century Symposium, UC San Diego, March 14-15, 2014, poster.

Hyun, James; Gimpel, Javier; Schoepp, Nathan; Mayfield, Stephen. "Recombinant protein expression of M-SAA in C. *reinhardtii* chloroplast in a greenhouse facility" 2014 Food & Fuel for the 21st Century Symposium, UC San Diego, March 14-15, 2014, poster.

Manandhar-Shrestha, Kalpana and Hildebrand, Mark. "Overexpression of DGAT2 gene increases accumulation of neutral lipids in the centric diatom *Thalassiosira pseudonana*" 2014 Food & Fuel for the 21st Century Symposium, UC San Diego, March 14-15, 2014, poster.

Golden, J. "Cyanobacterial synthetic biology, genetic tools, and production of renewable products, such as biofuels" California State University Fresno, Fresno, CA. April 2, 2014, invited seminar.

Golden, J. "Cyanobacterial heterocyst development, nitrogen fixation, and biofuels" Reed College, Biology Department, Portland, OR. March 7, 2014, invited seminar.

Barrera, Daniel. "Algal Chloroplast Produced Camelid VHH Antitoxins are Capable of Neutralizing Botulinum Neurotoxin." Algae Biomass Organization Summit Meeting; Sept. 29-Oct 2nd, San Diego, CA. Poster Presentation. 2014.

Ferreria-Camargo, Livia. "Increase of Protein Accumulation in C*hlamydomonas reinhardtii* Chloroplasts: DsbA Protein." Algae Biomass Organization Summit Meeting; Sept. 29-Oct 2nd, San Diego, CA. Poster Presentation. 2014.

Gimpel, Javier. "Production of Recombinant Proteins in Microalgae at Pilot Greenhouse Scale." Algae Biomass Organization Summit Meeting; Sept. 29-Oct 2nd, San Diego, CA. Presentation. 2014.

Mayfield, Stephen. Panelist for "DOE Bioenergy Technologies, Office: Report from Project Performers". Algae Biomass Organization Summit Meeting Sept. 29-Oct 2nd, 2014 San Diego, CA.

Mark Hildebrand. "Improvement of Lipid Accumulation in Microalgae by Mutagenesis and Metabolic Engineering." ABO Summit, San Diego, CA September Invited speaker. September 29 – October 2, 2014.

Anderson, M. "Synthetic Algal Promoters for *Chlamydomonas reinhardtii* engineering." Oral presentation at the Cal-CAB Student and Postdoc Symposium; Nov 21, 2014 La Jolla CA.

Barrera, Daniel. "Developing microalgae for the production of orally available recombinant therapeutic proteins." Oral presentation at the Cal-CAB Student and Postdoc Symposium; Nov 21, 2014 La Jolla CA.

Golden, J. "Broad-host-range vector system for cyanobacteria." Kyungpook National University, Department of Biology, Daegu, Korea. Nov. 21, 2014. Invited research seminar.

Golden, J . "Broad-host-range vector system for cyanobacteria." 3rd Asia-Oceania Algae Innovation Summit (AOAIS). Daejeon, Korea. Nov. 17-20, 2014. Invited research seminar.

Golden, J. "Genetic Tools for Diverse Cyanobacteria." Robert Haselkorn Symposium. Univ. Chicago, Chicago, IL. Nov. 8, 2014. Invited research seminar.

Ostrand, J. "High throughput sorting and analysis for increased recombinant protein accumulation in C. *reinhardtii*." Oral presentation at the Cal-CAB Student and Postdoc Symposium; Nov 21, 2014 La Jolla CA.

Ruben, B. "Exploring Cyanobacteria's Essential Genes." Oral presentation at the Cal-CAB Student and Postdoc Symposium; Dec 12, 2014 La Jolla CA.

Taton, Arnaud. "Improved broad host range molecular tools for synthetic biology and biotechnology in cyanobacteria" Pacific Rim Summit on Industrial Biotechnology & Bioenergy. Invited as a speaker for the Life Technologies' workshop entitled "Advances in Algal Synthetic Biology Technologies". San Diego, California, December 8-11, 2013.

Brueggeman, Drew. "Development and testing of herbicide resistance genes for *Chlamydomonas reinhardtii* and algae of potential commercial importance." Nebraska Coalition for Algal Biology and Biotechnology, February 25, 2013, seminar.

Mayfield, Stephen. Invited seminar at Washington University St. Louis. DOE biofuels Center, January 22, 2013.

Mayfield, Stephen. Invited seminar at Michigan State University. Department of Biochemistry, January 24, 2013.

Mayfield, Stephen. Invited talk. San Diego Science and Engineering Festival, March 20, 2013.

Mayfield, Stephen. Invited talk. Life Technologies, Carlsbad CA, February 19, 2013.

Mayfield, Stephen. Committee meeting and talk. California oversight committee meeting on Salton Sea, February 22, 2013.

Thomas Plucinak. "The power of FMDV 2A technology for production of multiple proteins from a single gene in *Chlamydomonas reinhardtii*." Nebraska Coalition for Algal Biology and Biotechnology, February 25, 2013, seminar.

Weeks, Don. "Use of TAL Effector Nuclease (TALEN) Technologies for Targeted Gene Knockout and Gene Replacement" Seminar: UNL Center for Plant Science Innovation, January 17, 2013.

Ma A, Golden J. "Riboswitch-Mediated Regulation of Gene Expression in Cyanobacteria." 2013 Food and Fuel for the 21st Century Symposium. April 19, 2013. Poster.

Ma AT, JW Golden. "Riboswitch-Mediated Regulation of Gene Expression in Cyanobacteria" Annual meeting of the San Diego microbiology group. May 11, 2013. Poster.

S Golden. "Developing Cyanobacteria as Platforms for Food and Fuel Production" Food and Fuel for the 21st Century Symposium. April 19, 2013. Talk.

A Taton, E Lis, DM Adin, G Dong, S Cookson, F Unglaub, T Swinney, E King, R Cook, NE Wright, SA Kay, SS Golden and JW Golden. "Development of *Leptolyngbya* sp. BL0902 as a new bioengineering platform and improved genetic tools for cyanobacteria. " 2013 Food and Fuel for the 21st Century Symposium. April 19, 2013. Poster.

Invited Speaker, M Hildebrand, AK Davis, K Manandhar-Shrestha, RM Abbriano, JE Polle, SR Smith, JC Traller, EM Trentacoste, R Roth, U Goodenough. "Metabolic and Cellular Organization in Evolutionarily Diverse Microalgae As Related to Biofuels Production" The Third International Conference on Algal Biomass, Biofuels, and Bioproducts, Toronto Canada, June 16- 19, 2013.

EM Trentacoste, R Shrestha, SR Smith, C Gle, A Hartmann, M Hildebrand & WH Gerwick. "Increased lipid accumulation without compromising growth: Metabolic engineering of lipid catabolism in *Thalassiosira pseudonana*" The Third International Conference on Algal Biomass, Biofuels, and Bioproducts, Toronto Canada, June 16- 19, 2013.

J Traller, S Cokus, D Lopez, M Pellegrini, M Hildebrand. "Genome and Methylome Of A Candidate Biofuel Organism: How 'Omic' Data Can Inform Approaches To Improve Productivity" The Third International Conference on Algal Biomass, Biofuels, and Bioproducts, Toronto Canada, June 16- 19, 2013.

SR Smith, AE Allen, M Hildebrand. "A global regulatory mechanism integrates carbon and energy metabolism in the diatom *Thalassiosira pseudonana*" The Third International Conference on Algal Biomass, Biofuels, and Bioproducts, Toronto Canada, June 16- 19, 2013.

Golden, J. "Two presentations: "Broad host range tools for engineering cyanobacteria" and "Cyanobacterial synthetic biology, genetic tools, and production of renewable products"." Uppsala University, Uppsala, Sweden, September 25-27, 2013.Invited research seminar and PhD Opponent.

Golden, Jim. "Improved genetic tools for cyanobacteria" American Society for Microbiology General Meeting (Denver, CO). May 18- 21, 2013.11th Workshop on Cyanobacteria, St. Louis, MO, August 7-11, 2013, poster.

M Khasi, M Kang, KW Nickerson, G Oyler. "Nuclear Encoded Expression of GFP in *Chlorella vulgaris* UTEX 259." 2013.

Golden, J West. "Improved genetic tools for cyanobacteria" Coast Bacterial Physiologist's Meeting, Asilomar Conference Grounds, Pacific Grove, California, Dec. 13-15, 2013, oral presentation.

Specht, EA. "Characterizing chloroplast gene regulatory elements to construct optimized synthetic regulatory regions." Talk, Quantitative Biology Winter Conference. Waikiki, Hawaii. February 18, 2013.

Mayfield, SP. "Green Algae for Bio-products Production" FF21 Symposium, UCSD, La Jolla, CA. April 19-20, oral presentation.

Rasala, BA Lee, P, Barrera, D, Haerizdeh, F, Peterson, T, Rosenberg, J, Oyler G, Plucinak TM, Weeks, D, Mayfield, S. "Genetic tools for microalgal research and biotechnology" Poster. FF21 Symposium, UCSD, La Jolla, CA. April 19-20, 2013.

Specht, E. "Genetic Manipulation of Chloroplast Gene Expression" FF21 Symposium, UCSD, La Jolla, CA April 19-20, 2013, poster.

Gimpel, Javier. "Analysis of heterologous regulatory and coding regions in algal chloroplasts." FF21 Symposium, UCSD, La Jolla, CA. April 19-20, 2013, poster.

Georgianna, DR, Davis, I W, Elich, T, Mayfield, SP. "Development of Functional Synthetic Promoters for C. *reinhardtii*" Poster. FF21 Symposium, UCSD, La Jolla, CA. April 19-20, 2013.

Muff, TJ, Mayfield, S. "Transcriptional Regulation in *Chlamydomonas reinhardtii*" FF21 Symposium, UCSD, La Jolla, CA. April 19-20, 2013, poster.

Tran, M, Camargo, L. "Disulfide bond formation as a rate limiting step in the accumulation of complex recombinant proteins" FF21 Symposium, UCSD, La Jolla, CA. April 19-20, 2013, poster.

Emily Trentacoste, Jenifer Ro Hull, Roshan Shrestha, Sarah R Smith, Corine Gle, Aaron C Hartmann, William H Gerwick, Mark Hildebrand. "Metabolic Engineering of Lipid Catabolism Improves Lipid Yields from Microalgae" FF21 Symposium, UCSD, La Jolla, CA. April 19-20, 2013, poster.

DP Weeks. "Use of TAL Effector (TALEN) technology for development of rice resistant to bacterial blight" FF21 Symposium, UCSD, La Jolla, CA. April 19-20, 2013.

GA Oyler. "A Vision for Integrated Algae Systems using *Chlorella*" FF21 Symposium, UCSD, La Jolla, CA. April 19-20, 2013, talk.

Mark Hildebrand. "Improvement of Lipid Accumulation in Microalgae by Mutagenesis and Metabolic Engineering." ABO Summit, Orlando, FL September 30 - October 3, 2013. Oral presentation.

Sarah Smith, A Allen, M Hildebrand. "A Global Regulatory Mechanism Integrates Carbon and Energy Metabolism in the Diatom *Thalassiosira pseudonana*." ABO Summit, Orlando, FL September 30 - October 3, 2013. Oral presentation.

Emily Trentacoste, R Shrestha, SR Smith, C Gle, A Hartmann, M Hildebrand & WH Gerwick. "Increased Lipid Accumulation without Compromising Growth: Metabolic Engineering of Lipid Catabolism in *Thalassiosira pseudonana*." ABO Summit, Orlando, FL September 30 - October 3, 2013. Oral presentation.

Mark Hildebrand. "Improvement of Lipid Accumulation in Microalgae by Mutagenesis and Metabolic Engineering." FF21 Symposium La Jolla, CA. April 2013.

Sarah Smith, A Allen, M Hildebrand. "A Global Regulatory Mechanism Integrates Carbon and Energy Metabolism in the Diatom *Thalassiosira pseudonana*." FF21 Symposium La Jolla, CA. April 2013.

Hildebrand, M. "Differences in Carbon Flux Pathways and Photosynthetic Processes in Evolutionarily-distinct Microalgae." Invited Speaker, The 22nd Annual Western Photosynthesis Conference, Asilomar, CA, January 3-6, 2013.

Weeks, Don. "Transcriptome analysis of *Chlamydomonas* subjected to carbon dioxide deprivation, " to USDA North Central 1168 Photosynthesis Conference, Michigan State University.

Hildebrand, Mark. "Development of Diatom Genetic Manipulation Tools;" oral presentation at the Food and Fuel for the 21st Century, April 22-23, 2012, La Jolla, CA, USA.

Golden, Jim. "Cyanobacterial strains, genetic tools, crop protection, and nutrient supply; " oral presentation at the Food and Fuel for the 21st Century, April 22-23, 2012, La Jolla, CA, USA.

Rosenberg JN, Wan M, Kobayashi N, Andlay G, Balasubramanian A, Betenbaugh MJ, Oyler GA. " A comparative analysis of *Chlorella* species' heterotrophic growth characteristics and lipid composition," ABO Algae Biomass Summit (Denver, CO), Biology Breakout Session: Analysis and Modification of Lipid Production, September 27, 2012.

Weeks, Don. "Massive changes in *Chlamydomonas* gene expression during activation of the CO2-concentration mechanism triggered by CO2 depletion." Algal Biofuels Symposium 2012, held in La Jolla, California; May 2012, oral presentation.

Golden. "Development of *Leptolyngbya* sp. BL0902 as a new bioengineering platform and improved genetic tools for cyanobacteria" 14th International Symposium on Phototrophic Prokaryotes (ISPP), Porto, Portugal, August 5-10, 2012, oral presentation.

Hildebrand, Mark. "Evolutionary-based Differences in Microalgal Cellular Organization and Processes as Related to Biofuels Production" Invited Keynote Speaker and Session Chair, The Second International Conference on Algal Biomass, Biofuels, and Bioproducts, San Diego, CA, June 10-13, 2012.

Taton, Arnaud. "Gene transfer in *Leptolyngbya* sp. strain BL0902, a cyanobacterium suitable for biomass and biofuel production; " oral presentation at the SD-CAB Student and Postdoc Symposium, September 23, 2011, La Jolla, CA, USA.

Golden, J. "July seminars on Cyanobacterial Biotechnology" China at the Hydrobiology Institute and Central Normal University (Wuhan), Qingdao Institute of Bioenergy and Bioprocess Technology (Qingdao), and the Shanghai Institute of Plant Physiology and Ecology (Shanghai), seminar, 2012.

Oyler, George. "Chlamy Chloroplast Expression VHH Antibodies and Protein Delivery Domains and VHH Antibodies for Oral Therapeutics, " ABO Algae Biomass Summit (Denver, CO), September 27, 2012.

Oyler: Noel EA, Kobayashi N, Barnes AL, Watson A, Rosenberg JN, Erikson GE, Van Etten J, Oyler GA. "Integrated algae growth on anaerobic digester effluent: phylogeny and lipid compositions of *Chlorella* spp., " ABO Algae Biomass Summit (Denver, CO), September 24-27, 2012.

Beth A Rasala, Phillip Lee, Jenny Ng, Dan Barrera, Thomas M Plucinak, Julian Rosenberg, Donald Weeks, George Oyler, Stephen Mayfield . "Genetic tools for microalgal research and biotechnology" Algal Biomass Summit, ABO meeting, Denver, Colorado, September 27, 2012.Poster presentation.

Rosenberg: Rosenberg JN, Wauchope AD, Jiang WZ, Kang M, Kobayashi N, Tremblay JM, Shoemaker CB, Weeks DP, Hildebrand M, Betenbaugh MJ, Mayfield SP, Oyler GA.. "Development of a single-chain antibody toolkit to interrogate and manipulate the microalgal cell, " Poster, ABO Algae Biomass Summit (Denver, CO), September 24-27, 2012.

Weeks, Don. "Massive Transcriptome Changes During Activation of the CO2-concentrating Mechanism (CCM) in Microalgae and Development of TAL Effector Nucleases (TALENs) for Targeted Gene Knockout for Yeast and Rice." Lawrence Berkeley Laboratory, University of California-Berkeley, December 18, 2012.

Weeks, Don. "Use of TAL Effector Nuclease (TALEN) Technologies for Targeted Gene Knockout and Gene Replacement." USDA North Central Conference, Reno, Nevada.November 17, 2012.

Weeks, Don. "Developing Surface Nanobodies Specific to *Chlamydomonas reinhardtii*." Algal Biomass Summit, ABO meeting, Denver, Colorado, September 27, 2012.Poster presentation.

Weeks, Don. "Massive changes in gene expression associated with activation of the CO2-concentrating mechanism in Chlamydomonas reinhardtii." 15th International Chlamydomonas Conference, Potsdam, Germany, June 7, 2012.

Kobayshi N, Noel E, Barnes A, Rosenberg J, DiRusso C, Black P, Oyler GA. "Comparison of triacylglycerol quantification by HPLC-ELSD, GC/MS, and LC/MS methods in *Chlorella*, " ABO Algae Biomass Summit (Denver, CO), September 24-27, 2012.

JW Golden. "Development of *Leptolyngbya* sp. BL0902 as a new bioengineering platform and improved genetic tools for cyanobacteria" Development of Microalgae Industrial Biotechnology: from animal food to bioenergy, French BioBeach. November 12 2012, San Diego, CA.

Rasala, Beth Mayfield, SP. "Genetic tools for microalgal research and biotechnology" SD-CAB Symposium 2012, Food and Fuel for the 21st Century. May 2012.

Rasala, Beth Mayfield, SP. "Production of industrial enzymes in microalgae to enable cellulosic biofuels" American Chemical Society Spring 2012 National Meeting and Exposition. March 2012. Platform presentation.

Specht, EA Mayfield, SP. "Genetic Manipulation of Chloroplast Gene Regulation" San Diego Center for Algal Biotechnology Annual Symposium, La Jolla CA, 13 May 2012, poster.

Kumaraswamy, Kenchappa. "Metabolic engineering of *Synechococcus* sp. PCC 7002 for improved utilization of fixed carbon. " Food and Fuel for the 21st Century, May 11-13,2012.

Rasala, BA and Mayfield, SP. "Development of Molecular Genetic Tools for Microalgae" SD-CAB Symposium 2012, Food and Fuel for the 21st Century. May 2012. Platform presentation.

Weeks, Don. "Algal and Agricultural Biotechnology: The Next Wave " Seminar: Nebraska LEAD Program. October 25, 2012.

Brueggeman, Drew. "Massive changes in gene expression associated with CO2 deprivation in *Chlamydomonas reinhardtii*. " UNL Research Fair, November 16, 2012.

Yuan, J Kendall, Alyssa. "Life cycle assessment of second and third generation biofuels: a review of feedstocks, processes and environmental implications" Growing the Bioeconomy Conference, Banff, Alberta, Canada, 2012.

JW Golden. "Multiple Seminars: Heterocyst Development and Cyanobacterial Biotechnology" Hydrobiology Institute(wuhan), Central Normal University (Wuhan), Qingdao Institute of Bioenergy and Bioprocess Technology (Qingdao), and the Shanghai Institute of Plant Physiology and Ecology (Shanghai), July 1-12, 2012. Invited presentations.

JW Golden. "Cyanobacterial strains, genetic tools, crop protection, and nutrient supply" Food and Fuel for the 21st Century, La Jolla, CA. May 11-13, 2012, oral presentation.

J Golden. "Outreach presentation to 5th & 6th graders at SciFri." Del Mar Heights School, Del Mar, CA, March 30, 2012.

Golden. "Developing Cyanobacteria for Production of Industrial Products and Fuels" Speaker, Keystone conference on Biofuels, Singapore, 3/1/2011.

Golden. "Prospects for Renewable Biofuel Production by Cyanobacteria" 3rd Thomas Hunt Morgan Lecture speaker, University of Kentucky, Lexington KY, April 21, 2011.

Golden. "Breakout session on alternative fuels" National Academy of Sciences annual meeting, "Micro-algae for the Production of Biofuels and Bio-products," Washington DC, May 1, 2011.

Dismukes. Seminar at ACS National Meeting (Denver, CO, August 2011).

Taton, Arnaud. "Gene transfer in *Leptolyngbya* sp. strain BL0902, a cyanobacterium suitable for biomass and biofuel production" oral presentation at the SD-CAB Student and Postdoc Symposium, September 23, 2011, La Jolla, CA, USA.

Dismukes, C. NSF Algal Biofuels Workshop. http://www.engr.colostate.edu/NSFalgaeworkshop/index.html (Arlington, VA; November, 2011).

Mayfield, Stephen. Invited Seminar at Agilent. Santa Clara, CA; November 2011.

Mayfield, Stephen. Oral presentation, Algal Biomass Organization, Minneapolis, MN; October 2011.

Mayfield, Stephen. Invited talk. Atlantic meets the Pacific Forum, La Jolla, CA; October 2011.

Mayfield, Stephen. Invited talk. Berlin Algae Conference, Berlin, Germany; December 2011.

Mayfield, Stephen. Invited talk. Environmental Entrepreneurs Breakfast, La Jolla, CA; October 2011.

Mayfield, Stephen. Microorganisms for Biofuel Production from Sunlight. Invited talk, Bielefeld, Germany; September 2011.

Weeks, Don. Invited talk. Li-COR Corporation, Lincoln, NE; December 12, 2011.

J Golden. Invited talk. NSF Algae Workshop, Colorado, November 2011.