
NAABB
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FULL FINAL REPORT
SECTION III

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SECTION III
Individual Project Summaries

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Work Funded by U.S. DOE-EERE Bioenergy Technologies Office, DE-EE0003046

Systems-level Analysis of N-starvation Induced TAG Accumulation in a *Chlamydomonas* Starchless Mutant

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Algal-derived oil has gained much attention in the past decade as a possible renewable energy source due to algae's higher yield compared to land plants and its lack of competition with human feedstocks. However, several barriers exist in generating an economically viable algal-derived biofuel. Chief among these are gaps in our fundamental knowledge of algal cell physiology, genomics, metabolism, and regulation of metabolic pathways.

Chlamydomonas reinhardtii, a reference alga, is capable of accumulating intracellular lipids, which can be converted to biofuel apparently under any condition in which the cells experience physiological stress, such as nutrient deprivation, salt, or high light. In particular, deprivation of nitrogen has been intensely studied for this purpose in recent years. As such, *Chlamydomonas* serves as an excellent reference organism to elucidate metabolic pathways and enzyme function with the intention of translating the findings to more industrially relevant strains of algae. A related area of research is based on the observation that starchless mutant strains, such as *sta6*, hyper-accumulate lipids when stressed. Since, when placed under duress, *Chlamydomonas* generates starch first and lipids second, the generation of "obese" cells containing a higher triacylglycerides (TAG) content per dry weight in *sta6* presumably represents a channeling of carbon into lipid production.

The primary task of the Merchant and Pellegrini laboratories in the context of the National Alliance for Advanced Biofuels and Bioproducts (NAABB) project is to employ genomics and computational analyses to elucidate the underlying molecular mechanisms leading to differential lipid accumulation in general and specifically in two stressed strains of *Chlamydomonas*, *sta6* versus *cw15*, its parent. These approaches have several key advantages. First, since lipid production occurs under stressed conditions, mRNA levels of genes associated

with these biosynthetic pathways should be enriched when deprived of nitrogen. A comparative analysis of *sta6* and *cw15* should therefore illuminate the metabolism permitting *sta6* to hyperaccumulate lipid relative to *cw15*, aiding gene discovery. Second, a global, time-course approach enables similarly expressed genes to be grouped, facilitating regulon annotation. Finally, the wealth of data provided by deep-sequencing methods contributes to the overall accuracy of the *Chlamydomonas* genome sequence and gene models.

Towards this goal, we have generated high-quality RNA from *sta6* and *cw15* under nitrogen deprivation in time-course experiments, from 0 h to 48 h. We have subsequently analyzed the transcriptomes by comparing each dataset at each time point to the time zero and by comparing strains at each particular time point. We identified a large number of differentially expressed genes between each comparison. In particular, enzyme activity and metabolite assays validated an observed enrichment of mRNA levels corresponding to genes associated with the glyoxylate and gluconeogenic pathways, suggesting increased hexose/hexose-6-phosphate levels in *sta6*.

Sequencing the genome of *sta6* revealed the precise nature of the *STA6* mutation, as well as exposing a number of other significant mutations, totaling 7 genes. It was therefore questionable as to whether the loss of *STA6* or another mutation caused the different gene expression profiles in *sta6* and *cw15*. To investigate this, we prepared mRNA from nitrogen-deprived *sta6* and the three *STA6*-complemented strains, *STA6*-C2, *STA6*-C4, and *STA6*-C6 at 0.5 h, 4 h, and 48h, analyzed by RNA-Seq methods. Analysis of these datasets verified genes encoding enzymes of the glyoxylate cycle and gluconeogenesis were differentially expressed as a result of the *STA6* mutation. We subsequently independently validated this result at the proteome and metabolome level by comparing enzyme activity and metabolite level between *sta6* and the *STA6*-complemented strains.

On the basis of these results, we hypothesize that increased metabolic flux through these pathways is instrumental in enabling higher lipid production in *sta6* compared to *cw15*. Although a valid mechanism would seem to be increased acetate assimilation in the *sta6* strain, which is a notion apparently supported by increased expression in *sta6* versus *cw15*, all strains assimilated acetate at comparable rates. Increased reductant production by increased turnover in the pentose phosphate pathway suggests the possibility that enzyme cofactor level rather than carbon availability is a significant limiting factor in lipid synthesis.

One of the problems associated with interpreting algal omics-based data sets is the high proportion of proteins of unknown function. Indeed, with near 50% of coding regions in *Chlamydomonas*, the best studied alga, having little or no functional annotation, this has become a rate-limiting step in placing metabolic context on transcriptomic data. One method to help overcome this is to employ comparative genomics. To aid with this, we have built the algal annotation genome tool, available at http://pathways.mcdb.ucla.edu/algal/index.html. This web-based database enables the annotation of algal genes, and this has been useful for the annotation of other algal genomes.

Whilst *Chlamydomonas* remains an excellent reference organism, it is unlikely to become a production strain. To date, lipid accumulation can only be induced when cells are placed under duress, which is coupled with the inherent caveat of arresting cell division, ultimately limiting biomass and TAG production. We have therefore forged collaborations within the NAABB consortium to sequence the transcriptome of *Botryococcus braunii*.

Finally, we have investigated the nutritional requirements for optimal *Chlamydomonas* growth. By defining the concentrations of micronutrients necessary to achieve optimal growth rate, we are able to maximize biomass production whilst minimizing cost of media ingredients.

Determining the Effects of Genetics and Environment on Algal Biofuel Model Species

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Our project goals were (1) to identify components of the media or interactions between components that are the key drivers of algal growth and oil production and (2) to use genetics to identify genes involved in promoting growth and oil production in reduced-nutrient media.

Goal 1: Identify components of the media or interactions between components that are the key drivers of algal growth and oil production.
Elements are essential components of algal growth media and are significant components of the production costs. However, the levels of each element in the environments where algae can grow vary widely. If the concentration of each element is conceptualized as one dimension of a multidimensional space, the potential growth space is vast and gets even larger when the various fertilizers are considered. Complicating efforts to identify optimal media for growing algal biofuel production strains, elements can interact with each other through direct chemical interactions or indirectly through their effect on the organism. We have taken an unbiased approach to identifying important components of the growth media. By moving from single-factor analysis to looking at the multidimensional matrix of elements, we are able to identify interactions between elements that are critical for the growth of algal strains.

Goal 2: Use genetics to identify genes involved in promoting growth and oil production in reduced-nutrient media.
The parallels between crop plant domestication and algal biofuel species domestication suggests that large breeding programs will be necessary for major gains in multiple traits. It is therefore critical that we develop quantitative genetics and breeding approaches in algae. *Chlamydomonas reinhardtii* is one of the few algae with a sexual cycle that is well understood and easily manipulated. Given the vast array of other genomic and strain resources, it is a natural choice for a test system and should allow for facile downstream gene identification.

Aim 1: Exploring the media growth space of algae strains. We used "design of experiments" methodologies to screen algae growth in response to different media compositions. For *Chlamydomonas*, we screened two strains, *CC125* and *CC1952*, in a media space with different levels of seven macronutrients (Na^+, K^+, Cl^-, SO_4^{2-}, PO_4^{3-}, NO_3^-, and NH_4^+) and acetate (components of standard *Chlamydomonas* tris-acetate-phosphate, or TAP, medium) from 0.1–100 mM. A total of 160 growth media, representative of an even sampling of the entire growth space, were selected for the experiment. We used the same approach to screen *Auxenochlorella protothecoides* (previously *Chlorella protothecoides*) in a media space that varied NO_3^-, NH_4^+, urea, and NaCl.

Most of the conditions in the initial *Chlamydomonas* screen did not support growth and none of the medium supported growth as well as TAP media, which is an incredibly rich medium. However, we were able to get both strains to grow in Medium 1 which contained 75 times less N and tenfold less P than TAP. Since it is impossible to vary the levels of the individual components of the media independently and maintain the pH, we allowed the pH to vary in our experiments. This also has the advantage of matching the wide range of pHs in available water sources for algal biofuel production. In both rounds of the *Chlamydomonas* experiment, we observed growth across a wide range of pHs. We were also able to determine that several nutrients considered to be macronutrients, such as potassium, could be omitted entirely without preventing growth, as the micromolar concentrations present in the micronutrient solutions were enough to support growth.

Aim 2: Quantitative genetics and selection for improved growth and lipid accumulation. We started with two strains designed to maximize the available genetic diversity in the population, *Isolo10* and *CC1952*. *Isolo10* is a derivative of the common lab reference strain *CC125* that the Dutcher lab cleaned up through repeated backcrosses to improve the crossing efficiency. We have chosen *CC1952* as the other parental strain because it is highly polymorphic (1 SNP every 47 base pairs) with *CC125*. To maximize QTL mapping potential, we crossed *NIT1* and *NIT2* back into *Isolo*, and backcrossed 10 times to generate the *NIT1NIT2*-complemented *CC125* strains *Napoleon* (*mt+*) and *Dynamite* (*mt-*). We then used *Napoleon10* (the tenth backcross) to generate a new recombinant population. To prevent gametogenesis in *Chlamydomonas* during selections, we introgressed the *nic7* into the *Napoleon* background. This allows the populations to be treated with 3-acetylpyridine to kill off any progeny carrying the *nic7* allele, thus eliminating one mating type and preventing further mating within the population.

We attempted two different types of selections, one for growth and one for high lipid. To select for growth in reduced-nutrient media, we tested a population from the *CC1952* x *nic7* cross in a medium identified by the growth screen (medium 1) and in TAP, a conventional rich media, using serial transfers between flasks. For lipids, in collaboration with Taraka Dale at Los Alamos National Laboratory, we sorted the population using fluorescence-activated cell sorting (FACS) to isolate the fractions of nitrogen-depleted *Chlamydomonas* cells that demonstrated the highest fluorescence intensity when stained with the vital dye BODIPY 505/515, a neutral lipid marker. Technical challenges we were unable to solve due to time and resource limitations prevented us from successfully completing a selection for growth or lipid accumulation before the

end of the funding period. The Danforth Center Phenometrics photobioreactors should all have turbidostat modules shortly after the funding period ends, and we hope to attempt another selection in an abiotic stress environment at that time. We are also hopeful that technical issues with staining of *Chlamydomonas* can be resolved to allow for selections for high lipid.

We created a population of 384 lines by crossing *CC1952* x *Napoleon9*. We did multiple rounds of the cross and dissected tetrads from individual zygotes to ensure that each line was unique. Because the NAABB grant is ending, we are distributing the population to anyone interested in using it. The Baxter lab has committed to genotyping 192 lines, and Michael Purugganan and Kourosh Salehi-Ashtiani have agreed to genotype an additional 192. These lines will be an enduring resource for the algal biofuels and *Chlamydomonas* communities.

Genomic and Transcriptomic Resources for Analysis of Algal Production Candidates

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Although much effort has been expended to characterize growth phenotypes and the lipid-generation potential of many algae, knowledge of the genetic and genomic basis that defines and controls their physiological behavior was sorely lacking. The high degree of algal diversity prevented the extrapolation of genetic, physiological, and biochemical properties observed in common model organisms (such as *Chlamydomonas*) to the algae of interest. Furthermore, only a handful of algal genomes have been sequenced to high quality and those that have are not viable candidates for employment in biofuel production systems. Therefore, to effectively inform and populate genetic engineering pipelines, genomic and transcriptomic sequencing and analysis projects were implemented for the most promising biofuel production strains identified by NAABB investigators.

With respect to algal genomes, our team in collaboration with several NAABB members sequenced and assembled eight algae species from three phyla. We also generated preliminary gene models and functional annotations for *Nannochloropsis salina*, *Picochlorum* sp., and *Auxenochlorella protothecoides* to enable construction of metabolic pathways. In addition, to identify potential genomic mutations responsible for the observed phenotypes, we resequenced a low-phosphate-adapted mutant of *A. protothecoides* and a hyper–lipid-accumulating natural (non-genetically modified) mutant of *Picochlorum* sp.

In terms of gene expression and phylogenetic profiling, we sequenced 220 transcriptome samples for 11 NAABB investigators at seven institutions and we processed 18 16S rRNA samples for phylogenetic analysis. We completed many of the gene-expression studies during nitrogen deprivation or other stress conditions to monitor changes in gene expression during lipid-induction time-course experiments.

To improve assembly, data interpretation, and analysis, we customized informatics pipelines to enable processing of algal samples. To enhance analysis, we successfully constructed a beta version of a web-based omics viewer to facilitate integrating the genomics, transcriptomics, and proteomics data generated by various consortia members. When fully developed, we envision that this viewer will be publicly available, allowing the algal research community to continue to mine large omics datasets to gain a systems-level knowledge about any sequenced production strain.

Proteomics Analyses of Model Algal Strains

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The goal of this task was to utilize proteomics based on liquid chromatography (LC) and mass spectrometry (MS) to identify proteins responsible for important algal strain characteristics identified by NAABB Algal Biology investigators. One major proteomics project was with Dr. Peter J. Lammers at New Mexico State University. Differentially expressed proteins are expected to be involved in lipid production and provide gene targets for genetic engineering of improved algal strains. For this task we performed proteomic analysis of *Nannochloropsis* time-course samples grown in a photobioreactor. The time-course samples had corresponding fatty acid methyl ester analysis performed at New Mexico State University. For proteomics analysis of the *Nannochloropsis* time course series samples produced by Lammers at New Mexico State University, a total of 14 LC/MS injections were performed for each of four sample days (Day 1, Day 8, Day 15, and Day 22). We identified more than 1700 proteins and 4000 peptides more than once in the analysis. In statistical modeling of relative protein expression, we analyzed 445 proteins by spectral counting methods and 510 proteins by mixed-effect statistical modeling of mass spectral abundances.

Because of the limited amount of genome similarity of *Nannochloropsis* to other annotated model organisms, a significant number of open reading frames identified in the proteome analysis did not have a homolog in the UniProt database. The majority of the identified proteins that have increased expression over time are those involved in glycolysis, including glyceraldehyde-3-phosphate dehydrogenase, fructose bisphosphate aldolase (also involved in gluconeogenesis and the Calvin cycle), and enolase. A component of the pyruvate dehydrogenase complex, which converts the end product of glycolysis to acetyl-CoA, also demonstrated an increase in expression in comparison to the Day 1 sample. Glutamate decarboxylase, which is also increased in the Day 22 sample, is a component of the γ–aminobutyrate (GABA) shunt, which in plants has been associated with responses to stress.

The majority of proteins with decreased expression are involved in photosynthesis, including photosystem Q protein, chlorophyll a-b binding protein, photosystem II reaction center protein, fucoxanthin-chlorophyll a-c binding protein, photosystem II chlorophyll a apoprotein, and chlorophyll a-b

binding protein. Ribulose bisphosphate carboxylase (RuBisCO) expression is also reduced in comparison to Day 1. In *Chlamydomonas*, oxidative stress has been reported to reduce RuBisCO expression. On the whole these results suggest that *Nannochloropsis* metabolism at Day 22 is drastically shifted from the early-stage growth as photosynthesis machinery appears to be down-regulated and enzymes in glycolysis are up-regulated.

We performed an additional time-course analysis on samples provided by Dr. Scott Twary at Los Alamos National Laboratory. Over several weeks we completed proteomic analysis of a set of time-course samples from both *Nannochloropsis* and *Nannochloris* following lipid production. The overall goal was to identify proteins differentially expressed under lipid and non-lipid productive growth states. For proteomics analysis of the *Nannochloropsis* time-course series samples produced by Twary, we performed six LC/MS injections for each of six samples (harvested July 30, August 1, August 3, August 6, August 7, and August 12 of 2011). We identified more than 900 proteins and 2150 peptides more than once in the analysis. In statistical modeling of relative protein expression, we analyzed a total of 250 proteins by spectral counting methods and 375 proteins by mixed-effect statistical modeling of mass-spectral abundances. As observed in the analysis of Lammers' *Nannochloropsis* samples, a significant number of predicted open reading frames with increased abundance in the later time-point samples did not have any identified homologs by BLAST analysis present in the UniProt database. Four proteins observed with increased expression in later time points were identified as sharing homology to glutathione reductase, peroxiredoxin, and superoxide dismutase, which are involved in responses to cellular oxidative stress. An additional two proteins with increased expression in later time-point samples have similarity with peptidyl-prolyl cis-trans isomerase enzymes. These enzymes have been shown to have increased expression in plants under stress and to influence gene expression in eukaryotes.

A majority of the proteins with reduced expression in later harvest time points are involved in protein synthesis machinery or regulation of transcription. Of interest is the open reading frame with reduced expression that is homologous to adenosylhomocysteinase. In yeast the reduced expression of this enzyme correlates with an increase in triacylglyercides. An open reading frame with homology to ketol-acid reductoisomerase also has reduced expression in comparison to the initial sample. This protein is a member of the branched-chain amino acid biosynthetic pathway. While sampling times were not identical between the Los Alamos National Laboratory and New Mexico State University experiments, we observed this open reading frame to have reduced expression in later time-course samples in both analyses.

The final major proteomics project is a collaboration with Dr. Judy Brown at University of Arizona to examine protein differences between low-phosphate-adapted strains of *Chlorella protothecoides* and *DOE1412* and their parental strains. This project is currently underway. We also performed several smaller proteome projects, including experiments with Dr. Rose Ann Cattalico at University of Washington to determine protein components of lipid bodies in *Chrysochromulina* sp., identification of extracellular tryptic digested proteins of *C. protothecoides* with Dr. Amanda Barry at Los Alamos National Laboratory, and analysis of *Dunaliella salina* with Dr. Juergen Polle at Brooklyn College.

Lipid Production in Salt-tolerant Algae: Transcriptomic Analysis and Adaptive Evolution of *Picochlorum* sp. and *Nannochloropsis salina*

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The NAABB techno-economic analysis continues to show that increasing the productivity of algae will have the greatest impact on the economic viability and sustainability of the algal biofuels industry. In addition NAABB's Biomass Assessment Tool guides us to the most economically favorable biofuel production scenario: year-round algae production in temperate coastal regions using seawater. Our focus was on two salt-tolerant strains *Picochlorum* sp. and *Nannochloropsis salina CCMP1776*. Both strains are salt tolerant and have been cultured in up to double the salinity of seawater. In addition, *N. salina* in particular and *Picochlorum* to a lesser extent have been cultured successfully in outdoor ponds and outdoor photobioreactors by NAABB partners. The physiology described in more detail in this report is ideal for making fuels; specifically, under nutrient stress conditions they convert CO_2 to triglycerides at an accelerated rate, which allows maximum lipid yield while minimizing the demand for nitrogen and phosphorous.

First we developed a photobioreactor that can be used to reproducibly culture algae and conduct careful biochemical analyses that were used to characterize phenotypes. Our bioreactor is a computer controlled 5 liter chemostat. This chemostat controls and logs the temperature, pH, dissolved [O_2], and rate of addition of gasses CO_2, O_2,and N_2. For illumination, we use a bank of 24 fluorescent lights placed approximately 3 cm from the surface. The photosynthetically active radiation (PAR) measured at the center of the reactor, looking all directions, was 1200 $\mu E/m^2/s$. The pH of the growth medium was 8.2 and during growth, the pH was used to control the addition of CO_2, which ensured that both the pH and the concentration of the carbon substrate ([CO_2] + [HCO_3^-] + [CO_3^{-2}]) were constant throughout the experiment. The photobioreactor was sampled automatically using a robot. These samples were used to determine $OD_{750\ nm}$, total lipid as fatty acid methyl esters (FAMEs) and biomass dry weight. We also developed protocols and routinely measured both the intracellular and media concentrations of nitrate and phosphate. Samples were also analyzed by flow cytometry to measure cell number, cell size, and autofluorescence. The fluorescent dye BODIPY was used to measure total neutral lipids.

Representative data from photoautotrophic growth of *Picochlorum* and *N. salina* are presented. Although algae grown in our photobioreactors grew under significantly less light then is available outdoors, we achieved initial growth rates equivalent to 26 $g/m^2/d$ for *N. salina*. Similar growth rate was achieved for *Picochlorum*. Each of these marine algae increases its lipid accumulation rate when nitrogen is depleted from the growth medium. Under nitrogen depletion, the rate of lipid accumulation increases 3-fold in *Picochlorum* cultures. More significantly, when nitrate is depleted from the growth medium of *N. salina* cultures, the lipid accumulation rate increases 6.3-fold from 24 mg/L/d to 151 mg/L/d. Under these conditions *N. salina* cultures accumulate lipids to approximately 50% of the biomass.

Systems biology analysis of increased lipid biosynthesis in Picochlorum *and* N. Salina

Very high quality genome sequences of *Picochlorum* and *N. salina* were produced, the details of which are reported elsewhere in this report. We obtained parallel transcriptome and proteome analysis of algal strains during the initial rapid growth phase, as nitrate was exhausted from the growth medium and during the rapid lipid biosynthesis phase. Analysis of the transcriptome data for *Picochlorum* shows that during depletion of nitrogen from the growth medium there is a clear induction of key genes encoding for lipid biosynthesis and an induction of genes required for starch biosynthesis. This transcriptomic profile suggests that upon nitrogen depletion from the growth medium, *Picochlorum* stores energy and carbon as starch and triglycerides. This result suggests that in *Picochlorum* significant carbon flow might be redirected to lipid biosynthesis by knocking out the genes for starch biosynthesis.

Our analysis of the *N. salina* transcriptome suggests genes that could be constitutively overexpressed to accelerate induction of lipid biosynthesis. These genes include the lipid droplet protein, which is highly induced during nitrate depletion and the onset of rapid lipid accumulation. Genes encoding for one of three acetyl-CoA carboxylase (the initial step in lipid biosynthesis) and diacylglycerol acyl transferase (DGAT) (the final step in triglyceride biosynthesis) are also induced during the high-lipid-production phase of growth. Finally our transcriptome experiments suggest that repressing the expression of 3-ketoglutarate dehydrogenase could minimize acetyl-CoA oxidation and lipid catabolism, which would have the effect of increasing overall lipid production.

Adaptive evolution of high-lipid producing Picochlorum *sp.*

Using fluorescence-activated cell sorting, we have isolated a population of *Picochlorum* that shows a ≥ 70% increase in lipid accumulation and a 140% increase in rate of lipid accumulation during nitrogen depletion, relative to the unsorted parent population. These measures of lipid biosynthesis have been validated by gas chromatography–mass spectrometry FAME analysis and the population has been stable for more than 100 generations (more than a year). Improved lipid accumulating performance is also observed under nitrogen replete conditions. We sequenced the genome of the sorted and parent population and sequenced the transcriptomes of the sorted and parent populations, under nitrogen replete and deplete conditions. Very few SNPs were observed in the open reading frames of the *Picochlorum* genome when comparing the genome of the sorted population to the parent population. However, a large number of changes were observed in the transcriptome. Changes in transcriptome levels (sorted relative to parent) were observed in numerous metabolic pathways, including fatty acid metabolism, the TCA cycle, glycolysis/gluconeogenesis, amino acid metabolism, starch and sucrose metabolism, and purine and pyrimidine metabolism.

Assessment of Microbial Biocontaminants in Ponds and Genetic Engineering Approaches to Protecting Algal Crops

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Cultivation of algae in algal raceways and open ponds is envisioned as the most economical route for algal biomass and biofuels production. In the coming decades, if the projected scales of biofuels are to be generated, algae have to be cultured in several thousand acres of land for the desired biomass yields. Like most of the plant crops, algal cultures in open ponds are susceptible to a number of environmental factors, including biological agents that enter the ponds. Our goal is to examine and understand the nature of biotic factors such as bacteria, viruses, invasive algal species, fungi, and herbivores in algal ponds that impact the algal biomass yields. It is broadly accepted that the biological agents that are present in the algal ponds could be beneficial, harmful, or unobtrusive to the cultivated algal culture. Entry of invasive species into algal culture can have deleterious effects on algal cultivation, result in lowered biomass yields, and even cause pond crash resulting in crippling loss to biofuels producers. For instance, it has been documented that certain bacteria, virus, fungi, or herbivores such as rotifers can cause demonstrable losses to the algal crop. One of the major and imminent challenges in cultivating algal crops in an open environment is therefore protecting the algal crop from invaders.

Furthermore, our investigations are also important because we hope to determine if any potential human, livestock, or plant pathogens can propagate in the algal pond ecosystem that could cause serious problems to cultivators and the environment. If this is not addressed actively, in the long run it could pose serious sustainability problems for the algae production industry. We also undertook identifying economically viable strategies to reduce and eliminate contaminants without incurring additional cost in mitigating biomass losses. Furthermore, we proposed to investigate molecular approaches to contain genetically modified algae propagation outside the algal ponds.

As part of the Algal Biology Team in the NAABB consortium, we carried out census analysis on organisms that enter and possibly thrive in algal ponds. With this knowledge, we aimed to determine economically viable alternatives for algal crop protection from harmful contaminants through bioengineering approaches. To understand the biological agents that co-inhabit algal ponds, we designed and conducted experiments with both laboratory grown cultures of *Chlamydomonas reinhardtii* and *Auxenochlorella protothecoides UTEX25* (representative fresh water cultures grown in nutrient-rich media) and raceway-grown cultures of *Nannochloropsis salina* (a representative marine species grown at Texas AgriLife–Corpus Christi, provided by Dr. Tzachi Samocha). Based on the rRNA gene-based identification of species of contaminants in the *UTEX25* culture, we identified different bacterial and fungal species, with bacterial population reaching 10% of the algal culture at the end of seven days. Importantly, many representative bacteria belonged to the genus *Pseudomonas*, with a few of them being opportunistic pathogens.

For the *N. salina* contaminant survey, we undertook both rRNA sequencing and more extensive chip-based analysis of species distribution (PhyloChip, Lawrence Berkeley National Laboratory). Our surveys indicated that an array of organisms ranging from bacteria, fungi, and viruses inhabit these raceways. Significantly, in our analyses, only one known species of human pathogen was identified. It is unclear if this strain was actively propagating in the open ponds or happened to have entered the pond at the time of sampling.

Our pond survey studies conclusively proved that large numbers of bacteria can coexist with algae in open pond cultures and the occurrence of algae-invading opportunistic pathogens is certainly a looming problem. To address this, we explored the use of antimicrobial peptides (AMPs) and other biomolecule production in algae for improving their innate defense against bacteria and rotifers. In this effect, we successfully screened and identified one or more AMPs that kill bacteria and biomolecules that eliminate rotifers but not our algae of choice for biomass production. To achieve economically viable means for crop protection, we have successfully developed bioengineering tools for expressing these agents in the chosen green algae. Various lines of algae expressing these biomolecules have been generated to test the viability of these transgenic algae lines in the presence of deleterious bacterial and rotifer species. Based on a similar approach, new biomolecules or gene products can be identified to test their applicability in eliminating other invasive contaminants. Furthermore, we have also undertaken the task of characterizing gene-switch mediated regulation of chloroplast genes in the model organism *C. reinhardtii* to determine its applicability in genetically modified algae containment. The regulation of the gene-switch is currently coupled to a small molecule effector that regulates the expression and/or translation of the gene coupled to this element. If this strategy is proven successful, we hope to extend this idea into other green algae that are genetically engineered for biofuels or other value-added-product production, thus averting their proliferation in the natural environment.

Investigating Natural-selection Approaches to Obtain Strains with Improved Robustness.

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Algae can reproduce at least 100 times more oil per surface area than traditional land crops and on a lab scale we can convert the oil into drop-in fuels. However, we now face the challenge of performing this conversion on a large commercial scale. One cost-reduction strategy involves adapting the algae to grow with fewer inputs without compromising lipid yields.
Growing algae under suboptimal conditions can promote adaptation through selection (or adaptive evolution) and thereby create optimized strains that could outperform native algal strains under selected conditions. A prime target for adaptation is lowered-phosphate use because phosphate shortages are anticipated due to rapid depletion of the phosphate supply globally.
Continuous cultivation of *Auxenochlorella protothecoides* in a circulating,

replenishing chemostat system with constant selective pressure imposed by the stepwise reduction of the total phosphate concentration showed promising signs of adaptation beginning approximately 4 months after culture establishment under low phosphate selection.

The low-P adapted strain out-performed the wild type *A. protothecoides* (positive experimental control) under the same low-phosphate growth conditions, producing approximately 25% more total biomass. The robustness of this adapted strain was evaluated for total biomass and lipid content. We used RNAseq for gene expression (collaboratively with Dr. Starkenburg) and proteome analysis (collaboratively with Dr. Panisko; in progress) to identify the biochemical and genetic/genomic bases for adaptation, respectively.

The RNAseq expression analysis identified five significantly misexpressed transcripts showing responsiveness to the lowered phosphate availability. That suggests compensatory genetic modifications ensued to permit more efficient growth (similar biomass production with less phosphate supplied) compared to the wild type. Proteome analysis is in progress with this strain, compared to the wild type alga (Panisko lab).

Pilot-scale testing is planned to evaluate performance traits, including fitness (growth and cell division), total biomass, and lipid production in the outdoor low-cost Aquaculture Raceway Integrated Design (ARID) UA algae cultivation raceway.

Molecular Monitoring of Algae and Associated Bacterial Communities for Quality Control of Laboratory, Scale-up, and Cultivation Operations

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To facilitate long-term, successful monoculture production of algae, identification and monitoring of the algal-bacterial community has become essential to maintain quality control at key steps in the pipeline, from primary culturing through scale-ups, growth-cultivation phases, and pre-harvest. Therefore, the second aim was to develop, validate, and implement molecular diagnostics tools implementing polymerase chain reaction (PCR) amplification and DNA sequencing of candidate bacterial/chloroplast 16S rDNAs and algal 18S rDNAs. Using published reference sequences for identified bacteria or algal spp., the closest match to our sequences was determined to establish identity (high thresholds, e.g. ~1.0–1.5% divergence) or closest relatives (lower thresholds, e.g. > 1–2 % divergence). The monitoring of bacterial, plastid, or algal nuclear rDNA genes (relative presence over run time, seasons, etc.) will allow for rapid assessment of the composition of the prominently present organisms in a community at a given time.

Using the 18S rRNA sequences, sequence-specific primers were designed for 18 algal species or strains in the NAABB project. Primers were validated to establish primer specificity and expected product length for the NAABB prioritized algal species, while providing, when possible, a diagnostic-size product for the particular alga as well as specific primers. Together this approach increases monitoring efficiency of the cultivated algal species and circumvents the need for DNA sequencing each time to achieve a rapid turnaround time for monitoring needs.

In addition, 16S rRNA sequence analysis even of most "pure" laboratory cultures (18 tested herein) has revealed that a suite of bacteria is associated with most algal cultures even when grown in "pure" culture. Axenic culture for most algae cultured in our hands is not free of bacteria despite rigorous selection. The extent and types of bacteria are now being monitored to establish baseline community profiles that might represent "healthy" compositions of organisms, in relation to different algal species.

Clearly it is not possible to detect or identify every prokaryotic or algal species in a scale-up or cultivated unit of any significant size using this method and relatively small sample size (20 16S rDNA clones) or to determine the exact composition of the total bacterial community (18S rDNA), given the prohibitive time and cost of doing so. However, implementation of the 16S rDNA assessment approach, together with monitoring algal populations with algal-specific primers (the expected species), proved to provide valuable snapshots of the community composition (together with cell counter/hemostat counts of sizes and shapes) over the time of the run. Implementation also provides valuable information about the commonly occurring organisms associated with a given culture and cultivation system such that when baseline perturbations are detected, it may provide early warning of a flux in the system. That flux could, if caught early, be remedied upon closer inspection to determine the cause and abatement plan.

From these studies we hope to learn if such monitoring can provide a measure of a "healthy" cultivation system, such that when the system is perturbed, the diagnostic results provide a reliable indicator of impending or existing problems that are not easily recognized by cell count methods. Cell count methods often miss impending contaminations until the competing organism has reached competitive numbers that could result in displacement of the cultivated alga. These diagnostics have been used to monitor several runs in the raceway in which *Chlorella* sp. *DOE1412* is cultivated, and testing identified a foreign algal that predominated midday-to-late in one run and a concomitant shift in the bacterial community. Upon the initial recognition of a contaminant species-specific *Scenedesmus* primers were developed and included in the diagnostic panel to learn more about the ecology of possibly endemic, perhaps seasonally abundant algal species. In this same run the bacterial 16S rRNA primers identified *Vampirovibrio,* a bacterium that attacks and consumes certain algal species, indicating a clear target for the treatment program to rid the cultivation system of these two invaders and restore health and stability. This is but one example of the kinds of biotic stresses that are expected to be encountered in monoculture systems, similarly to cropping systems producing food or fiber, and for which pest and pathogen management becomes an inherent requirement for achieving consistent quality and yield.

Lipid Productivity Gene Identification and Engineering of Improved Strains

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The goal of this project is to develop an efficient method to identify genes in *Chlamydomonas* whose expression can be modified to increase production of triacylglycerols and other lipids. We have focused on developing an inducible RNAi system that contains RNAi targeted against every gene. This is accomplished by generating a random library of genomic DNA that is converted into a microRNA (miRNA) precursor that can be converted by the cell into miRNA. We successfully made the library and deep sequencing confirms that it has a very high diversity with over 10^6 different genomic inserts that contain at least one match to nearly every gene in the *Chlamydomonas* genome. The library currently exists in two vectors, one in which the miRNA is constitutively expressed and another in which it is driven by the *Nit1* promoter and inducible by nitrogen starvation. Tests of the *Nit1* vector have shown that it has the desired property of expressing the miRNA only under inducible conditions where it specifically knocks down the target gene. Screens are ongoing to select strains with desired phenotypes from the entire library from which the miRNA inserts can be sequenced *en masse* to identify the gene knockdowns responsible for those phenotypes.

The ultimate goal is to use this system to identify genes that up- or down-regulate the production of TAGs and other lipids, work that is ongoing. After we have identified genes that are important for lipid production through these genome-wide screens, we will construct strains with multiple expression variants simultaneously to determine if we can get additive effects. The goal is to create strains with either consistently or readily inducible high levels of TAGs and lipid production that can be effectively used in high-production settings. In addition we hope to learn more about the general biochemical pathways that are involved in lipid production and turnover, information that can be transferred to other algal species that may have more beneficial characteristics than *Chlamydomonas* and be more useful for production.

Lipid-body Production in Microalgae

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The two goals of our sponsored research by the NAABB have been (1) to understand and develop the potential of the chlorophyte microalga *Chlamydomonas reinhardtii* as a biofuel producer and (2) to evaluate the potential of additional candidate production strains of interest to the NAABB consortium. The primary investigative tool has been quick-freeze deep-etch electron microscopy (QFDEEM), and the projects have entailed productive collaborations with NAABB-supported laboratories headed by T. Devarenne, J. Jaworski, P. Lammers, S. Merchant, M. Pellegrini, M. Park, J. Polle, and R. Sayre and with Department of Energy–supported laboratories headed by C. Benning, M. Hildebrand, and M. Posewitz.

We have focused our studies on maximizing triacylglycerol (TAG) production and, in one alga, long-chain hydrocarbon production, these being familiar starting materials for diesel and kerosene/jet fuel production by the petrofuel industry. Recently, studies have explored the potential of hydrothermal liquefaction and related technologies to generate starting materials for biofuel products from crude algal biomass. In this context, low-cost biomass might be projected to be more important than biomass composition; that said, a high TAG content in any biomass can only enhance the value of its product.

When we joined the NAABB consortium, we had just demonstrated that the *sta6* strain of *C. reinhardtii* carrying a deletion in a gene required for starch synthesis produces two to three times the level of TAG as its parent starch-producing strain when both are subjected to nitrogen starvation. During the NAABB-supported period, we discovered that the augmented TAG levels in *sta6* can be attributed to the fact that it produces TAG-filled lipid bodies (LBs) in both the cytoplasm and the chloroplast, whereas they are only produced in the cytoplasm in wild-type cells. We went on to develop a protocol (the "acetate boost") that further amplifies LB augmentation, generating "obese" *sta6* cells that are 70% TAG on a dry-weight basis and produce approximately 1.3 g TAG per liter of culture, exceeding all published yields for a microalga. Importantly, the obese cells float on water, a possible adjunct for harvesting, and they lack cell walls, a key adjunct for breakage and drying. Using RNA sequence analysis, we identified the few genes whose expression is up-regulated with acetate boost. They prove to encode enzymes in the glyoxylate, gluconeogenesis, tricarboxylic acid, and pentose-phosphate pathways.

Our current model is therefore as follows: (1) Nitrogen starvation stimulates the up-regulated expression of genes encoding enzymes involved in TAG biosynthesis, as observed by several laboratories, generating an elevated steady-state level of TAG production. (2) The final TAG yield then depends on how long the cells stay alive in TAG-production mode, and the acetate boost prolongs nitrogen-starved cell lifetime by approximately 8 days, apparently by stimulating the operation of key metabolic pathways. Metabolomic analysis of this transition will be of great interest.

In conversations with downstream algal biofuel investigators, we have learned that to date there has been little success in growing large-scale cultures of *C. reinhardtii*, for reasons that are not clear. Moreover, several laboratories, including our own, reported that *C. reinhardtii* requires exogenous acetate for robust TAG production, a drawback for several reasons. In fact, however, on-going experiments in our laboratory indicate that the acetate requirement can be bypassed by increasing light intensity and bubbling 3% CO_2. And while *C. reinhardtii* is the only oleaginous algal species with a defined sexual cycle, a positive feature for combining traits, it currently lacks homologous knockout and exogenous gene-expression capabilities, important for genetic engineering. Given these drawbacks, we elected to evaluate a number of additional microalgae, including *Auxenochlorella*, *Dunaliella, Hematococcus, Ochromonas, Symbiodinium, Thalassiosira*, and *Cyclotella*, and focused on three groups: *Nannochloropsis*, *Cyanidioschyzon merolae*, and *Botryococcus braunii*.

Nannochloropsis is a small marine stramenopile that grows photoautotrophically to high density in large facilities. Several genomes have been sequenced, and homologous knockout and exogenous gene expression is reported. QFDEEM of four *Nannochloropsis* species has elucidated their mode of LB formation. We found that one species, *N. gaditana*, can augment its TAG content to the point that it can be designated obese: the cells float and most contain a single enormous mega-LB, where mega-LB formation entails a unique pathway of ER and chloroplast-thylakoid autophagy. The downside of *Nannochloropsis* is that it possesses a highly impermeable (recalcitrant) cell wall that is very difficult to break or deconstruct, impeding product extraction. A class of materials generically called algaenans has been identified in the *Nannochloropsis* wall and also in the walls of *Scenedesmus* and *Chlorella* strains that display robust pond growth. This concordance suggests that algaenans may confer both robustness and recalcitrance. Our lab has initiated studies of algaenans, which are reportedly cross-linked long-chain hydrocarbons, with the goal of understanding their composition and biosynthetic pathways, information that may allow us to engineer strains that preserve the robustness and lose the recalcitrance.

Cyanidioschyzon merolae is a small red alga that grows photoautotrophically to high densities in high-sulfur media at pH 2 and 40°C, an environment that is presumably hostile to most putative predators. Its small genome is sequenced and it can be subjected to homologous knockout and exogenous gene expression. By QFDEEM and biochemical assays, it produces almost no LB/TAG during log phase, but a marked increase can be induced with nitrogen starvation and salt addition. Further engineering of this trait is now in progress.

Botryococcus braunii is a colonial freshwater trebouxiophyte that is currently totally unsuitable for development because it grows very slowly and has poor genetic tools. However, it produces copious amounts of a product that is highly suited to fuel generation, a long-chain liquid hydrocarbon called botryococcene, under photoautotrophic conditions. *B. braunii* would be the organism of choice if its growth rates could be enhanced and/or if its genes for botryococcene production could be productively expressed in a faster-growing alga (or other organism). Our extensive QFDEEM, histochemical, and biochemical studies, recently published, have generated numerous insights into the organization of *B. braunii* and its botryococcene-producing/retaining mechanisms, and these are expected to inform such future endeavors.

Hydrocarbon Biosynthesis Gene Identification and Extracellular Matrix Characterization in the Green Microalga *Botryococcus braunii*

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This study developed transcriptomes from the three chemical races, A, B, and L, of the green colony-forming microalga *Botryococcus braunii*, which produces large amounts of liquid hydrocarbons that can be converted into petroleum-equivalent fuels. The transcriptomes were used to identify genes from all three races that have proposed roles in hydrocarbon biosynthesis. Several of the genes have been cloned and are in the process of being characterized for activity of the encoded enzyme. Another aspect of our studies was to learn more about the structure and composition of the *B. braunii* extracellular matrix to better understand how *B. braunii* may be engineered for future use.

The three chemical races of *B. braunii* are defined based on the type of hydrocarbon they produce. Race A produces fatty acid derived alkadienes and alkatrienes, Race B produces the isoprenoid-derived triterpenes known as botryococcenes, and Race L produces the isoprenoid derived tetraterpene known as lycopadiene. All three oils have been found as major constituents of petroleum deposits suggesting *B. braunii* was a large contributor in geologic times to the formation of these deposits.

For our transcriptome studies we isolated total RNA from several days throughout the 30-day culture cycle of *B. braunii* with a focus on the early time periods when the hydrocarbon biosynthetic rate is maximal. We did this to optimize the chances of having the transcriptomes containing gene sequences directly related to hydrocarbon biosynthesis. The isolated RNA was analyzed by several methods to confirm integrity/quality and presence of some known genes related to hydrocarbon production. Once the quality of RNA was established it was sent to Los Alamos National Laboratory for library construction and sequencing using Illumina technology. The resulting sequences were assembled into contigs by fellow NAABB member Istvan Molnár.

For Race B, the main genes involved in botryococcene production have been previously identified, thus we used the transcriptome to reconstruct the entire pathway for botryococcene production through the isoprenoid pathway. We identified all main isoprenoid pathway genes and constructed additional pathways of importance, including triacylglycerols and starch.

The Race A transcriptome was used to identify potential genes in this race that are involved in alkadiene/triene biosynthesis. This first step in alkadiene/triene production is the elongation of the fatty acid oleic acid (18:1 *cis*-Δ^9) and/or its isomer elidaic acid (18:1 *trans*-Δ^9). This elongation is similar to that which takes place in the production of waxes for the leaf cuticle in land plants. Thus, we searched the Race A transcriptome for contigs similar to the fatty acid elongation genes from land plants and identified six candidate genes. These genes are currently being cloned for future characterization.

For Race L, the biosynthesis of lycopadiene is predicted to require a gene similar to the squalene synthase (SS) gene. Thus, the Race L transcriptome was screened for contigs that are similar to SS. Two Race L contigs were found that have significant homology to SS and they have been cloned for characterization of the enzyme activity of the encoded protein. For these enzyme characterization studies an *in vitro* cell free enzyme assay was developed for the production of lycopadiene. This assay entails using a Race L protein extract with NADPH, the substrate geranylgeranyl diphosphate (GGPP), and analyzing the reaction products by gas chromatography–mass spectrometry. Two molecules of GGPP will be condensed to produce lycopadiene. This assay was successful in production of lycopadiene and will be used with bacterial protein extracts from cells expressing the cloned SS-like genes to test the activity of the encoded proteins.

Finally, we undertook a collaboration with fellow NAABB member Ursula Goodenough to study the structure and composition of the *B. braunii*, Race B extracellular matrix (ECM). The Goodenough lab used cryo-electron microscopy for their portion of this study, while the Devarenne lab used histochemical microscopy and biochemistry to identify the composition of the ECM. It was found that the *B. braunii* colonies are completely surrounded by a polysaccharide fibrillar sheath. These sheath fibers are composed primarily of arabinose and galactose connected by unusual linkages between individual sugars such as 2, 3 and 2, 4 connections. This may be to help reduce the possibility to degradation of the polysaccharides by bacteria. This sheath appears to function in retaining the liquid hydrocarbons within the ECM. Thus, removal of the sheath fibers would allow for release of the hydrocarbons from the ECM, facilitating easy hydrocarbon collection under culturing conditions.

Overall, these studies have offered new insights into the genes needed for hydrocarbon biosynthesis in *B. braunii* as well as the makeup of the ECM to identify engineering targets.

Optimizing Culture Conditions for Hydrocarbon Production

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Our work focused on isoprenoid production to biofuels. The research epicenter began with the colonial micro green alga, *Botryococcus braunii* var Showa (Race B), which is known to be a prolific producer of the highly branched, unsaturated hydrocarbons known as botryococcenes. This freshwater alga has been reported to produce greater than 50% of its dry cell weight in botryococcenes, mostly accumulating in the extracellular matrix (Figure 1). These compounds range in size from C31–C36, are derived exclusively from a terpenoid biosynthetic pathway and have been shown to be convertible to gasoline, jet fuel, and diesel using existing infrastructure currently employed by the petrochemical industry (Figure 2). Although this alga is attractive as a source of biofuels, the scientific community generally recognizes it grows too slowly, with doubling times on the order of days, for practical use as a production strain. Therefore, our primary goal was to unravel the molecular details associated with hydrocarbon production in *B. braunii* var Showa and, ultimately, genetically manipulate an algal production strain that is (1) fast growing and (2) demonstrates increased carbon flux through the isoprenoid pathway to desired end-products (i.e., botryococcenes).

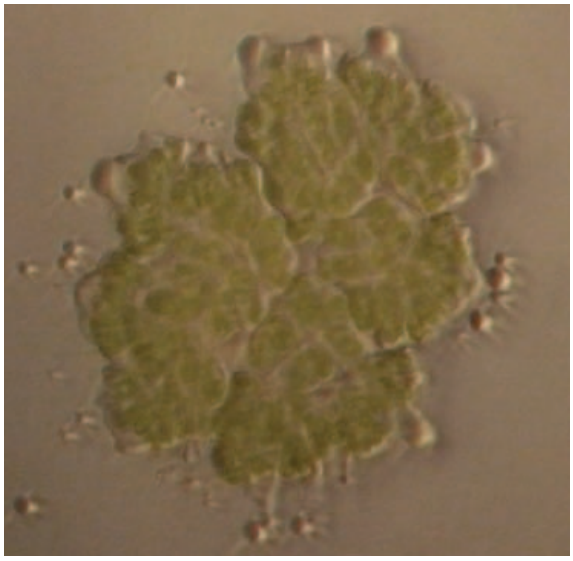

Figure 1.

Initially, we began our pursuit with our colleagues manually annotating the *B braunii* var Showa transcriptome (Istvan Molnar, University of Arizona, and Timothy Devarenne, Texas A&M University). They identified all the precursor genes encoding for enzymes to isoprenoids and narrowed in on likely candidates that address bottlenecks toward botryococcene biosynthesis. Briefly, gene candidates included *dxs I, II, III* (deoxyxylulose phosphate synthase), *dxr* (methylerythritol phosphate synthase), *ispH* (hydroxydimethylallyl diphosphate synthase), *fps* (farnesyl diphosphate synthase) and, importantly, *sqs1, 2, 3* (squalene synthase-like), which were found to be responsible for the head-to-head coupling of two farnesyl units to provide the C30 botryococcene. Based on these findings our team opted to engineer isoprenoid-specific genes into the proof-of-concept green alga *Chlamydomonas reinhardtii* and the cyanobacterium *Synechococcus elongatus PCC7942* and determine the extent of increased hydrocarbon production as a result of overexpression of the respective enzyme. The Sayre laboratory provided vector design and transformation protocols into the *C. reinhardtii CC-4147* strain and the Fox team is developing vector design for insertion into the *PCC7942* genome.

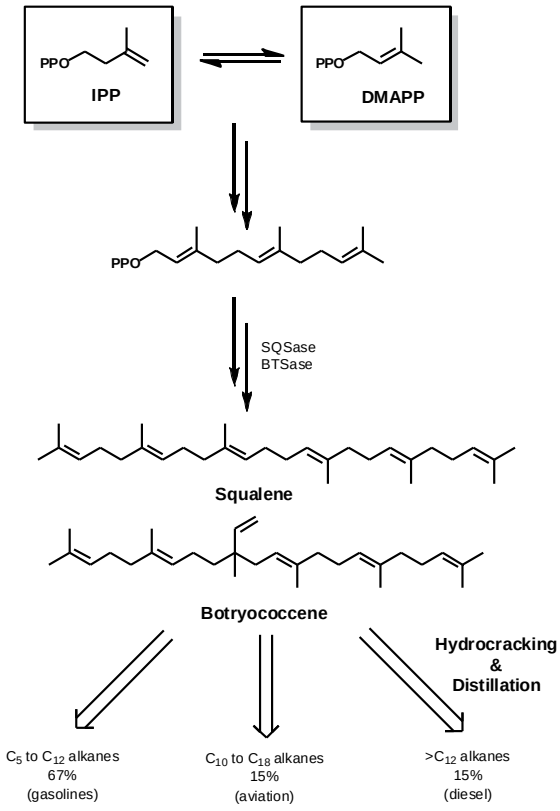

Figure 2.

To rapidly assess carbon flux through the methylerythritol phosphate (MEP) pathway to isoprenoids, we designed synthetic schemes to isotopically labelled precursors. Specifically, we made significant progress toward the synthesis of [1-^{13}C]-deoxy-D-xylulose for feeding studies into multiple algal strains. We designed our first route to proceed through the intermediate Weinreb amide, which enables facile incorporation of the 1-^{13}C label at the penultimate step in the synthesis. However, in our hands yields were prohibitively low for use in subsequent feeding studies. We turned our attention to an alternative approach where the 1-^{13}C label was introduced into a known aldehyde intermediate. Following oxidation of the secondary alcohol and deprotection, 1-^{13}C-DX was provided in less than 10% overall yield on a low milligram scale. There were sufficient quantities to perform a couple feeding studies to examine carbon distribution through the MEP pathway but postincubation and organic extraction results were inconclusive. Scale-up of the synthesis will be necessary to assess DX uptake and assimilation into downstream isoprenoids in comparative analysis between wild-type and genetically modified algae.

In parallel to the previously described projects, our team also focused on synthesizing, characterizing, and implementing biocompatible solvent for extraction of isoprenoids from both algae in a manner that does not significantly reduce culture viability (i.e., reagents capable of "milking" the cultures for hydrocarbon compounds). A panel of ionic liquids designed for optimal isoprenoid solubility were synthesized in gram quantity and their utility was assessed as a function of both (1) culture biocompatibility and (2) extractive efficiency of targeted long-chain isoprenoids (e.g., C_{30} and C_{40}). Whereas several ionic liquids were tolerated relatively well in a prokaryotic model system (i.e., in a cyanobacterium), only one proved to be tolerated to a significant degree in *B. braunii*. Initial results indicate that the extractive does not cause an appreciable reduction in photosynthetic ability (as measured by O_2 evolution). In our assays, the ionic liquid, [C4MPy][Tf2N], appeared to reduce cell viability slightly more than hexane, although other authors have reported an effect on culture viability of hexane exposure comparable to our estimate of the ionic liquid.

Extractions of botryococcenes from ~1.3 mg dcw of *B. braunii* was also similar for both hexanes and [C4MPy][Tf2N] (approximately 25 +3 nmoles, or 10.5 +1.3 μg, extracted using hexanes while 20 +4 nmoles, or 8.4 +1.7 μg extracted using [C4MPy][Tf2N]). The average colony in our labs comprises about 130 μg per 1.3 mg dcw, and extraction efficiencies are approaching 10% after only 30 minutes of moderate mixing. Although extraction yields were comparable to hexanes, an upper limit for product recovery was not attained, unlike hexanes, thus providing a scaffold for future IL formulations. The use of [R3R'P][Cl] or [Bu4P][Cl] in the extraction process resulted in complete cell death of the *B. braunii* sample. Extraction with these ILs resulted in the complete depigmentation of *B. braunii*, likely not only from extraction of botryococcenes/carotenoids but also liberation of chlorophylls. Commonly used extractive methodology (such as extraction with linear alkanes) was observed by us (or reported by others) to be tolerated less well by *B. braunii*.

Genetic Engineering of *Auxenochlorella* to Enhance Lipid Production

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With the increasing world demand for biofuel, oleaginous algal species are being developed as renewable sources of oil. The unicellular green alga *Auxenochlorella protothecoides* is a photosynthetic aquatic species that is a member of the Scotielloideae subfamily of the family, Chlorellaceae. The alga is considered one of best species for genetic and metabolic engineering to produce biodiesel fuels due to the large oil content accumulation (< 55% of its dry weight) when heterotrophically cultivated.

The *Auxenochlorella* species synthesizes triacylglycerols (TAGs) as a storage compound that can be converted into renewable fuels. Although TAGs are assembled in the endoplasmic reticulum, their main constituents—fatty acids—are synthesized in the chloroplast. The regulation of this anabolic pathway is poorly understood. The paucity of available algal chloroplast genome sequences has been an important constraint to chloroplast transformation and for studying gene expression in TAG pathways.

Our initial goal was to devise a method for chloroplast enrichment to facilitate completing chloroplast genome sequences. Intact chloroplasts from the green oleaginous microalga were successfully enriched and the accompanying debris was decreased using sonication, followed by sucrose density gradient ultracentrifugation. Quantitative polymerase chain reaction analyses revealed the intact chloroplasts were effectively enriched by 2.36-fold from *A. protothecoides*, compared to the number in control samples. We anticipate that the approach will guide chloroplast enrichment efforts for additional oleaginous algal species.

Taking advantage of the devised method, we isolated total DNA from the enriched chloroplast fraction and performed DNA sequencing using the Illumina platform to yield a total of 3,032,536 reads. The chloroplast genome sequence was determined to be 84.5 kb in size that, when annotated, revealed 114 annotated open reading frames (putative coding regions). The genes include 32 tRNAs, 26 rRNAs, 21 photosystem subunits, 6 ATPs, 6 transcription and translation related genes, 5 cytochromes, 4 chlorophyll genes, 2 chloroplast division related-genes, 1 RuBisCO, 1 acetyl CoA reductase (*accD*) gene involved in the fatty acid biosynthetic pathway, and 7 others. The size of the cpDNA of *A. protothecoides* is relatively smaller than the cpDNA of *C variabilis* (124,579 bp) and *C. vulgaris* (150,613 bp) due to the loss of noncoding regions. Not only are some genes not present, but also the cpDNA of *A. protothecoides* shows high gene compactness (only 19% of entire genome is non-coding sequences), in contrast to *C. variabilis* cpDNA and *C. vulgaris* chloroplast genomes contained 46% and 53% noncoding sequences. Overall, the three algal chloroplast genomes shared many highly conserved genes; however, they each also had unique coding and noncoding regions. Gene synteny was conserved overall, but each genome likewise exhibited some variation. The phylogenetic analysis revealed a distinct *Auxenochlorella-Chlorella* sp. clade, in relation to the more divergent species included in the analysis.

Despite great potential of *Auxenochlorella* as an oil producer, a genetic engineering toolbox has not been developed for widespread use. Strategies involving genetic transformation of the *A. protothecoides* nuclear genome to knockout expression of key gene targets, and chloroplast transgenesis to achieve overexpression of different key targets, all involved in the lipid biosynthetic pathways were considered in the project. Chloroplast transformation can exploit the use of homologous recombination, which has distinct advantages over nuclear transformation, including (1) site-specific insertion of transgene, thereby reducing unintended phenotypic effects, (2) relatively higher heterologous protein production, and (3) regulation of chloroplast genes involved in fatty acid biosynthesis.

The complete genome sequences enable us to construct three overexpression plasmid vector cassettes flanked with the chloroplast regulatory sequences atpA, psbD/C, and rbcL and the fluorescent protein iLOV reporter. Each cassette is flanked with chloroplast-derived homologous sequences for chloroplast recombination. We tested the various constructions using glass bead, electroporation, and particle bombardment transformation. A method that employs glass beads was used successfully for nuclear transformation of *A. protothecoides* and *DOE1412* (a NAABB field isolate of *Chlorella* sp.) with the pGreen plasmid vector bearing the aphVIII gene encoding paromomycin resistance. However, transformation efficiency was 4/114 clones, or 3.5%, and 2/96, or 2%, respectively.

Upon validation, candidate genes involved in lipid biosynthesis and free fatty acid trafficking to the cytosol/or TAG assembly in the ER were selected for chloroplast overexpression to increase lipid accumulation. Several candidate gene targets were selected for *A. protothecoides* chloroplast genetic engineering. The list includes two *B. braunii* squalene synthase-like genes (ssl-1, HQ585058.1, and ssl-3, HQ585060.1), three isoforms of *B. braunii* 1-deoxy-D-xylulose 5-phosphate synthase gene (dxs-I, JF284350.1; dxs-II, JF284351.1; and dxs-III, JF284352.1), *B braunii* 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (lytB/hdmapp), *B braunii* Farnesyl diphosphate synthase (fdps), and *A. protothecoides* accD gene.

Transformation experiments using these candidate genes are underway. We anticipate the genetic engineering of *Auxenochlorella* species could provide an important step forward to enable increased use of algae for biodiesel production by providing essential molecular tools that will make possible the modulation of key enzymes in lipid biosynthetic pathways.

Dissection of Hydrocarbon Biosynthetic Pathways in *Botryococcus braunii* for Metabolic Engineering of Novel Biofuels

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The Molnár group at The University of Arizona contributed to the work of the integrated *Botryococcus* team within the NAABB consortium. The overall long-

term goal of this team was to investigate the possibility of the biological production of hydrocarbon-based drop-in fuels that are fully compatible with existing petrochemical infrastructure and to minimize extraction costs through identifying hydrocarbon-excretion mechanisms.

We targeted three chemistries to achieve the long-term goals. These were triterpenoid hydrocarbons (C_{30} botryococcenes and methylsqualenes), tetraterpenoid hydrocarbons (C_{40} lycopadiene), and long-chain, fatty-acid-derived alkenes (C_{23}-C_{33} alkadienes and alkatrienes). These compounds are true drop-in fuels that can completely replace the currently used fossil-based crude oils. This biocrude can be refined to gasoline, jet fuel, and diesel by existing technology already deployed in the petrochemical industry. Further, refined products from this biocrude are fully compatible with currently existing distribution (pipelines and pumps) and existing use infrastructure (engines in all transportation market segments).

The best model organism to study the biosynthetic pathways for these chemistries is the green alga *Botryococcus braunii*. As an added benefit, *B. braunii* strains accumulate their hydrocarbon biocrude products outside of the cell in an extracellular matrix material. This trait may permit relatively mild extraction procedures, thus allowing the recycling of "de-fatted" algal biomass ("milking"). This trait, if transferred to other algae, may also eliminate product toxicity issues for the producer organism.

B. braunii strains fall into three chemically distinct races, each producing one of the targeted chemistries listed above. The close phylogenetic relationship of these races may imply that the genomes of these strains are relatively similar in gene content, with the notable exception of the genes encoding the distinguishing hydrocarbon synthetic pathways. Thus, we hypothesized that comparative omics approaches may be utilized for the identification of relevant biosynthetic pathways. Since the genome of *B. braunii* is especially large and complex, we have elected to conduct our studies at the transcriptomic level. Thus, we have isolated mRNA and used next-generation sequencing technologies to assemble practically complete transcriptomes *in silico* for all three races of *B. braunii*. Next, we have used a large array of bioinformatic methods to annotate the functions of these transcripts and to reconstruct biosynthetic pathways and biosynthetic networks. We have concentrated on the pathways that affect hydrocarbon biosynthesis and export. In particular, we were interested in the biosynthetic pathways that:

- yield the universal terpenoid precursors DMAPP and IPP,
- provide C_{30} botryococcenes and C_{40} lycopadienes,
- provide C_{23}-C_{33} alkadienes and alkatrienes,
- contribute to the extracellular localization of hydrocarbons, and
- channel photosynthetic carbon and energy into nonhydrocarbon storage compounds.

We have used the Race B strain of *B. braunii* that produces large amounts of C_{30} triterpenoid hydrocarbons as our main model organism. We have reconstructed complete metabolic pathways for the biosynthesis of DMAPP and IPP and identified a possible anaplerotic (replenishing) route in addition to the expected main pathway (MEP/DOXP). We have identified a large contingent of genes that take part in the biosynthesis of prenyl chains (linear terpenoid "backbone" intermediates of different lengths) from DMAPP and IPP and those that use the prenyl chains to synthesize hydrocarbon oils. We have mapped all terpene metabolic pathways that divert the common IPP and DMAPP precursors from hydrocarbon biosynthesis. These pathways include those for meroterpenoid quinones, the side chain of chlorophyll, diterpenoid gibberellins, triterpenoid phytosterols, tetraterpenoid carotenoids, polyprenyl carrier molecules, and the prenyl chains of proteins.

We explored the production of the co-metabolite SAM necessary for hydrocarbon oil biosynthesis. We also listed and mapped genes taking part in competing storage compound biosynthesis, including those involved in fatty-acid-based triacylglycerol and starch biosynthesis. We have identified a large number of transporters, some of which may take part in the extracellular localization of the hydrocarbon oil products. Using the Race L strain of *B. braunii*, we have identified a novel gene and pathway that is a prime candidate for the biosynthesis of the C_{40} tetraterpenoid hydrocarbon oil lycopadiene. Finally, we have investigated several possible biosynthetic routes for the production of the very-long-chain fatty-acid–based alkene hydrocarbons of the Race A strain.

The biosynthetic and export genes identified may be used to improve hydrocarbon production in *B. braunii*. However, *B. braunii* strains grow slowly and cannot currently be manipulated by genetic transformation. Thus, a more relevant and immediate goal is to use these genes and pathways as genetic tools to improve the production of biofuels in other green algae that are amenable to genetic transformation and to large-scale cultivation. Successful completion of this transcriptomic study, as described in the detailed report, has provided NAABB consortium members and the scientific community at large with a substantially complete genomic picture of *B. braunii* and a list of sequenced genes that are especially interesting for algal metabolic engineering to improve biofuel production. To facilitate the utilization of the large amount of information generated, we have not only published our findings in a respected open access journal, but have also made our datasets available in the form of a publicly accessible website offering an advanced data mining environment.

Production of Limonene, a Volatile Monoterpene, in the Freshwater Algae *Chlamydomonas reinhardtii*

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Here we show our study aimed at developing an autotrophic production system for hydrocarbon-like terpenoids to satisfy the unique needs of U.S. Air Force aviation fuels. From a technical perspective, this study involved the introduction of terpenoid biosynthetic genes into model microalgae species to alter the downstream isoprenoid pathway for monoterpene and sesquiterpene biosynthesis. Specifically, the scope of this study aimed to establish a unique closed-culture system for the production of the volatile monoterpene limonene to serve as a "drop-in" replacement for JP-8 and other high-volumetric energy-dense jet fuels.

Stable nuclear transformation of rice osTPS23 (limonene synthase) into the model algal *Chlamydomonas reinhardtii* was achieved in this study and serves the basis for our genetic engineering approach. Expression of TPS is driven by the strong inducible/constitutive Hsp70A/RbcS2 promoter and targeted to the chloroplast using a stroma cTP signal in place of the rice cTP signal. For product determination, this study developed an optimized gas chromatography–mass spectrometry (GC-MS) analytical strategy for detection of limonene. This modified method detected limonene in the headspace of several transgenic lines and was also used in single-vial enzyme product-identification reactions.

The implication of limonene in the headspace of closed photobioreactors is significant on two accounts: (1) limonene is volatilized from algae into the airspace above the liquid culture—key for future bioseperation approaches harvesting limonene—and (2) introducing a secretory pathway into algae is not necessary for production of limonene. Wild type *C. reinhardtii* is not known to produce volatile monoterpenes, thus our report is novel in that it establishes a key discovery for any future hydrocarbon-like autotrophic production in microalgae.

Optimization of the metabolic pathway to enable the high accumulation of monoterpene and sesquiterpene molecules in the model algae *C. reinhardtii* is currently in progress. Achieving optimized limonene production will require a dual approach: first, key terpenoid biosynthetic genes (DXPS, GPS, FPS, LMS, SQS) should be engineered into *C. reinhardtii* using a similar strategy previously used to transform rice osTPS2; and second, each of the key biosynthetic genes is codon-optimized for expression in *C. reinhardtii.* Additionally, CO_2 enriched air—provided by our scaled-up algae growth/VOC screening system—could potentially allow higher accumulation of limonene by allowing the algae to replenish lost carbon flux.

Despite the potential for algae-based biofuels, many technical challenges remain for full realization of an algal autotrophic-based liquid hydrocarbon production system. Long-chain fatty acids derived from triacylglycerols (TAGs) represent the

majority of renewable algal-based biofuels—collectively known as biodiesels (fatty acid methyl esters). However, compared to petroleum-based fuels such as gasoline and diesel, biodiesel lacks certain thermochemical and thermophysical properties intrinsic to its petroleum counterparts, restraining its use in certain applications such as aviation fuel. Limitations related to biodiesel are primarily related to the presence of oxygen molecules in the ester bonds. Similarly, ethanol shares this same disadvantage. Limonene differs from biodiesel and ethanol in that it's a pure hydrocarbon—lacking oxygen— therefore offering similar fuel properties to petroleum based fuels.

Optimized Growth Conditions and Micronutrient Control and Analyzing Lipid Body Formation in Various Algae

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Oil production in algae is mediated by a complex array of cellular components that are coordinated in a hierarchical manner. Our laboratory uses a systems biology approach (omic tools) to identify fatty acid packaging mechanisms and novel lipid biosynthetic pathways that have evolved among diverse algal taxa. This basic information will serve as a platform for understanding and optimizing biosynthetic processes in algae of commercial value.

Lipid bodies represent newly recognized cellular components that are indispensible contributors to fatty acid biosynthesis. These organelles serve a multitude of functions including both protein and neutral lipid sequestration. Though lipid body formation represents the keystone in algal lipid production, little is known concerning the biogenesis of this organelle in different algal taxa. An evolutionary time line shows green (Chlorophyta) and brown (Chromophyta) algal lineages diverged about 1.3 billion years ago. It is well documented that green algae produce different fatty acids than those of brown algae, emphasizing the fact that the underlying metabolic processes that drive oil production and packaging that are found in these diverse algal lineages have specialized during evolution. Almost all information concerning lipid body biogenesis has been established using green algae such as *Chlamydomonas* and *Dunaliella*. In contrast, even though brown algae comprise a huge taxonomic assemblage of organisms, many of which produce copious amounts of oil and high-value bioproducts, few studies on lipid body genesis exist for this important phylogenetic cluster.

A new model system for lipid body analysis has been developed in this study. *Chrysochromulina tobin* (Haptophyceae) is a unicellular, naturally wall-less, golden-brown alga that is grown synchronously on a completely defined artificial medium. The alga has a high lipid content (40% of dry weight) and displays a broad range of fatty acids that are especially rich in polyunsaturated fatty acids. Fluorescent, electron, and confocal microscopy of the small 4 μm organism show

it contains only two lipid bodies. These organelles are intimately associated with the endoplasmic reticulum that appears to form a skin over the lipid body. Each lipid body is adjacent to a chloroplast (also only two per cell), and each lipid body is often near several mitochondria. We have documented a diurnal correlation between lipid body size, lipid profiles, and lipid quantity in synchronized *Chrysochromulina tobin* cultures that were maintained on a 12-hour light, 12-hour dark photoperiod.

Chrysochromulina tobin can grow mixotrophically. The small alga will actively hunt bacteria as a food source. For this reason, the initially obtained culture of *Chrysochromulina tobin* was highly bacterized. Flow cytometry and antibiotic treatment (streptomycin and hygromycin) were successfully used to generate axenic cultures of this organism. Comparative analysis of 18S *rn*A (nuclear), *psb*A and *rbc*L (chloroplast as well as *nad*5 mitochondrial) gene sequences show this algal to be a new species and the name *Chrysochromulina tobin* has been proposed. DNA sequencing (with Los Alamos National Laboratory) of the *Chrysochromulina tobin* genome (nuclear [~59 Mb], mitochondrial [25 kb], and chloroplast [104 kb]) represents only the second nuclear genome to be analyzed within the large haptophyte genus that comprises over 500 oleaginous species. Transcriptome libraries were developed (with Los Alamos) using synchronized cells sampled at 7 time points during the synchronous growth period when lipid body size is maximal (in the light) and minimal (in the dark). Data show large shifts in expression of genes critical to fatty acid synthesis. For example, 3-oxyacyl-(acyl-carrier-protein synthetase) and glycerol-3-phosphate dehydrogenase expression are respectively 10- and 30-fold higher in the light than in the dark. Proteomics (with Pacific Northwest National Laboratory) using liquid chromatography–mass spectrometry/mass spectrometry of whole cell and isolated lipid body preparations (searched against genomic and transcriptomic databases) identified *Chrysochromulina tobin* peptides with homology to proteins associated with lipid metabolism, such as dihydrolipoamide dehydrogenase, lipoamide dehydrogenase, and phospholipid scramblase. Antibodies to select proteins, micro-liquid chromatography–mass spectrometry technique, and flow cytometry using the lipophilic dye BODIPY 505/515 techniques developed in this study were used to gain insight into the relationship between stress response, protein sequestration, and lipid accumulation within isolated lipid bodies of *Synura petersenii*, *Isochrysis galbana*, *Emiliania huxleyi,* and *Ochromonas danica*. Transformation studies have been initiated. New plating technologies for wall-less cells have been devised and transitory introduction of genes into the organism has been accomplished.

Identification of Lipid Productivity Genes in Cyanobacteria

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This report breaks down and summarizes research funded by the NAABB and carried out at Matrix Genetics using photoautotrophic cyanobacteria, *Synechococcus elongatus PCC7942,* into four distinct topic areas: (1) lipid productivity, glycogen deficiency, and carbon partitioning; (2) neutral lipid production, (3) scalable production/biomass generation, and (4) expansion of the genetic toolbox.

Matrix Genetics' initial research plan and project scope focused heavily on genetic screening directed at identifying lipid production phenotypes from a full genome transposon mutant library of *S. elongatus*. The project evolved over the three-year NAABB funding period as screening efforts were de-emphasized due to an excessively high false-positive rate associated with the use of the lipophilic dye Nile Red. We modified our project focus to allow for expanding efforts in the areas of partitioning carbon and regulating carbon stores, strain construction, and strain cultivation. These modifications were designed to maintain project alignment with the Department of Energy Roadmap declaration that a better understanding of carbon metabolism and regulation in cyanobacteria "can not only serve to advance the understanding of how production of different carbon storage molecules are controlled in response to physiological conditions, but may also serve to guide the development of other types of algae for biofuels production." Furthermore, the synthetic and systems biology research undertaken by Matrix Genetics has enabled strain optimization efforts directed at enhancing neutral lipid production in *S. elongatus* in support of the Algal Biology milestone of transgenic strains incorporating best trait(s) demonstrated in culture.

In total, NAABB funds provided to Matrix Genetics helped support transcriptomic and metabolomic studies focused upon characterizing the alteration of carbon storage and partitioning in cyanobacterial mutants that are defective in glycogen synthesis. These studies provided a significant increase in knowledge concerning what was an unappreciated role for glycogen synthesis in regulating lipid production, stress-induced transcriptional programs, central carbon metabolism, and carbon partitioning in response to nutrient stress. As noted in previous NAABB reports, Matrix Genetics has generated one manuscript currently under review, and Matrix Genetics has filed a provisional patent application based upon this body of work (see listed below):

> Hickman J. W. et al. Glycogen synthesis is a required component of the nitrogen stress response in *Synechococcus elongatus PCC 7942* (under review at Algal Research).

A provisional application detailing work focused upon the decoupling of photosynthesis from biomass accumulation in *S. elongatus PCC7942* under conditions of nutrient stress was filed February 6, 2012. This provisional patent is titled "Modified photosynthetic microorganisms for continuous production of carbon-containing compounds."

In addition, NAABB funds partially supported efforts directed at reducing toxicity associated with expression of bacterial wax ester/DGAT and the production of neutral lipid in *S. elongatus*. The ability to produce neutral lipid in cyanobacteria while limiting toxicity associated with expression of enzymatically active bacterial wax ester/DGAT represents a potentially significant advance towards utilizing cyanobacteria as photocatalytic producers of lipid biofuel feedstocks. A provisional application detailing work focused upon elimination of cellular toxicity associated with wax ester/DGAT transgene expression in *S. elongatus PCC7942* was filed March 9, 2012. This provisional patent is titled "Modified diacylglycerol acyltransferase proteins and methods of use thereof." NAABB funds were also used to provide partial support for efforts focused on optimizing cyanobacterial biomass production in collaboration with NAABB partner Solix Biofuels and expanding molecular biology tools for application toward genetic and metabolic engineering of *S. elongatus*.

Mining the Lipid Secretory System of Plants: Investigation of a Tobacco ATP-binding Cassette Protein for Its Function in Neutral Lipid Transport

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Neutral lipids such as triacylglycerol that accumulate in algae are currently collected in a batch mode in which the algae are first harvested and the lipid then extracted into organic solvents. The economics of producing algal biofuels would be significantly improved if algae were capable of secreting the lipid into the growth media. There it would float to the surface where it could be collected by skimming and the algae would be able to grow continuously. Since algae do not secrete their oil, this project was intended to identify a system that could be engineered into algae to provide them with this capability.

Tobacco stigma secretes large amounts of lipid consisting of mostly triacylglycerols to the surface at a maximum rate two days prior to flower opening. A gene encoding an adenosine triphosphate binding cassette (ABC) protein, *NtWBC1*, was found to be specifically expressed in stigma and identified as a component for the lipid secretory system. We cloned *NtWBC1* from a tobacco stigma cDNA library and in that process identified a second, very similar ABC transporter, *NtWBC2*, which had not been previously reported. It is well established that lipid transporters are ABC transporters and have been extensively studied. Because in the case of tobacco there were two ABC transporters, it is possible that either *NtWBC1* or *NtWBC2* was operating as a homodimer or that *NtWBC1* and *NtWBC2* were working in concert as a heterodimer. Using an RNAi strategy on tobacco stigma, we have established that *NtWBC1* is a component of the stigma lipid secretion system. A similar study to analyze *NtWBC2* is incomplete and we cannot at this time draw any conclusions about its involvement in lipid secretion.

We have also attempted to demonstrate the secretion activity of *NtWBC1* and *NtWBC2* by heterologous expression in yeast under conditions that the yeast produce triacylglycerols. Whether these genes were expressed individually or in combination in the yeast, no secretion activity was detected. Based on these results as well as results in other labs attempting heterologous expression, we concluded that there are likely additional and as yet unidentified components to the lipid secretion system.

Transformation of the Production Algae *Nannochloropsis salina* and *Picochlorum* sp.

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The saltwater alga *Nannochloropsis salina CCMP1776* was the alga of choice for engineering. It had performed well in outdoor ponds during the Aquatic Species Program testing and other more recent efforts where it persisted well and produced a reasonable amount of lipid when provided a continuous nutrient supply. Preliminary studies at Los Alamos National Laboratory had also suggested that *N. salina* could also accumulate high lipid concentrations when subjected to nitrogen nutrient depletion. There were no literature reports of successful transformation of *N. salina*. A method was reported during the project for transformation of a closely related alga, *N. gaditana*; this method proved unsuccessful when these vectors were applied to *N. salina*. Therefore, we worked to develop a method for stable transformation of *N. salina.*

This effort capitalized upon the *N. salina* genome sequence obtained within NAABB to design and demonstrate *N. salina*–specific vectors that contained *N. salina* promoters and terminators. The transcriptome studies conducted by NAABB also guided our choice of the lipid droplet protein promoter because of its very high expression induced during culture conditions of nitrogen depletion. This effort demonstrated three promoters and a terminator that was effective with each of the promoters. In addition to the lipid droplet protein promoter, we tested the commonly employed *PsaD* and *tubulin* promoters and demonstrated them to be effective. We tested a set of selection agents and showed three to be effective: zeocin, puromycin, and blasticidin. We constructed four separate vectors to provide a set of combinations of promoters and resistance genes to generate a set of vectors to aid the gene stacking effort that followed. We also constructed a later-generation vector using the FMDV 2A sequence as a linker for multiple gene expression. We developed and demonstrated a method of homologous recombination of *N. salina* to provide an additional molecular biology tool. Having a method for homologous recombination allows the researcher to genetically change this alga without using foreign DNA or antibiotic resistance genes.

We developed two effective methods for stable transformation using these vectors. Electroporation and biolistic methods were effective, although the electroporation was the method of choice because of the greater recovery of transformed cells. The use of competent cells and the careful recovery process described in this report were essential to good recovery of transformed cells.

A set of target genes for overexpression was chosen for their potential to increase either biomass or lipid accumulation, based on the results of the NAABB transcriptome studies and literature reports from other efforts. These targets included a bicarbonate transporter gene and a combination of glutamine synthetase and glutamine phenylpyruvate transaminase genes to address biomass accumulation rate and carbon use efficiency. Genes for acyl carrier protein, lipid droplet protein, and diacylglycerol O-acetyltransferase and acetyl CoA-

carboxylase were tested for their potential to improve lipid accumulation. Because *N. salina* can easily be induced to accumulate very high concentrations of lipids after its nitrogen source is depleted, we also chose the global nitrogen metabolism regulator, the *Nit2* transcription factor. Simultaneous overexpression of multiple genes could be tested with the *N. salina*-specific bicistronic vectors we designed. Because *N. salina* could be breaking down its carbon storage compounds at night, we also designed and are ready to test a construct containing RNAi against the gene encoding for the key tricarboxylic acid cycle enzyme, α-ketoglutarate dehydrogenase. Impairing this enzyme will reduce respiration and thus reduce the use of stored carbon in the alga.

We have created a library of transformed *N. salina* strains and we have generated sets of transformed strains using constructs with each of these target genes. The genetic and phenotypic characterization of these strains will be required and will employ methods developed during this project. The growth and lipid production phenotypes will be characterized in our Los Alamos National Laboratory–designed photobioreactors and lipid production analyzed with our established methods.

Algal Strain Isolation and Lipid Productivity Screening

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The Algal Biology team of the NAABB was responsible for delivering novel strains of microalgae to the consortium for further strain testing and potential development. Within the Algal Biology team the laboratory of Dr. Juergen Polle performed the tasks of environmental sampling, isolation of new potentially valuable algae strains using high-throughput methods, screening of strains for lipid production, and first characterization of strains for growth and lipid productivity as the primary selection criteria in culture. The best-performing strains characterized in the laboratory/greenhouse were then forwarded to NAABB facilities of the Algal Cultivation Team for further testing in testbeds. During the project performance period, the laboratory of Dr. Polle provided multiple strains for testing to the university and national laboratory partners of the NAABB consortium. Examples of recipients of novel wild-type strains were:

- Texas A&M University testbed facilities located in Pecos, Texas, and Corpus Christi, Texas.
- Marine Science Laboratory of Pacific Northwest National Laboratory in Sequim, Washington.
- University of Arizona in Tucson, Arizona.
- New Mexico State University in Las Cruces, New Mexico.

In addition to forwarding strains to testbed facilities the Polle laboratory collaborated with several other groups within the Algal Biology Team, for example on genome sequencing, proteomics, and microscopy of algal strains.

Our approach of isolating new strains and first testing in the laboratory combined with later testing in outdoor settings using multiple different

cultivation systems proved successful. Not all strains coming out of the laboratory screening and laboratory testing performed in the outdoor testbed cultivation systems, but several algal strains could be successfully grown in outdoor testbed facilities. This result contrasts with the previously published Aquatic Species Program report from 1998, which indicated that no successful combination of indoor isolation/screening/testing with outdoor strain performance might be achievable.

Two major milestones were associated with this project. A total of at least 1500 strains had to be screened. In addition, at least 30 of the best strains were to be deposited in the algal culture collection at the University of Texas at Austin. Both milestones were completed by screening 1595 strains and by depositing 30 strains to UTEX. To achieve these milestones, we had to complete multiple tasks, including sampling, isolating strains, screening strains, and testing isolated strains. For comparative performance testing of any new wild-type strain, we used the "gold standard" production strain *Nannochloropsis salina CCMP1776*.

Chrysochromulina tobin (Haptophyceae): A New Model System for Lipid-body Analysis Using a Systems Biology Approach

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We have developed a toolbox of techniques to help both growers and laboratory-based scientists identify and develop high-performance non-GMO algal strains. Specifically targeting "non-conventional" algal taxa within the chromalveolata, a large taxonomic nongreen (chlorophytic) cluster that produces oil as a primary photosynthetic product, we have (1) developed new methods to support high-throughput lipid analysis using micro quantities of biomass, (2) established a new algal matrix standard for comparative analysis of productivity among growers, (3) generated new dyes for microscopic and flow cytometric studies, and (4) provided a technique for genetic fingerprinting of algal strains. We also analyzed a brief discussion of the importance of medium development and wastewater use. These results are summarized below.

Micro-method for gas chromatography–mass spectrometry (GC-MS) analysis of fatty acids

The development of high-throughput technologies that can be done in conventional laboratories is seminal to the selection and optimization of novel algal strains. It is well known that the quality and quantity of lipids in an algal cell are in continuous, dynamic flux, reflecting the response of the organism to environmental cues and culture age. Unfortunately, small cultures used in the laboratory for physical response experiments are often limited in the amount of biomass available for lipid studies—especially studies that have a time-course

component. To circumvent this problem, we have devised a method by which fatty acid analysis can be done using less than 250 micrograms dry algal mass (approximately 10 ml of culture for most algae). This method employs conventional boron trifluoride transesterification followed by a two-step phase separation. The technique uses inexpensive and commercially available microvolume glassware and bench-top equipment. Gas chromatography–mass spectrometry (GC-MS) analysis is performed relative to internal standards by single-ion quantitation.

To demonstrate the utility of our method, we present data on the lipid profiles of 29 algal representatives within the stramenopile clade of the chromalveolata cluster. This technique will contribute to the biofuel community by reducing the time and labor costs associated with analyzing algal fatty acids. Importantly, this method not only has laboratory applications, but also could enable algal field studies of lipids or provide rapid analysis of organisms other than microalgae.

A natural matrix standard for the algal oil industry

Expanding efforts in both commercial and research facilities will require the screening and monitoring of candidate algal strains for lipid synthesis. Algal oil recovery and its quality are impacted by constraints of both biology (algal cell type and growth physiology) and technical ability (recovery and extraction methodologies). Unfortunately, and unlike other well-established food and oil commodities, no universal reference standard exists for the algal oil industry. A laboratory-optimized strain of *Chrysochromulina tobin* is proposed as a natural matrix reference standard for analyzing algal fatty acids. The alga is amenable to this purpose because:

(1) As a soft-bodied organism, it is susceptible to many disruption and fatty acid extraction techniques.
(2) It has a high fatty acid content (about 40% dry weight).
(3) Its growth response and lipid profiles are highly reproducible.
(4) Unlike many algae that have limited fatty acid distributions, *Chrysochromulina tobin* cells contain a broad representation of both saturated and unsaturated fatty acids ranging from C:14 to C:22.

As a proof of concept, *Chrysochromulina tobin* was used as a reference standard for comparing 20 taxonomically diverse algal cultures that were grown under identical physiological conditions and analyzed for fatty acid content using the micro-GC-MS analytical technique.

Fluorescent dye applications

Selective staining of target regions in cells is an ongoing challenge. Fluorescent probes provide a convenient method of reporting on the structure as well as the presence and quantity of important biomolecules such as lipids. Fluorescent dyes that also have nonlinear optical properties may enable improved assays of the biochemistry and biology of specific cellular regions. We are developing biocompatible optical probes that interact with specific cellular targets with high affinity, photostability, and brightness but low toxicity.

The ability to assess lipid concentration in living algal cells frequently, rapidly, and with precision without time-consuming extraction, conversion, and

measurement of the target product is a valuable asset in assessing strain performance. We have developed the fluorescent lipophilic dye BODIPY 505/515 for both cytological and flow cytometric application. This dye allows measurement of neutral lipid levels in live algal cells, while requiring small sample size. We determined the saturation values of BODIPY 505/515 dye for 20 algal strains representing a broad range of taxa. Lipid production for algal cultures sampled over a growth cycle (logarithmic to stationary phases) show that oil accumulation as a culture ages is taxon dependent—some strains accumulate large oil reserves while others have significantly depleted oil levels. DANPY-1 is a prototype fluorescent probe that we have synthesized in collaboration with colleagues in the University of Washington chemistry department. This new probe provides high contrast for microscopic imaging and fluorometric analysis of DNA in intact algal cells. A modification of this dye is being constructed to function in cellular lipid assessment.

Algal population fingerprinting

Many algal cells of the same species are indistinguishable morphologically, yet contain genetic variation that confers distinct physiological capabilities, including the ability to sustain robust growth or to generate copious amounts of oil. Such cryptic speciation presents a significant problem when trying to identify high-potential strains or to determine if genetic drift has occurred in a large-volume-culture facility. To distinguish among algal strains, we have developed a molecular fingerprinting technique. For this study, we used the raphidophyte alga, *Heterosigma*, an organism that occurs worldwide. To probe genetic diversity of 50 cultured and field-collected strains of this alga, we used sequences from five mitochondrial genes (cytochrome C oxidase subunit 1 [*cox1*], and NADH dehydrogenase subunits 2, 4, 5, 7 [*nad2, nad4, nad5*, and *nad7*]). We identified 23 mitotypes. In the course of this study we observed that it is significantly important to use high-fidelity Taq when doing strain assessment to avoid sequencing artifacts. Strong genetic data was generated showing that medium choice will drive strain selection after initial culture.

Medium development

Commercial growers and laboratory scientists often have significantly different goals with respect to algal culture maintenance. Growers want a culture that can withstand a potentially heavy eco-cohort load (e.g., bacteria) while laboratory researchers want an axenic culture (no contaminating organisms) that allows molecular analysis of underlying biosynthetic pathways. Our study shows that the growth profile and lipid content of an organism grown axenically or with its natural bacterial population have different fatty acid production rates and fatty acid profiles. Several algal taxa were robustly grown with high fatty acid production in media made with a wastewater stream that originated from a salmon aquaculture facility.

Environmental Photobioreactor for Assessing Algal Performance

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Microalgae are increasingly valued biotechnological platforms for activities ranging from antibody expression to biofuel production. However, because light, not reduced carbon, is the main energy source, large-scale cultivation of microalgae poses unique challenges. Previously available laboratory-scale photobioreactors do not attempt to imitate the dynamic environmental conditions found under production conditions. Our work thus had three major aims: (1) to produce a new type of environmental photobioreactor (ePBR) that simulated key environmental parameters, (2) to use the ePBR to explore the importance of environmental light fluctuations in controlling the efficiency of light capture in algal and cyanobacterial water columns, and (3) to test the utility of the ePBR for predicting in the laboratory the productivity of algal strains under natural or production conditions.

For the first aim, we sought to simulate the abiotic features of pond conditions that influence algal photosynthesis and growth the most: light intensity and quality, temperature, gas exchange, and natural dynamics thereof. We present here the ePBR (Figure 1), a laboratory-scale platform for growing algae under simulated natural environments. We have designed a columnar vessel to mimic a water column in an algal production pond by using collimated white light from a high-power LED to reasonably reproduce both the intensity of sunlight and the light gradient throughout the water column. The ePBR provides programmable computer control over light, mixing, temperature, and gas flow while autonomously measuring optical density and pH. Scripting allows the ePBRs to create complex environments and react to their own data. Additionally, the system is scalable for parallel and matrix experiments.

As a demonstration of the ePBR utility, we present multiple studies investigating uses for the ePBR system. First, we compared the batch culture growth of *Chlamydomonas reinhardtii* wild type *CC125* and state-transition mutant *stt7-9* under simulated laboratory and field-like conditions. We observed that the growth rate of the state-transition mutant in the ePBR was significantly less than the *CC125* strain, but neither strain was significantly hampered by the weather simulation compared to their growth under static conditions. Secondly, we have incorporated saturation pulse chlorophyll fluorescence measurements with the ePBR system. Here we monitored PSII activity of *Chlorella sorokiniana* grown under square-wave light conditions versus sinusoidal light. We determine that cells grown under nonstatic conditions show different energy capture regulation (Figure 2).

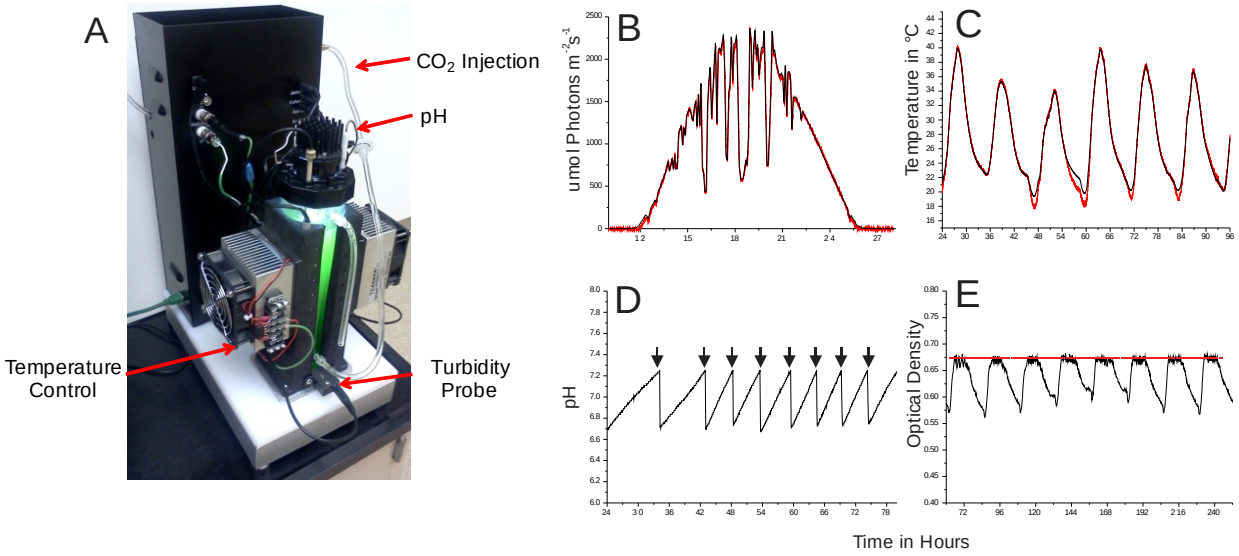

Figure 1. The environmental photobioreactor is a modular unit capable of simulating an outdoor water column. Panel A: Labeled view of a single ePBR unit. Panel B: The ePBR can simulate dynamic environmental conditions within the culture vessel. Solar data from Altus, Oklahoma, was programmed into the ePBR at 5-minute intervals, while actual light intensity output from the reactor was recorded with a data logger connected to a PAR meter. Panel C: Culture temperature as programmed into the reactor and temperature as recorded by the ePBR. Panel D: The ePBR can control pH via CO_2 injection, as marked by arrows. Panel E: When run in turbidistat mode, the reactors can maintain a constant (or target) optical density.

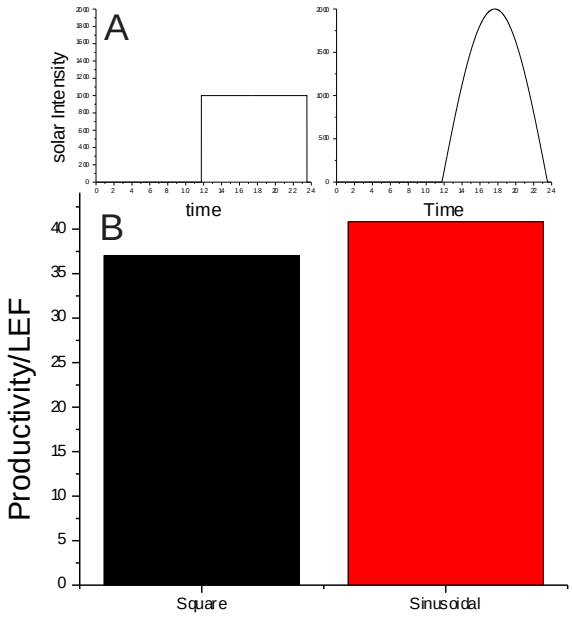

Figure 2. Panel A shows experimental lighting regimes for cultures of Chlorella sorokiniana*. Lighting was either square (left) or sinusoidal wave (right). Panel B gives estimates of linear electron flow rates throughout the diurnal cycle of Chlorella sorokiniana grown under either sinusoidal or square wave lighting regimes. Estimates of biomass productivity as a function of linear electron flow rates.*

Third, we have begun competition studies using *Fremyella diplosiphon* wild type and phycobilisome mutants to understand what conditions may be conducive to cyanobacterial small antenna mutants (Figure 3). Finally, to demonstrate the industrial utility of the ePBR system, we have initiated environmental playback simulations of outdoor algal biomass production from closely monitored pond growth.

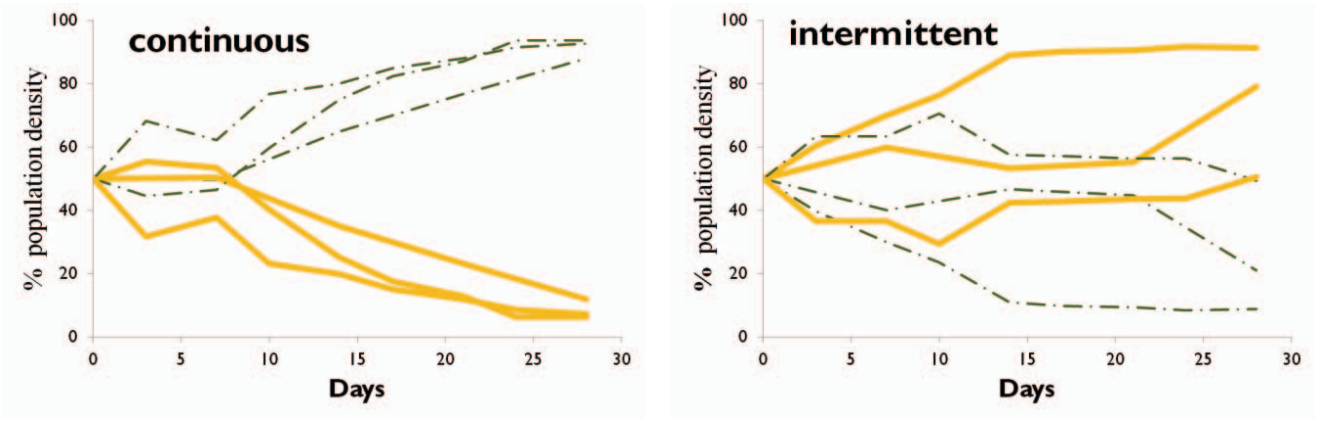

Figure 3. (A) Population density of the wild type (WT) Fremyella diplosiphon in green and the mutant lacking phycobilisomes (PBSs), GOLD in yellow, under continuous light conditions. (B) Population density of the WT in green and GOLD in yellow, under intermittent light conditions (one minute on, one minute off).

Strain Improvement via Flow Cytometry Cell Sorting

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Flow cytometry, coupled with the lipid-specific fluorescent dye Nile Red (NR), is a rapid approach to identify and select algae cell subpopulations with enhanced lipid content. This technology has been successfully applied to *Nannochloropsis salina* for biodiesel production, with total lipid content doubled in sorted daughter cells relative to that of the parent cells. A significant correlation of NR signal was observed between polar to neutral lipid ratio and docosahexaenoic acid (DHA) per cell for the marine dinoflagellate, *Crypthecodinium cohnii*, which was used to select cells with high DHA content.

Cellana uses natural, indigenous strains of marine microalgae isolated from coastal habitats in Hawaii. The goal of this project was to select favorable subpopulations of *Nannochloropsis oceanica* using flow cytometry and cell sorting for large scale production. The fatty acid profile of *Nannochloropsis* sp. is suitable for biofuels production as it is mainly composed of saturated (C16:0) and mono-unsaturated fatty acids (C16:1). In addition, *Nannochloropsis* sp. is also a good candidate for producing the high-value omega-3 fatty acid eicosapentaenoic acid (EPA), which often was found to be abundant in phospholipids.

For this project, we sorted *N. oceanica KA19*, newly isolated on the Big Island of Hawaii, by flow cytometry to select cells with increased content of total lipids and high-value long-chain omega-3 fatty acid (i.e., EPA).

KA19 culture maintained at Kona Demonstration Facility (KDF) was brought to the University of Hawaii for cell sorting. To enhance neutral lipids, we performed cell sorting 2.5 to 3 days into the stationary phase. For phospholipids, we sampled near the end of the exponential phase. Prior to sorting, we added glycerol at a final concentration of 0.1 g/ml. We added the fluorescent dye Nile Red to the

mixture and incubated it in the dark for 5 minutes. We then diluted the sample 1:30 and sorted it in the flow cytometer with an excitation wavelength of 488 nm and emission wavelengths of 575 nm and 660 nm for neutral lipids and phospholipids, respectively. We sorted the cells into 24-well plates and grew the cells in f/2 medium with 133 $mE/m^2/s$ of light in a cycle of 12 hours of light and 12 hours of dark. Temperatures ranged from 24°C to 26°C. We repeated the process for a total of three generations of daughter cells.

We brought the sorted cells back to KDF along with the parent cells to verify the enhanced characteristics. We compared the parent culture to the following cultures enhanced by flow cytometry: second-generation neutral lipids, third-generation neutral lipids, and second-generation phospholipids. We repeated the experiment for two rounds, with the first round conducted after cells were maintained for over 80 generations and the second for over 140 generations. We grew each treatment in triplicate 1 L polysterene bottles and provided aeration, ambient sunlight, and temperature control via a water bath. Each treatment began the experiment with nearly identical cell density and nutrient availability. Treatments were nutrient depleted on Day 4 and Day 3 for experimental rounds 1 and 2, respectively. We monitored cultures over the course of 7 days until the experiment was terminated. We monitored cell density and lipid composition at various stages throughout the growth curve.

During Round 1, the sorted cells of second-generation neutral lipids reached significantly higher cell density at the end of the experiment compared to all the other sorted cells (ANOVA, $p < 0.05$), indicating variability within the *KA19* population during the Round 1 experiment, despite the fact it was newly isolated from a single cell. Total fatty acid methyl esters (FAMEs) were higher for all sorted cells than the parent cells brought back from the University of Hawaii. However, the parent culture used for the Round 1 experiment later was found to be contaminated with *Chlorella*-like alga. Therefore we conducted a second round of experiments to compare sorted cells with parent cells maintained at KDF, which was free from contamination. We observed no difference among treatments for Round 2. The time elapsed between the last round of cell sorting and the Round 2 experiment was greater than 140 generations for *KA19*, so the selected characteristics in sorted cells were likely lost, if they were expressed at all.

Diagnostics for Community Composition and Competitiveness and Cost Analysis

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The work at Solix focused on developing molecular tools to monitor the culture for competing algal species in the Solix growth-system panels. The work initially focused on using molecular beacons to quantify the level of *Nannochloropsis salina* (the production strain) in the culture as well as the level of the major competitor strain identified to date, which was a *Tetraselmis striata*. Beacons were developed that showed specificity for each strain when tested in pure cultures. The beacons were also specific when tested against laboratory-produced mixed cultures with defined levels of contamination present in the sample. However, in cultures where the mixtures fell below one contaminant for every 100 targets the beacons could not accurately quantify the level of contaminant in the mixture. Further research indicates that a number of issues complicate the use of beacon technology as a quantitative contamination assay.

After the work with molecular beacons, the effort was switched to developing real-time polymerase chain-reaction (qPCR) markers and cleaved amplified polymorphic sequence (CAPS) markers. For the qPCR work, we developed primers specific to *N. salina* and *T. striata*. The primer set for *N. salina* is specific for the target species while the primer set developed for *T. striata* will amplify *Chlorella* sp. in addition to the target species. The assay developed allows for the detection of the contaminant at levels 1000-fold lower than the beacon assay.

For the CAPS markers, we developed a set of universal primers targeted at a region of the 18S ribosomal sequence and tested them against a wide variety of algal species. The species used were fairly diverse and include members of the class Trebouxiophyceae, Chlorodendrophyceae, Eustigmatophyceae, Bacillariophyceae, Coccolithophyceae, Pavlovophyceae, Cryptophyceae, Porphyridiophyceae, and Chlorophyceae. The primer set has been shown to amplify a target sequence for all but one species it has been tested against. For a subset of the species (seven of the 15 species tested to date), we cloned and sequenced the amplified products and used the sequence information to develop CAPS markers. The seven species can all be distinguished from each other using a single restriction enzyme (HaeIII). While CAPS markers do not provide a quantification of any contaminating species, the system is fairly robust and could be deployed in a production setting.

Utilization of Wastewater Nutrients for Algal Growth—Metals Toxicity

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The following collection of hypotheses and postulates motivated the program of experiments and analysis conducted here:

- The American Southwest is ideal for development of an algal biofuels industry from the perspective of climate, if problems related to water availability can be solved.
- Treated municipal wastewater that is unsuitable for human consumption can be used to satisfy water needs for growth of microalgae.
- The same waters carry macronutrients in sufficient quantity to significantly offset the cost of fertilizer for growth of microalgae and prevent an unnecessary competition for fertilizers between the biofuels industry and more conventional agriculture practices.
- The levels of metals in most conventionally treated municipal wastewaters will not impede the growth of at least some metals-tolerant microalgae at either levels present in wastewater effluent or concentrates in waste streams from the treatment of wastewater such as centrate or pressates from sludge dewatering.
- Metals toxicity is likely to be greater in continuous cultures of microalgae than in batch cultures since continuous culture methods produce very low concentrations of growth-limiting nutrients.
- Even when evaporation and recycling cause salts (and metals) to accumulate in waters used for algal growth, select algal species will be capable of unimpeded growth and lipid production.
- There are no other known toxicants in treated wastewater that will eventually impede the growth of a wastewater-dependent algal biofuels industry.

The program of experiments described and the accompanying analysis of data conducted to date have shown that *Nannochloropsis salina*, a saltwater species selected on the basis of its growth rate and overproduction of lipid, readily tolerates metals at levels present in Tucson area wastewater. Among the metals present at relatively high concentration, including zinc, copper, cobalt, lead, and nickel, zinc was the primary source of observed toxicity in combinations of metals concentrated beyond those levels present in wastewater effluent. Nevertheless, the zinc EC_{50} for *N. salina* grown on normal growth media was about 100 times the level normally found in treated Tucson municipal wastewater. Zinc was no more toxic to *N. salina* grown in continuous culture than it was in batch growth experiments. Experiments conducted to date suggest that wastewater metals will not degrade algal growth rates, at least for metals-tolerant species, at reasonable levels of accumulation that will follow from water recycling in the biofuels

industry. Metals toxicity experiments were successfully conducted in 96 well plates, potentially increasing the convenience of toxicity experiments of this type. *N. salina* has been shown to grow adequately on normal growth medium, without available nitrogen or phosphate, when either wastewater or centrate from sludge dewatering was provided as a nutrient source. Mixtures up to 50% v/v were non-toxic in batch cultures, and adding centrate that was ≤ 25% v/v to the normal growth medium actually accelerated growth. The source of toxicity that was observed at higher volume fractions has not been identified, although it appears to be unrelated to metals concentrations.

Algal yields of biomass and lipid were not lowered by growth in mixtures of wastewater and normal growth media. However, those yield factors indicate that consumptive use of nutrients from wastewater will not lead to a sustainable algal biofuels industry of great consequence since there is insufficient wastewater (and nutrient) available in any location to motivate such an industry. There is simply not enough nutrient in municipal wastewater to supply the nutrient requirements of a robust biofuels industry unless nitrogen and phosphorus can be efficiently recovered from lipid-extracted algae—or whole algal cells if other methods are used to convert biomass to useable energy. This was shown on the basis of straightforward stoichiometric calculations using 5% of the United States demand for transportation fuels as a realistic target for the biofuels industry. To date, experiments have shown that a combination of vigorous sonication followed by anaerobic digestion can significantly increase the rate and extent of methane recovery—itself an energy benefit—and nitrogen recovery as ammonia from whole algal cells. The same process is likely to increase energy and nutrient recovery from lipid-extracted algae. After a 23-day digestion period, 40% more methane and 60% more ammonia were recovered from sonicated algae than from the whole-cell control.

Photobioreactor Development for CO_2 Supplementation

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Traditionally, photobioreactors (PBRs) had been designed and operated to maximize volumetric biomass productivity, with little regard to the energy input to the cultivation process or the carbon dioxide (CO_2) utilization efficiency. Energy-efficient supply of carbon and light to sparged algal photobioreactors has recently been recognized as a limiting factor for algal biofuels to be widely accepted as an energy-efficient alternative to other fuels.

The goal of this research was to optimize the algal cultivation process to maximize biomass productivity per unit energy input and net energy gain. Towards the end, the following tasks were completed: (1) developed and validated procedures to minimize the energy input for sparging of PBRs and maximizing net energy yield, (2) developed and evaluated an airlift-driven open raceway configuration to maximize CO_2 utilization efficiency and maximize biomass productivity per unit energy input, and (3) developed and validated an internally illuminated PBR to maximize utilization of light energy input. In all

these tasks, laboratory-scale studies were conducted and the results were compared with literature reports to demonstrate the improvements in energy utilization, CO_2 utilization, and net energy yield. Mathematical models were developed and validated in all cases to make the results more general.

Minimizing energy input for sparging of PBRs

Sparging serves multiple functions in PBRs such as maintaining the cultures in suspension, mixing the dissolved nutrients throughout the PBR, supplying carbon to the cultures by transferring gaseous CO_2 to the broth, and stripping of the oxygen generated by photosynthesis. Sparging is an energy-consuming process; the extent of sparging is governed on one hand by the biomass density to be maintained in suspension, and on the other hand by the amount of CO_2 to be supplied to maintain the desired growth rate. This study developed and validated a procedure to identify the dominant one of the two and minimize the energy input to accomplish that using *Nannochloropsis salina* as a model species. Continuing the above, this study proposed and validated a procedure to maximize the net energy yield by maximizing the energy output in algal cultivation, measured in terms of lipid productivity, using *N. salina* as a model species.

Development of airlift-driven raceway

Traditional paddlewheel-driven raceway reactors for algal cultivation have been sparged with CO_2-enriched air and operated at shallow depths and low biomass densities to ensure adequate light penetration. At shallow depths, the residence time of the CO_2-enriched bubbles is too short for efficient transfer of CO_2 to the broth. As such, the CO_2 utilization efficiencies suffered and hence biomass productivity. In this research, it was hypothesized that an airlift-driven raceway configuration could enhance CO_2 utilization efficiency by increasing the residence time of the CO_2-enriched bubbles and, hence, improve biomass productivity and the net energy yield in cultivation. Laboratory studies were conducted in a 20 L prototype version of the airlift-driven raceway to validate the hypothesis. Results of this study were compared with literature reports on open raceways and engineered PBRs to demonstrate higher biomass productivity per unit energy input and higher CO_2 utilization efficiency.

Development of internally illuminated PBR

In this research, it was hypothesized that internal illumination could be more energy-efficient than the traditional external illumination. Theoretical analysis of an internally illuminated PBR showed that it could have a shorter light path length, have a smaller footprint, and support higher biomass density and that it could achieve higher biomass productivity per unit energy input than a traditional externally illuminated bubble column of equal surface area per unit culture volume. This hypothesis was validated under laboratory conditions at 20 L scale. Results of this study were compared with literature reports on a variety of PBRs to demonstrate the advantages of the internally illuminated PBR.

Polycultures and Effects of Environmental Parameters on *Nannochloropsis salina* Production and Invading Organisms

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Several limitations impede algal biofuel from attaining cost-effective, commercial viability. These include the need for optimized production systems and stable, resilient algae cultures that are resistant to invading organisms. Within these systems, major contaminants have included unwanted bacteria, fungi, algae, and grazers.

In production systems, algae exhibit growth with a lag, logarithmic, and linear growth phase followed by the steady state when algae have reached carrying capacity. At steady state, growth is halted, but some algae, like marine microalgae *Nannochloropsis salina*, accumulate lipid due to environmental stressors. Here we examine environmental parameters that promote growth and lipid accumulation of *N. salina* while keeping invading organisms at a minimum. Furthermore, we test productivity and stability (resistance and resilience after disturbance by a grazer) of algae polycultures compared to monocultures.

First, we reviewed specific growth rate, population productivity, and lipid productivity based on 192 publications of *N. salina*. Specific growth rate was reported by 30 publications, often using exponential growth equations, and 14 publications stated biomass productivity. Direct comparison among productivity estimates is impossible due to differences in calculations or omission of equations. Less than 5% of publications directly reported lipid productivity. We extracted growth data from 30 publications using Plot Digitizer software and tested best fit with exponential and logistic equations. The logistic equation represented growth data better than the exponential one (higher R^2, lower AICc value). Maximum sustainable yield (MSY) was a more useful measure for harvest rates than specific growth rates *per se*. Interestingly, MSY displayed a closer linear relationship with carrying capacity measures ($R^2 = 0.78$, $p < 0.001$ and $R^2 = 0.55$, $p < 0.001$ for algae density and biomass, respectively) than growth rate ($R^2 = 0.30$, $p < 0.001$ and $R^2 = 0.10$, $p = 0.09$).

Then, in a series of experiments conducted in open aquaria in a greenhouse, we determined optimum salinity, pH, temperature, and nitrogen source to maximize *N. salina* growth and minimize other algae competitors and predators (rotifers and ciliates). We found that *N. salina* grows fastest at salt concentrations between 22–34 PSU, pH 8–9, temperature 22–27°C, and urea as a nitrogen source. Invaders were reduced at a salinity of 22 PSU, pH above 8, temperatures above 32°C, and ammonium chloride as a nitrogen source. While *N. salina* still showed optimum growth at 22 PSU salinity and pH above 8, the higher temperatures and ammonium chloride had negative impacts on growth. Follow-up experiments investigated if additional stressors would trigger an increase in lipid accumulation by growing algae at optimum environmental conditions but then

changing parameters at steady state. Salinity was the only parameter that showed a significant increase in lipid, when increased from 22 PSU to 34 PSU upon reaching stationary phase. An experiment using the central composite design followed by a response surface model was conducted to study interactions between all parameters. This experiment identified pH as the most sensitive parameter to influence *Nannochloropsis* growth rate and lipid accumulation. A consistent pH of 8 ensured highest productivity.

In a laboratory experiment, we studied differences in algae monocultures versus polycultures. Polycultures consisted of two, four, and six species, while each of the six species was grown individually in the monocultures. Polycultures were assembled from three big (potentially ungrazable) algae species and three small, fast-growing species. We demonstrate that growing several algae species together in polycultures (more than four species) will ensure a doubling of productivity and make algae cultures more resistant and resilient to disturbances by predators.

In conclusion, we propose to apply concepts of logistic growth, carrying capacity, and MSY calculations to estimates of maximum lipid productivity, similar to commonly used density and biomass calculations. Furthermore, we recommend to grow *N. salina* at a salinity of 22 PSU, pH of 8, temperature around 25°C, and with urea for maximum growth rate and to limit undesired organisms. Upon reaching steady-state phase, salinity may be increased to 34 PSU to enhance lipid accumulation. Growing algae in polycultures (four to six species) instead of monocultures has the potential to more than double the biomass productivity and increase resistance and resilience to grazers.

Commercial Algae Cultivation in Oil and Gas Produced Water

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Water conservation and nutrient costs are critical determinants in commercial algal biofuel production. In this project we demonstrate the feasibility of using produced water for cultivating algae in open ponds in an arid climate.

Produced water is a byproduct of oil and gas extraction: it is contained in the same geological formations as the hydrocarbons and is unavoidably brought to the surface along with the oil and gas. Our primary objective is to convert this waste stream into cultivation medium, thereby replacing fresh or brackish groundwater consumption and enabling a sustainable algae industry on land that is extraordinarily well suited for large-scale algae cultivation. The sheer volume of produced water generated in the mature oil and gas industry, 1.09 trillion gallons in 1993, makes it capable of harboring an algae-feedstock industry that can approach the renewable fuel standards for advanced biofuels.

Of critical importance to making algae cultivation commercially feasible are transportation costs. Produced water is readily available where land is cheap. Locating algae farms adjacent to disposal wells minimizes the cost to transport the water and in fact the oil producer may even pay the algae farmer to take the water, reducing the rate of disposal-well re-injection and prolonging the time when the disposal formation reaches saturation and must be closed down and a new well

drilled. Co-locating with the disposal well also provides for convenient flushing of the ponds when needed. The goal of this project is to demonstrate how this can work.

The original scope of work for this project was to provide up to 2,400 gallons per day of treated produced water from oil and gas production to the algae pond test bed (location to be determined). The treated produced water will sustain algal cultivation. We used a multistage water treatment system consisting of filtration, flocculation, and oxidation technologies to rectify produced water from a single-source oil and gas well. The treated water was then discharged to lined, open cultivation ponds and mixed with nutrients to grow algae.

Siting an algae facility is one of the most important factors that will ultimately lead to the success and sustainability of the project. We selected a site in Jal, New Mexico, because of the availability of recycled nutrients from dairy farms, municipal wastewater plants, potash mines, and gas plants; inexpensive flat land; abundant sunshine; lack of predators; ideal quality and large quantity of produced water; and infrastructure for efficiently moving production goods and finished products.

We initially constructed four 900-gallon raceways. We cultivated algae cultures in produced water in two of the raceways and fresh water in two of the raceways to compare the success of the cultures. Subsequently we added nine ponds on a one-acre footprint and expanded the entire facility to a one hectare footprint to include a harvesting and processing building and to determine the cost of scaling.

Following the Aquatic Species Program insight that "the best approach for successful cultivation of a consistent species of algae was to allow a contaminant native to the area to take over the ponds," we have been cultivating native algal strains at our Jal facility in produced water since August 2011. Genetic characterization indicates that the algae are predominantly *Scenedesmus* sp. and *Tetracystis* sp., with *Nannochloropsis salina 1776* and *Chlorella* in lesser populations.

We sought to project a pathway towards commercialization and anticipated scaling issues that will help reduce our OpEx and CapEx. Our present operating expense to treat a 42-gallon barrel of water for resale is approximately $1.95. Our capital expense for water treatment is approximately $727,000, the ponds alone costing $327,000: a significant gap from the $45,000/acre published CapEx. Another significant gap in commercial sustainability is with growth rates. According to the report from the Aquatic Species Program, 50 $g/m^2/day$ should be achievable. On average we achieved an annual growth rate of 11 $g/m^2/day$.

An unexpected opportunity arose during the project: Algae bio-remediates the produced water so that post-harvest it can be sold for use in enhanced oil recovery techniques such as hydraulic fracturing. The price of water in the Permian Basin oil fields of SE New Mexico and West Texas is currently running between $1 and $3 per barrel. With additional research and development we believe we can lower costs to meet the demand for water at this price point.

Commercial viability for algal biofuels requires capital-intensive infrastructure investment. While public funding can help offset inefficiencies initially,

ultimately algal biofuels production must be competitive to be sustainable. Given current supplies and extraction efficiencies for fossil fuels, this is a tall order. Eldorado Biofuels recognizes that we are in a commodity market and are focused on driving down costs by using waste streams wherever possible and infrastructure synergies with existing industry, rather than pursuing valuable coproducts.

Algae facilities can be commercially viable at today's energy prices by utilizing algae treatment for produced water disposal and reuse in the oil and gas industry and by accounting for the external cost of greenhouse gas emissions in fossil fuel powered energy production.

Sensing and Control for Industrial Algae Production and Harvesting

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Algae are regarded as a promising feedstock for renewable energy production. To make the algae-to-biofuel production enterprise scalable and profitable, two technological breakthroughs should be achieved. First, high-throughput techniques for characterizing chemical composition of algae can accelerate the identification of optimal strains for scale-up cultivation and the selection of proper conversion techniques for fuel production and biomass utility. Second, automated sensing and control systems can realize real-time monitoring and cultivation decision-making for production-scale ponds. Overcoming both technology hurdles relies on the development of rapid and cost-effective sensor technologies that can measure key algae parameters such as growth rate, neutral lipid content, and protein content.

There are four specific research objectives in this project: (1) Improvement and test of an algae optical density (OD) sensor for determining biomass concentration and algae growth rate; (2) algal neutral lipid quantification and sensor development based on Nile Red staining and fluorescence (NRf); (3) near-infrared (NIR) and mid-infrared (MIR) spectroscopic analysis to characterize algal biomass compositions including total neutral lipids, protein, ash content, and heating value; and (4) lipid sensor development based on infrared attenuated total reflectance (IR-ATR).

We improved the OD sensor in the following four aspects. First, the sensor body was redesigned for better optical measurement geometry. Second, we implemented a constant-current LED driver circuit. Third, we implemented a thermo-electrical temperature control module to maintain the temperature of the photodetectors. Finally, we built a reference flow cell into the sensor body for reference measurement. We conducted two field tests, one in a lab-scale raceway and the other in a large-scale open pond. The tests showed that the sensor-based OD readings were highly correlated with the lab-spectrometer–based OD readings (R^2 = 0.98 and 0.96). Both software and hardware components of the sensor performed well during the tests with no need for human intervention. We concluded that the OD sensor can be used reliably to monitor algae growth in the production ponds.

We used two algae types for the NRf study, *Nannochloropsis* (*N. salina* and *N. oculata*) and *Botryococcus braunii* (races A and B). Experiments were conducted to elucidate the influence of different factors including excitation/emission wavelengths, incubation time, and temperature. We found that the combination of 490/590 nm gave the maximum fluorescence response for both algae types. The fluorescence signals continued to increase until three minutes after Nile Red addition and then leveled off. Temperature has a minor effect on the fluorescence signals. The most confounding factor for the NRf method was algae species. Within each species, the correlation between the NRf signal and neutral lipid content was quite high (R^2 = 0.96 and 0.88). However, the two regression lines cannot be merged together. A prototype NRf sensor was built based on inexpensive optoelectronic components, and the test showed that the sensor output was highly correlated with the bench-top fluorometer (R^2 = 0.89) with both algae types.

We analyzed dry algal biomass samples by the NIR and MIR-FTIR spectrometers to characterize their chemical compositions including ash content, energy value, total neutral lipids, and protein content. The samples used in this study were freeze-dried algal biomass samples of different species. The NIR and FTIR spectra were calibrated with partial least squares regression to develop calibration models for each chemical variable and then applied on a validation set for model evaluation. The result showed that for both NIR and FTIR the energy value and protein content can be predicted successfully, with R^2 generally higher than 0.85. The model performance for total neutral lipids and ash content is only fair, with R^2 ranging from 0.45 to 0.69. In general, the models developed with FTIR spectra performed slightly better than those developed with the NIR spectra. It is concluded that both techniques have the great potential to be used in downstream algal biomass utilities such as lipid-extracted algae for animal feeding and soil amendments.

For the development of an IR-ATR neutral lipid sensor, we investigated the following important parameters for the sensor design: (1) the usefulness of the C-H stretching at around 2900 cm^{-1} for lipid quantification and (2) the speed of thin-film development as the algae droplet evaporates on the ATR crystal. We found that the absorption depth at 2900 cm^{-1} was highly correlated with the neutral lipid content (R^2 = 0.90). However, it took more than one hour for the algal thin films to form on the ATR crystal. This indicated some sort of heating mechanism is needed. The overall sensor design was completed with the inclusion of a zinc selenide crystal, an IR LED (central wavelength 3400 nm), a lead sulfide photodiode, an electrical heater, and a sensor housing. Electronic circuits were designed to pulse LED light and amplify and condition the signals from the photodiode (proportional to lipid content). Lab tests were conducted using vegetable oil as a calibration standard and showed that the prototype sensor performed well.

Hydrodynamics of Open Ponds

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Computational Fluid Dynamics (CFD) can be employed to obtain the flow pattern in the raceway pond. Once the basic flow pattern is known, many important flow characteristics such as dead-zones and velocity channeling can be obtained. CFD can also be employed to obtain/infer pressure drop, hydraulic energy consumption, mass transfer, etc. in the pond. Algae growth can be simulated once the details of growth kinetics are known and are appropriately combined with flow pattern information from CFD. To this end, we have set up an extensive program for CFD simulations of raceway ponds. The geometry and operating details of the 360 m^2 raceway pond from Samalkota (Andhra Pradesh, India) were obtained. The CFD simulations were carried out for many different cases. This report outlines results for specific cases described subsequently. Key conclusions from the CFD simulations are:

1. The CFD simulations for flow in a (360 m^2) raceway pond showed the overall flow pattern with possible locations of dead-zones.
2. Vertical mixing in a raceway pond is seen to be very poor from simulations. The same was confirmed through a cold-flow, tracer study conducted on the 360 m^2 raceway pond.
3. The CFD results presented here were obtained for mean velocity of 0.3 m/s in the raceway pond and needs validation using some experimental measurements of flow pattern. Similar results were obtained for the mean velocities of 0.1 m/s and 0.2 m/s.
4. The top (free) surface of the fluid in the raceway pond is treated as a flat surface in simulations. In reality, the free surface shows a wavy characteristic mainly due to action of the paddlewheel. The turbulent mixing due to the wave interface is difficult to capture using CFD and hence the mixing in the raceway pond captured by CFD simulations is likely to be conservative.
5. The pressure drop in the raceway pond obtained from CFD modeling correlates well with the empirical Manning's equation (with Manning's constant ~0.2).

In addition to the CFD modeling, a detailed study was carried out on modeling algal growth kinetics and a report has been submitted to NAABB on it. This report provides a comprehensive description of the algal growth kinetics model that has been developed. Section 1 gives an overview of the various phenomena that affect the growth kinetics of the algal cells. Section 2 describes a detailed photosynthesis model along with the effects of nitrogen limitation and oxygen inhibition. Section 3 describes the effect of CO_2 limitation. This section also describes a dynamic model for predicting the strategy for CO_2 sparging for maintaining an optimum pH, providing adequate CO_2 to the cells, and, at the same time, minimizing CO_2 costs. The results of the simulation of various aspects of the comprehensive kinetic model are presented in Section 4.

Finally, the CFD simulations and findings, when combined with the model for algal growth kinetics can together help us modify and optimize the raceway pond design for achieving optimal biomass productivities.

Screening Microalgae for Optimum Productivity in Outdoor Ponds: Biomass Growth Modeling and Climate-simulated Culturing

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Light and temperature are the main determinants of biomass productivity of microalgae in photobioreactors and ponds operated under well-mixed and nutrient-replete conditions. For a pond at a given geographic location, the daily and seasonal fluctuations of sunlight intensity and water temperatures are determined by the prevailing climatic conditions at that site. The biomass productivity of a specific strain grown in this pond culture is then to a large degree determined by how the maximum specific growth rate is affected by sunlight intensity and water temperature. Thus, in order to optimize biomass productivities in outdoor ponds, it is not only necessary to identify geographic locations that have high annual solar insolation and climatic conditions that maintain pond water temperatures at elevated levels for most of the year, but also microalgae strains that exhibit high growth rates within the annual temperature range of the pond culture.

To accelerate the transition of promising microalgae from the laboratory into outdoor ponds, we developed and tested an integrated strategy for screening strains for high biomass productivity potential. For a given strain, the strategy consists of the following three-step approach: (1) measuring the biomass light-absorption coefficient and the maximum specific growth rate as a function of light intensity and temperature; (2) predicting, using a newly developed biomass growth model, the biomass productivity in outdoor ponds in different geographic locations and seasons; and (3) validating the growth performance in the optimum pond location by conducting culture experiments under climate-simulated conditions in state-of-the-art indoor LED-lighted and temperature-controlled raceways specifically designed and built for this project.

Using *Nannochloropsis salina* and *Chlorella* sp. *DOE1412* as model strains, we found that *Chlorella* exhibits much higher maximum specific growth rates at the optimal temperature (μ=5.9 day^{-1} at 31°C) and greater thermal tolerance (T_{max}~45°C) than *N. salina* (μ=1.1 day^{-1} at 26°C, T_{max}~35°C). Measurement of the maximum specific growth rates as a function of light intensity at different temperatures revealed that *Chlorella* is strongly photo-inhibited at lower temperatures (≤ 22°C) and only slightly at higher temperatures, most likely because cellular mechanisms that repair damage inflicted by high photon flux densities are less effective at lower temperatures. No photo-inhibition was found for *N. salina*. The light intensity at which photosynthesis saturates was about the same for both strains, 250 μmoles/m^2-sec.

The rate of biomass loss during incubation in the dark was about the same, around 1% per hour, for both *Chlorella* (31°C) and *N. salina* (23°C). When *Chlorella* was incubated for 24 hours at 31°C, about 35% of the initial biomass was lost. Oxygen consumption rates due to respiration in the dark are highly temperature dependent. The biomass-normalized oxygen uptake rate increased linearly with temperature for *Chlorella* and *N. salina*, exhibiting almost the same slope, and declined at the highest tolerated temperature that was tested. The maximum dark-respiration oxygen-uptake rates of both *Chlorella* (at 36°C) and *N. salina* (at 29°C) were found to be about 30% of the maximum photosynthetic oxygen production rates that were observed above saturating light intensity. This is in agreement with other studies reporting that biomass-specific dark-respiration rates are up to 32% of the strain's maximum specific growth rate.

Using these species-specific laboratory measurements together with sunlight intensity and pond water temperature data that were periodically measured during an outdoor study in Arizona, our biomass growth model successfully predicted the biomass growth of *Chlorella* in three outdoor raceways. The model slightly overestimated the biomass concentration at the beginning of the experiment but correctly predicted the slowdown of biomass growth at the end, which resulted from increasing losses of biomass due to dark respiration at night, as indicated by the ever more pronounced zigzag shape of the predicted biomass concentration profile. The model also successfully predicted the steady-state biomass concentrations that were measured during semi-continuous culturing mode at two different dilution rates.

To determine whether we could repeat the *Chlorella* outdoor pond culture experiment that had been conducted in Arizona using our indoor LED-lighted and temperature controlled ponds in Washington State, we operated the indoor raceway cultures under climate-simulated conditions by subjecting them to the sunlight and water temperature fluctuations that were recorded during the outdoor study. Based on data collected from a batch culture experiment, the biomass concentration (OD_{750}) in the two indoor climate-simulation ponds increased at the same rate as in the three outdoor raceways in Arizona during the first 15 days but rates then diverged, most likely because various operational mishaps that occurred in the outdoor cultures were not replicated indoors. The steady-state biomass concentrations measured in the climate-simulation ponds at two different dilution rates were comparable to those measured in the outdoor ponds. These findings demonstrate that it is possible to replicate the performance of outdoor pond cultures in state-of-the-art indoor LED-lighted and temperature-controlled ponds operated under climate-simulated conditions.

Microalgal Cultivation and Productivity Assessments with Saltwater, Freshwater, and Wastewater Systems

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Cultivation test-bed projects funded by the National Alliance for Advanced Biofuels and Bioproducts (NAABB) at New Mexico State University focused on five major topics and most involved developing cross-cutting process teams whose participants were NAABB investigators from outside the Cultivation Team. The project objectives are listed below with cross-cutting team members identified in parentheses:

1) Determination of biomass and lipid productivities of *Nannochloropsis salina CCMP1776* cultivated in outdoor photobioreactors (Ron Parsons and Tracy Yates of Solix Biosystems).

2) Development of physiological and DNA-based diagnostic tools (Ron Parsons of Solix Biosystems and Stephen Chisholm and Scott Fulbright of Colorado State University).

3) Assessment of algal productivities and associated bacterial communities when utilizing wastewater as a source of nutrients (Shawn Starkenburg of Los Alamos National Laboratory).

4) Assessment of proteomic, lipidomic, and metabolomic changes in outdoor production cultures of *N. salina CCMP1776* (Tanner Schaub of New Mexico State University, Ellen Panisko of Pacific Northwest National Laboratory, and Shawn Starkenburg of Los Alamos National Laboratory).

5) Analysis of the potential of extremophilic red algae from acidic hot springs for mixotrophic cultivation on municipal and animal wastewater (Ursula Goodenough of Washington University and Shuguang Deng of New Mexico State University).

Objective 1. *Nannochloropsis* biomass and lipid productivities
The New Mexico State University test bed operated one 4,000 L outdoor, temperature-regulated photobioreactor (PBR) and four 3 m^2 paddle wheel raceway systems identical to those used at the Texas A&M Corpus Christi test bed. The photobioreactor was from Solix Biosystems. The project Principal Investigator Peter Lammers started the period of performance at Solix but relocated to New Mexico State University five months after the project start date. Work supervised by him at both Solix and the university contributed to topics (1) and (2) above.

The first Cultivation team deliverable was to demonstrate cultivation methods for greater than 5 gdw/L biomass at more than 50% lipid content with low nutrient consumption in a small-scale closed system. Data demonstrating these metrics has been published with yields exceeding 5 gdw/L with 50% lipid using the Solix PBR system. Moreover, these results have been confirmed in both small and large scale PBR systems with efficient use of nutrients and CO_2.

Objective 2. Physiological and DNA-based diagnostics
Physiological methods for direct assessment of microalgal growth parameters were developed and published based on a sensitive methodology for determining the flux of both oxygen and carbon dioxide in production cultures.

DNA diagnostics for evaluation of invasive microalgae were developed in a collaboration among New Mexico State University, Solix Biosystems, and Colorado State University. This project required the development and testing of diagnostic polymerase chain reaction (PCR) primer sets as well as post-PCR processing and diagnostics. We showed that the presence of invasive, contaminating microalgae is to be expected in scale-up outdoor cultivation operations. Primers were designed to amplify an approximately 1500-nucleotide region of the 18S rRNA gene from four major classes of algae: Bacillariophyceae, Chlorophyceae, Eustigmatophyceae, and Prasinophyceae. These amplicons can be sequenced for definitive identification of strains, including closely related species from the same genus, or they can be digested with a restriction enzyme recognizing a four-base sequence to generate allele-specific fragmentation patterns (CAPS markers) for rapid, inexpensive characterization of strains and cultures. We also compared two strategies for culture monitoring for their ability to detect weedy algae at low abundance in elite cultures. We showed that the real-time qPCR method is more sensitive, specific, and accurate than flow cytometry for detecting weedy algae at low abundance in cultures. This work provides molecular tools to detect and monitor algae population dynamics and clarifies the utility, strength, and limitations of these assays. The use of these tools will be particularly useful for quality control purposes during production of inocula for large-scale cultivation of an elite algal strain.

Objective 3. Municipal wastewater as N and P source
Wastewater from municipalities, dairies and feedlots, swine and poultry production, and aquaculture represents an environmental burden and a source of needed resources for algal cultivation. Modern wastewater treatment is not only energy intensive, accounting for 60–70% of the energy consumption by municipalities, but tertiary treatments to remove nitrogen most often convert this valuable resource into valueless, inert nitrogen gas. Photosynthetic algae are theoretically capable of converting wastewater nitrogen and phosphorous into biomass for downstream processing but virtually no information is available about the fate or effect of wastewater bacteria on microalgal cultures at the microbial community level.

To estimate algal production process parameters and risk issues associated with wastewater bacteria we tested both wastewater and sterilized wastewater and we monitored algal phenotypes, biomass and lipid accumulation, nutrient use efficiency, and the changes in bacterial diversity. A 13-day experimental growth period included two stages, pre- and postsupplementation with nitrogen and phosphorous nutrients. *N. salina* effectively used nutrients from either wastewater or standard control substrates with the quantitative and qualitative process parameters closely reflecting initial and enhanced nutrient conditions. The OD680:750 ratios have been shown to be a promising monitoring tool for the parameters governed by the algal nutritional status. No significant differences were noted between the wastewater and the standard control media cultures in

the measured dry weight biomass, lipid quantity and quality as described by fatty acid methyl esters, and final bacterial diversities as described by 16S rDNA metagenomics. Process parameters were consistent with previously published reports on *N. salina*. The wastewater used in this study may provide a sustainable option for microalgae biofuel production while minimizing microbial risks.

Objective 3.a. Municipal wastewater viral monitoring and bacterial risk analysis

Wastewaters harbor complex microbial consortia including enteric viruses. Appropriate evaluation protocols are required to understand and monitor associated health risks. Commonly virus titers are assessed by host-specific plaque assays. The accuracy of such assays is critically dependent on the assumption that viral particles are uniformly distributed in samples. Complex charged and hydrophobic loci on algal cells may affect this assumption. We verified the likelihood of *N. salina* to affect partitioning of viruses between planktonic and attached compartments by employing a common test surrogate for enteric viruses, the MS2 bacteriophage. First, we verified the immediate (within 30 min) partitioning of spiked virus between supernatant (planktonic) and centrifuged algal pellet (fixed compartment) for algae of variable ages, and thus physiological states, grown in standard medium, f/2, or in sterilized advanced secondary treated wastewater. Second, co-incubation tests estimated MS2 persistence during algal growth. Both infective (plaque assay) and total MS2 (quantitative PCR) were quantified. Little immediate partitioning occurred with most of MS2 recovered in the supernatant. Incubation in wastewater or f/2 drastically reduced MS2 viability (< 8 hours) although the MS2's RNA signature persisted up to 312 hours. While algae and wastewater organics marginally enhanced virus viability this was at levels of no practical significance.

In summary, our results indicate that partitioning of viral particles between planktonic phase and retention on algae varies with the age of the algal culture. Presence of algae, as well as presence of wastewater organic compounds, enhanced recoverability of viable or total MS2, mitigating the negative effect of abiotic stresses associated with the mineral-only growth substrate. Underestimation of total virus load may occur when viruses are incubated in wastewater or f/2 in the absence of algae.

We examined the relationship between algal growth substrate and culture age with the associated bacterial diversity in enclosed photobioreactors. Algal cultures in both wastewaters and the standard NM-f/2 synthetic substrate contained distinct microbiomes. The diversity in wastewater reflects the source and the wastewater treatment conditions. Genetic fingerprinting indicated that the NM-f/2 consortium closely reflected known isolates from marine waters or the ubiquitous Pseudomonades. As the algae grew in wastewater, the bacterial population diversity shifted to reflect more closely the algal inoculum over the original wastewater source. This indicates that algae and the associated culture conditions can effectively control the bacterial diversity in their immediate environment. Algae will shift the diversity from risk relevant fecal populations to bacterial populations known to associate with the degradation of organic substrates often found in marine ecosystems.

Objective 4. Omics-based analysis of production cultures of *N. salina*
A major cross-cutting team collaborated in the detailed enumeration of biochemical changes in outdoor PBR cultures of *N. salina CCMP1776*. In our project report we describe the lipid composition of the microalga *N. salina CCMP1776* during the transition between growth and stationary phases with a shotgun lipidomic and proteomic approach provided by Fourier-transform ion cyclotron resonance mass spectrometry. We assign unique elemental compositions to more than 500 ion signals at mass measurement accuracy < 300 parts-per-billion rms for time-course samples collected during different phases of the algal growth cycle. With tandem mass measurement, we describe acyl-chain composition for molecules of each of the major observed lipid classes. We report the observation of the betaine membrane lipid class monoacylglycerol-trimethyl-homoserine (MGTS), previously unreported for this species. Sulfoquinovosyl diacylglycerol (SQDG) compounds dominate the negative ion mode mass spectra and show a marked increase in relative abundance between Days 1 and 7, with the acyl chain composition 16:1/16:0 being most abundant. We also observe esterified sulfate and doubly esterified sulfate lipid species. Elevated levels of 20:4 and 20:5 fatty acids observed in sulfate esters suggest a possible role in the biosynthesis and/or storage of eicosapentaenoic acid (EPA). EPA content was near 3% of dry biomass at both high and low total lipid content. Phospholipids were observed at low overall abundance with little growth-phase-dependent abundance change. Proteomic changes across > 500 proteins are quantified for each time point and clustered with changes in metabolites to provide details on biochemical profiles during batch growth of this organism.

Objective 5. Mixotrophic acido-thermophilic red algae for wastewater treatment
As noted previously, wastewater treatment is an energy-intensive and inefficient method for capturing the nutrient/fertilizer value inherent in wastewater. *Galdieria sulphuraria* is a mesothermophilic and mixotrophic red alga found worldwide in acidic hot springs. The algal cultivation team at New Mexico State University investigated the utility of this organism for biofuel production based on two factors: (1) resource assessment concerns based on the scarcity of water in the U.S. desert Southwest and (2) the need for thermo-tolerant algae resistant to solar heat gain if closed photobioreactors were used to minimize evaporative water loss. The robust mixotrophic capabilities of *G. sulphuraria* also suggested a strong potential for the use of *G. sulphuraria* in wastewater treatment for removal of biological oxygen demand (BOD) nitrogen and phosphorus.

Thus far, we have studied two strains from Yellowstone National Park. Results show the productivity of *G. sulphuraria* rivals that of the best strain from the NAABB prospecting effort, *Chlorella* sp. *DOE1412*. The laboratory growth rate of *G. sulphuraria CCMEE5587.1* is virtually identical to *DOE1412* while maximum photosynthetic rates reported in the literature for these two strains are also virtually identical. Bicarbonate ions are absent at the cultivation pH of 2.5 giving rise to ~1% of the inorganic carbon relative to neutral pH. Nevertheless, we observed no difference in growth rate when CO_2 in the gas phase was varied between 1% and 10% by volume in air. *G. sulphuraria* is being cultured outdoors in 400 L raceway systems enclosed in plastic bags to provide solar heat gain to achieve temperature that promotes maximum growth rates. Linear productivities

are observed at 0.11 g/L/day with temperatures varying between 25–50°C. To put this in perspective, *N. salina* growth in open raceways at the same location is typically 0.01–0.02 g/L/day and 0.12 g/L/day in an expensive, temperature-controlled PBR. Nitrogen deprivation experiments have demonstrated the presence of lipid bodies and starch granules in the cytoplasm of *G. sulphuraria* cells by freeze-etch electron microscopy. Finally, hydrothermal liquefaction extraction experiments were performed in the Deng laboratory at NMSU on *G. sulphuraria* and *C. caldarium* biomass producing 20–32% biocrude oil.

Test-bed Utilization and Large-scale Biomass Production at Texas AgriLife Research

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The Texas A&M AgriLife Research pilot plant in Pecos, Texas, is a large-scale algae biomass production facility that has been in operation for more than five years. Currently the facility has nine open ponds that cover around 1200 square meters of land and use several paddlewheel designs as well as several alternative open-pond system designs. During these five years, substantial quantities of algae biomass have been produced on a consistent basis from many types of algae strains to further research in biofuels as well as animal feed.

For this project the pilot plant was tasked with large-scale production of algae biomass using an open pond system. Large-scale production was conducted for five algae strains starting with *Nannochloropsis salina CCMP1776* serving as the baseline strain and four other strains selected by the Algal Biology Team within the NAABB consortium (Table 1). We collected data for each algae strain to efficiently compare all five strains grown in Pecos and to compare the data across the consortium. Some of the data parameters accumulated include productivity, strain robustness, harvest technique, electrical usage, media cost during production runs, and lipid percentage as well as ash percentage. All data were distributed on a monthly basis to the Sustainability Team within NAABB. Once we harvested and froze the algae biomass, we shipped the samples to several downstream researchers, mainly Valicor, to further their research and processing methodologies.

Table 1. List of NAABB Large-scale Production Algae

Species ID #	Texas AgriLife ID	Scientific Name	Growing Season
CCMP1776	08-AM	*Nannochloropsis salina*	Pecos Summer
DOE0043	12-AM	*Desmid* sp.	Pecos Spring
UTEX 998	43-AM	*Nannochloropsis oculata*	Pecos Summer
DOE1095	19-AM	*Chlorella* sp.	Pecos Winter
DOE1412	11-AM	*Chlorella* sp.	Pecos Summer/Fall/Winter
Pecos Native	26-AM	*Kirchneriella* sp.	Pecos Winter

Table 2. Harvest Summary for Pecos Facility

Production Year	Number of Harvests	Harvested Biomass (kg)	Average Lipid Percentage	Harvested Lipids (kg)
2009	23	224.7	8.0	21.56
2010	47	780.0	14.0	95.00
2011	48	610.0	16.8	102.48
2012	65	876.0	22.4	196.22

The four algae strains selected by the Biology Team for large-scale production were isolated from samples collected from many native sources within the United States. To use these wild strains to their full potential the Pecos team had to optimize their growth media. We put each strain through a four-step test where we introduced fertilizer-grade nutrients to the algae cultures and observed the response. We created media recipes for each strain and used them during large-scale biomass production of each strain. Comparisons done on cost and productivity showed the new media recipes cost 90–95% lower than standard media while productivity was matched or surpassed in some strains compared to standard media.

We observed several problematic aspects to growing algae in large-scale open systems during the three-year NAABB production schedule (Table 2). The most challenging parameter was high ash content in harvested biomass that resulted in low efficiencies or the impossibility of further downstream processing of the algae biomass. We tested and implemented several techniques to lower the ash content to the accepted levels for further down-processing. Other major challenges observed were contamination and algae culture stability.

An integral part of large-scale biomass production in Pecos was facilitating other consortium members with their dewatering techniques. Consortium members such as Kaselco, Pacific Northwest National Laboratory, and Pall were able to bring their large-scale systems to Pecos and collect all the necessary data to compare their technologies versus the in-line centrifuge that was chosen as the baseline dewatering technique.

Optimization of Temperature, Energy Use Efficiency, and Hydrodynamics in the ARID Raceway

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Our research focused on evaluating the temperature characteristics of the Aquaculture Raceway Integrated Design (ARID) raceway, improving the hydraulic design of the raceway, developing an algae growth model of the raceway, and developing a 3-D computer model of serpentine channel flow.

Diurnal and seasonal temperature variations are one cause of decreased algae production in open ponds. The ARID system maintains temperature in the optimal range by changing the water surface area between day and night. We focused our first project on calibrating a temperature model of the ARID raceway. In this research, we made several improvements to the energy balance model of the ARID raceway: an evapotranspiration algorithm, albedo, a wind speed adjustment, a method to calculate thermal resistance of canal covers, and variable pump on and off times. The model accurately simulated the temperature changes in the ARID raceway during the winter experiment. The experiment also showed that the ARID raceway remained 7–10°C warmer than conventional raceways.

The second project, which included funding from NAABB and the Arizona Center for Technology and Innovation, focused on improving pumping efficiency and channel hydraulics. We installed a serpentine flow pattern, in which the water flows through channels instead of over barriers, in the ARID-HV (high velocity) raceway in Tucson and Phoenix, Arizona. We evaluated constructability, reliability of components, drainage of channels, and flow and energy requirements of the ARID-HV raceway. Each of the energy inputs to the raceway (air sparger, air-bag blower, canal lift pump, and channel recirculation pump) were quantified, some by direct measurement and others by simulation. We recommended an Archimedes screw pump for future raceways, but did not install one. The potential energy use of an ARID raceway, with an Archimedes screw pump, is less than for a conventional raceway.

Our third NAABB-funded research project was the development of an algae growth model for the ARID raceway. We incorporated Michael Huesemann's algae growth model into the temperature and light model of the ARID raceway. The accurate estimates of light transmission and temperature enabled an accurate prediction of algae growth for various raceway configurations, depths, and operational schemes. The model showed that shallow channels (0.1 m) had nearly the same aerial production rate (gm^{-2}) as deeper flow (0.3 m). This is an advantage for ARID raceway design because the canal can be much smaller, decreasing construction costs. A smaller canal would also decrease the cost of lifting the algae into channels in the mornings. Monthly production rates of ARID and conventional raceways were simulated with the model. We ran the model for Tucson and showed that the ARID raceway had much higher production than a conventional raceway in winter, significantly higher production in spring, similar production in the last month before the monsoon (June), and lower production during the monsoon months (July and August). This, combined with lower energy use in the ARID raceway, means that the ARID raceway has a much higher annual energy productivity than conventional raceways.

The fourth NAABB-funded project was the development of a computational fluid dynamics model of serpentine channel flow. We developed two innovations with the model. First, a small spillover flow over the air bags can induce spirally forwarding vortices in channels and improve mixing. Second, spillover flow in corners can eliminate dead zones.

CULTIVATION

Development of Cost-effective Production Techniques for the Culture of Mono and Mixed Microalgae Biomass

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Researchers continuously look for ways to economically increase microalgal biomass and lipid production. The studies included in this report were designed to compare the productivity of different stocking densities, mixed cultures, local algal cultures, alternative nutrient sources, nutrient regimes, harvest strategies, carbon dioxide optimization, water reuse, and nitrogen-phosphorus ratios in 12 outdoor 3 m^2 fiberglass raceways. *Nannochloropsis salina* produced significantly more biomass when stocked at a higher initial biomass compared to cultures stocked at a lower initial biomass. We do not attribute this difference in biomass to lower contamination as we noted no difference in contamination levels between the two stocking densities at any sampling period during the trial.

In the mixed-culture experiments we stocked *N. salina*, *Phaeodactylum tricornutum,* and a mixture of the cultures to achieve an initial biomass of ~0.23 g/L ash-free dry weight (afdw) at 5 cm depth during each season. The mixed culture raceways during the fall and winter produced significantly more biomass (afdw/L/m^2) than both monocultures. The mixed culture raceways during the spring and summer produced significantly more biomass than *P. tricornutum* monocultures but were not significantly different from the *N. salina* monocultures. The significant increase in productivity in the mixed-culture raceways suggests the mixed cultures are better able to utilize ambient outdoor conditions when they are most limiting in the fall and winter.

Our efforts to optimize biomass production led to the discovery that local algal species may be better suited for growth in our climate than the same algal species obtained from a culture collection. A local *Phaeodactylum* (isolated from Corpus Christi Bay, Texas) produced significantly higher biomass than the *Phaeodactylum CCMP*. This work suggests the importance of optimizing the algal species for the exact growing conditions. To potentially lower the cost of nutrients, we examined the ability of *Amphora* sp. to remove nitrogen compounds from superintensive shrimp raceways effluent. Total NO_3-N uptake levels ranged from 1–9 mg/L NO_3-N/d and were highly dependent on starting nitrate level. *Amphora* grown in diluted effluent water (initial NO_3-N level 38 mg/L) produced significantly more biomass than *Amphora* grown in seawater supplemented with the same concentration of nitrate (obtained from sodium nitrate) and non-diluted effluent water. This study showed that effluent from intensive shrimp culture can provide nutrients for microalgal growth.

Our work with mixed cultures continued with a comparison of a local strain of *Nannochloropsis DOE916* to *N. salina CCMP1776* in mono and mixed culture. The *N. salina CCMP1776* monoculture produced significantly more biomass than the *DOE916* monoculture or the mixed cultures of *N. salina CCMP1776* and

DOE916. These results demonstrate that not all mixed cultures will perform better than their representative monocultures. In the harvest strategies experiment, we compared two harvest techniques, batch and continuous, over a 30-day period. Batch culture treatments continued to produce more biomass over the course of the study than the continuous cultures, with peak growth and time between harvests remaining consistent. The peak biomass of the continuous culture treatments began to drop after Day 15. Productivity was 12.8 $g/m^2/d$ for batch cultures, compared to 10.9 $g/m^2/d$ for the continuous cultures.

In the nutrient regime study, ODI-mix with ammonia as the nitrogen source, f/2, and ODI-mix with NO_3 as the N source were added on an equal N basis for optimal production of *N. salina*. No significant differences in production (afdw [g/L]) were found between treatments. The ability to get the same production from the ODI-mix with ammonia and nitrate compared to the more expensive f/2 media greatly enhances the ability to produce biomass at reduced costs. Additional work to optimize the ODI-mix was undertaken to determine whether our in-house nutrient formulation was limiting in phosphorus. We compared high (13.2 ml/L) to low (0.024 ml/L) concentrations of phosphorus in both 1x ODI and 2x ODI solutions. This would also allow a second comparison of the ODI formulations. Results suggested that the increase in phosphorous in the nutrient mixture did not significantly improve production in the low phosphorus. The 2x low-phosphorous treatment had a final production value of 12 $g/m^2/d$ compared to 9.3 $g/m^2/d$ of the 2x high-phosphorous treatment.

In our recycled seawater experiment we compared the effects of utilizing recycled media (without chemical treatment) versus new chlorinated seawater after harvest. We also compared continuous and batch harvest techniques. An old (originally started October 5, 2010) culture of microalgae was saved from the previous study and compared to a new culture that was brought up in the indoor laboratory (December 16, 2010) to determine the effects of long-time outdoor cultures as well as to continue the comparison of two *Nannochloropsis* species: *N. salina* and *N. OZ1*. Growth of the old culture treatments began to lag after the second week of the study and by the end of the experiment the old and new treatments had similar final production numbers. The recycled media treatments quickly fell behind in biomass production after the third harvest (January 8, 2011, 23 days into the experiment), most likely due to bacterial build up.

To optimize carbon dioxide usage, we stocked *N. OZ1* into the outdoor raceways and monitored biomass yields. The treatments included a 24 hr/d CO_2 treatment, application from the hours of 8:00 a.m. to 5:00 p.m., and no direct CO_2 addition. A fourth treatment compared to the 8:00 a.m. to 5:00 p.m. treatment involved increasing the salinity to 45 ppt. Growth with 24 hr/d CO_2 was marginally better and more consistent than the 8:00 a.m. to 5:00 p.m. CO_2 treatments at 5.435 and 6.01 $g/m^2/d$, respectively. No CO_2 introduction showed less stability and slower growth with final growth at 6.25 $g/m^2/d$. The high salinity treatment showed stunted growth compared to the normal seawater salinity treatments at 3.75 $g/m^2/d$. In another study of nutrient formulations, two *Nannochloropsis* species, *N. salina* and *N. OZ1,* and two different nutrient formulations, ODI mix and the standard f/2, each were applied once a week. The *N. salina* had more consistent growth, but the *N. OZ1* grew at a faster rate. During the experiment

the ODI nutrient formula resulted in higher and more stable growth at an average of 4.168 g/m^2/d between species. The f/2 treatments ended the experiment at an average of 2.26 g/m^2/d between species.

In another study related to nutrients supplementation, we compared nutrient enrichment of once per harvest at inoculation versus weekly nutrients enrichment beginning at inoculation and continuing until harvest. Harvest occurred when cell count reached 110–120 x 10^6 cells/ml. We also tested the effects of extending treatment 10 days past nutrient depletion. Normal *N. OZ1* batch culture produced the most biomass (13.2 g/m^2/d) with the biomasses of the extended batch treatments only producing 10.3 g/m^2/d and 9.3 g/m^2/d for *N. OZ1* and *N. salina,* respectively. These studies show our ability to increase productivity, optimize the harvest process, and reduce costs, which are vital to the growth of the microalgae industry.

Algal Biomass Production and Optimization Using Cellana's ALDUO™ Cultivation Technology

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The goal of this project was for Cellana to generate algal biomass from one or more high-performing strains utilizing the ALDUO™ cultivation technology. Cellana biomass was supplied to NAABB consortium members, who conducted a range of downstream investigations. The ultimate objective is to achieve a commercially viable microalgae biorefinery that generates fuel feedstocks, protein-based feeds, and high-value coproducts.

Cellana's ALDUO™ large-scale cultivation system used a hybrid system of photobioreactors (PBRs) and open ponds. Each production system consists of six 25,000 L PBRs and three 450 m^2 production ponds. All fluid transfers—including inoculations, nutrient additions, and harvest volumes—were operated and monitored by a remote process-control system.

We conducted strain screening and optimization work in a mid-scale cultivation system, a stand-alone system of 24 PBR and pond simulators, each of 200 L capacity. Cultures in this system are exposed to ambient environmental conditions of light and temperature, each measured continuously; pH is monitored and controlled continuously by CO_2 addition. Enclosed plastic container "bags" are used to scale up from laboratory cultures to production in the large PBRs. For each experiment, pond simulators were inoculated with light-acclimated, exponentially growing bag cultures, and then grown through nutrient depletion as a batch culture. Cellana's experiments have shown that algal strains grow in the miniponds at rates similar to those achieved in large ponds; the mid-scale system models the production system in a cost-effective manner.

Cellana produced biomass from three different strains at large scale, including the high-lipid strains of *Nannochloropsis KA19* and *Pavlova C870* and the low-lipid strain *Tetraselmis KA33*.

Among the three strains produced at large scale, *KA19* was optimized at the mid-scale system for pH, salinity, total nitrogen (TN), and optimal pond cycle. Biomass productivity showed no differences at pH levels ranging from 7.5 to 8.3. However, *KA19* biomass productivity was significantly higher for 15 ppt and 20 ppt ponds, compared to that for the 35 ppt pond (ANOVA, $p < 0.05$). Higher TN, in the range of 1000 μm to 1800 μm, led to higher production of biomass and higher content of protein, but lower content of lipid, carbohydrate, and total fatty acid methyl esters in the biomass. The net gain of biomass was negligible by increasing TN from 1800 μm to 2000 μm for a four-day pond run. The content of total saturated and mono-unsaturated fatty acids was 83% and 70% for treatment with TN at 1000 μm to 2000 μm, respectively. Polyunsaturated fatty acids were slightly higher in treatments with higher TN levels on Day 4 for *KA19*.

We incorporated data obtained from the mid-scale screening and optimization system into the large-scale production when feasible. For example, the *KA19* biomass was produced at TN level of 1800 μm to strike a balance between biomass yield and lipid content in the biomass. We set the maximum pH at 8.3 to avoid unnecessary loss of CO_2. However, we could not achieve a lower salinity level in a large pond because of limited access to fresh water.

For *KA19* under the conditions tested, we found biomass productivity to be highest at Day 3. Biomass productivity then decreased over time as biomass density continued to increase until Day 7-8. Therefore, the optimal harvesting time was Day 3 for maximum biomass productivity and Day 7-8 for maximum biomass yield per harvest per pond. Due to the high energy and capital cost to harvest *KA19* via centrifugation, ponds were harvested on Day 7-8 for higher yield per harvest.

Specific growth rate was negatively correlated with stock density of the culture measured as optical density, which we observed for culture both in open ponds and closed PBRs. We speculate that self-shading as the culture became more concentrated caused this phenomenon. This once again suggested the importance of maintaining the optimal stock density to maximize the performance of *KA19*. To keep the culture at a dilute condition, daily continuous harvesting requires an energy-efficient harvesting strategy with appropriate equipment.

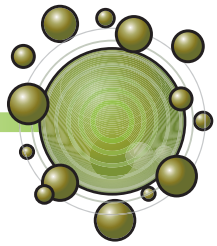
To sum up:

1. Cellana has successfully grown three algal strains utilizing the ALDUO™ technology at large scale, with > 50 kg of *Nannochloropsis oceanica* biomass, 50 kg of *Tetraselmis* sp. biomass, and 4 kg of *Pavlova pinguis* biomass supplied to consortium members.
2. The ALDUO™ technology allowed Cellana to produce these three strains, as well as others not reported on here, continuously. In particular, successful rearing of the challenging strain *C870* suggests the potential of the ALDUO™ technology for expanding the catalogue of algal strains available to the industry.

3. The advantages of ALDUO™ were further verified by the contamination issues encountered after deviating from it by using an open pond only for production.
4. Using Cellana's mid-scale screening and optimization systems, we examined a few factors (salinity, pH, TN, and pond cycle) affecting growth and productivity. We applied the data at a large-scale production system.
5. We obtained highly comparable results between mid-scale and large-scale, including biomass productivity, biochemical composition (including lipid content, fatty acids profile, and eicosapentaenoic acid content), and optimal harvesting time. The results further confirmed that Cellana's mid-scale system serves as a powerful research tool with results transferable across scales.

Energy-efficient dewatering technology will help to increase the yield of nonsettling strains like *Nannochloropsis* while reducing the operational cost.

HARVESTING AND EXTRACTION



Acoustic Harvesting and Extraction

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Realization of a viable algal biocrude industry requires determined development of new technologies that will lead to significant reductions in harvesting and extraction (H&E) costs. Conventional technologies applied to large-scale cultivation scenarios are too energy- or cost-intensive to compete with current crude oil prices. We have determined that acoustic technologies for harvesting algae and extracting and separating biomaterials offer several advantages including the distinct possibility of lowering H & E costs by orders of magnitude. Acoustic technologies have no moving parts to wear or break and require no chemical additives that must be removed from the water. Furthermore, we believe that acoustic harvesters can be designed for placement directly in ponds to avoid transporting vast quantities of dilute algae.

Despite these advantages, significant challenges for acoustic technologies remain in the design and operation of energy-efficient devices and in their scale-up. Acoustic technologies require more research investment and more time for development before lower costs at large scale will be achieved. In this report, the framework for comparison of acoustic and conventional technologies is described and true energy requirements based on the acoustic devices assembled for this project are reported.

The objectives of the acoustic H&E task were to:

1) Measure relevant acoustic properties and determine optimum operating conditions.
2) Assure operational robustness and compatibility with upstream and downstream processes by investigating the use of acoustic fields at laboratory scale for algae concentration, disruption, and lipid extraction and for separating biomaterials from the cultivation water.
3) Provide quantitative technical and economic analysis of scale-up by Month 18 for a technology down-select process.
4) (After a successful down-select at Month 18) develop and demonstrate a prototype device capable of harvesting algae feedstock from cultivation waters at a rate of 100 to 1000 liters per hour, and compare performance of the scaled-up harvester with projected feasibility estimates.

We emphasized harvesting and extraction for the first half and harvesting alone for the last half of the project. We applied physical models to measure properties relevant to system performance and to probe the design space of acoustic harvesters. We measured the properties of the piezoelectric resonators and layered vessels so we could better understand energy delivery to the liquid layer and overall energy efficiency. As a precursor to scale-up, we investigated the factors contributing to harvester operation and performance and we estimated and reported system limitations and requirements.

We assembled laboratory-scale acoustic harvesters, extractors, and separators from off-the-shelf materials and tested them using *Nannochloropsis salina* and *Chlorella protothecoides* (recently renamed *Auxenochlorella protothecoides*). We developed a variety of assays to quantify process performance and determine the quality of biomaterials, including viability and lipid content of individual cells using flow cytometry. The power source was augmented so that we could accurately measure and quantify the true power flowing into each acoustic device. Tests conducted with these laboratory-scale devices demonstrated that algae passing through the harvester were as viable and healthy as the original algae, while postextraction viability was more dependent on species. Only 1% of *N. salina* remained viable following lipid extraction compared to 11% of *A. protothecoides*. Energy costs for the partially optimized processes were 1 to 4 cents per gallon of biocrude for acoustic harvesting (compared to 62 to 300 cents per gallon for centrifugation), 21 cents per gallon of biocrude for acoustic extraction (compared to 92 cents per gallon for solvent extraction), and less than 1 cent per gallon of biocrude for acoustic separation.

Based on these very promising results, we selected the acoustic harvester to undergo scale-up and field testing in the last half of the program. Detailed design of the more than 10-fold scaled-up harvester was achieved using a 1-dimensional layered resonator model and other physical models. We assembled 12 modules into a large-scale acoustic harvester system. We conducted field tests in September and early October 2012 in an outdoor facility at Los Alamos National Laboratory using *N. oculata* provided by Solix Biosystems' Coyote Gulch facility. The energy densities achieved in the scaled modules were 100 times higher than in the laboratory-scale harvester at the same power-density input, and multi-module operation came at a relatively low energy cost. However, performance of the pilot-scale harvester was more limited than our predictions. Energy density is derived from the acoustic standing wave and is directly proportional to the force acting on the algae to achieve concentration. Although these initial measurements indicated that we were achieving much higher energy densities for a given power input, data from the field tests indicated that energy density decayed exponentially in the pilot-scale harvester along with performance. We believe that the control strategy of the system must be modified to be more adaptive to the complex and dynamic phenomena occurring in the pilot-scale harvester before this promising technology can be successfully implemented.

Despite technological unknowns and uncertainties at the onset of this project, the acoustic H&E team achieved several important successes. These include:

- Demonstrating the feasibility of acoustic H&E with respect to favorable energy balance, low capital and operating costs, and effectiveness with multiple types of microalgae.
- Showing that acoustic harvesting had no measureable effect on algae viability and health and that the quality of lipid and protein coproducts remains high after extraction.
- Designing and assembling a pilot-scale acoustic harvester and conducting an outdoor field test. The scaled acoustic harvester system provided a 100-fold improvement in energy efficiency over the laboratory-scale harvester, exceeding expectations.

The following challenges need to be addressed before this promising technology can be successfully implemented in a large-scale acoustic harvester.

- Gain an improved understanding of the factors that affect energy-delivery efficiency from the power source to the resonator.
- Gain an improved understanding of the complex phenomena that affect the operation of a large-scale harvester, including the dynamics of the acoustic properties of algae during the cultivation period.
- Develop an adaptive control strategy to track high-efficiency modes of operation in a complex and dynamic environment.

Novel Membranes for Algae Harvesting by Filtration

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Microalgae harvesting is one critical enabling process for commercialization of algae-based products such as fuels, chemical feedstock, and animal feedstock. The harvesting process has direct impacts on the production cost of algae and the sustainability of the cultivation process. Before the National Alliance for Advanced Biofuels and Bioproducts (NAABB) was started, however, there was little information about membrane-harvesting studies, and harvesting techniques used in the laboratory experiments were not economical for practical industrial application.

Membrane filtration is a physical process based on size exclusion. Thus, porous membranes can be used for harvesting various algae species by tuning the membranes' surface pore size. Membrane filtration has other unique performance attributes, such as little or no addition of foreign chemicals and materials, reuse of the water filtrate, low-energy consumption, and a modular-type unit that is readily scalable. Fresh culture can be concentrated by cross-flow filtration first and the resulting concentrate is further dewatered by membrane belt filtering to produce a wet cake. In cross-flow filtration, as the culture flows over the membrane surface, water is removed from the other side of the membrane sheet under a pressure gradient. Thus, the culture should have reasonable viscosity to be pumped around. A certain cross-flow linear velocity needs to be maintained to avoid accumulation of the algae on the membrane surface. The cross-flow filtration can be used for concentrating dilute algae cultures to slurry by rejecting the bulk water (> 95%). The resulting algae slurry can be further dewatered by using membrane technologies. The membrane belt filter is a commercial process for dewatering the slurry. One example is dewatering gypsum slurry produced from power-plant flue-gas desulfurization processes.

The goal of this project was to develop and demonstrate advanced membrane filtration technology as an energy-efficient and economically viable method for harvesting microalgae from fresh culture solutions. We aimed to:

- Achieve 20- to 100-times concentration (from 0.05 to 0.10 wt% solid to 1 to 5 wt% solid).
- Generate new information and knowledge that is not available in the public domain and that is necessary for substantiating an analysis of process economics.
- Develop new membrane materials and/or structures and demonstrate their significant performance advantages over conventional filtration membranes.

The project work focused on developing novel membranes comprising a metal sheet that is thin and porous, such as porous nickel alloy sheets, and on evaluating representative commercial membrane samples and filters. The key innovation of this project was the development of novel ceramic-modified thin (about 50 μm thick) porous metal sheet membranes with high permeation flux and biofouling resistance. Algae harvesting studies were conducted in this project with *Nannochloropsis salina* and the *DOE1412* strain of *Chlorella* sp., the two species cultivated within NAABB.

One main challenge to an economical harvesting process with membranes is to obtain sustainable, high membrane flux. A number of polymeric and ceramic-modified porous metal membrane samples were evaluated on the laboratory-bench cross-flow filtration testing apparatus. The 50 μm-thick porous nickel (Ni) sheet membrane was found to be so resistant to algae adhesion that a spent membrane surface looked similar to a fresh one. This membrane showed stable and high permeance (> 200 L/bar/m^2/h) for concentration of fresh *N. salina* by 45 times, and its flux was a few times higher than the polymeric membrane sheets evaluated under the same conditions. With aged *N. salina* cultures, however, filtration fluxes of all the membranes tested declined with time within an 8-hour batch testing period. The Ni membrane was regenerated by washing with clear water. We scaled up the Ni sheet membrane preparation so that a number of 12.5 cm x 12.5 cm membrane sheets were produced and cross-flow membrane modules comprising 18 membrane sheets were assembled.

A mobile filtration testing unit was built that could be readily shipped to cultivation sites for testing of new membrane modules. The membrane module was tested with *Chlorella* sp. *DOE1412* at Sequim, Washington, and Pecos, Texas. The module integrity was demonstrated in 10-day runs under various conditions. All the testing runs showed gradual decline of flux with time for this algae species. Average flux and permeance were about 60 to 40 L/m^2/h and 120 to 80 L/m^2/bar/h, respectively, which was about 2 to 3 times the flux of a membrane bioreactor module used for wastewater treatment. The spent Ni sheet membrane looked similar to the fresh one under scanning electron microscope. It appeared that the flux decline was likely caused by adsorption of some soluble carbohydrate species in the culture in the membrane pores. At the start of this project, we expected that all microalgae species would be filtered out using membranes of submicrometer pore sizes. This expectation was confirmed and

the algae cells can be completely blocked using a membrane of uniform pores at an appropriate pore size level, such as 200–700 nm. However, the results suggested that there might not be a universal membrane for all algae species due to variations in the content of soluble matters other than salts and algae cells among different cultures. Those soluble matters can pass through exterior pores of the membrane and gradually accumulate in the membrane pores, which results in decline of filtration flux. Their accumulation would be affected by surface adsorption on the pore surface and porous structures such as tortuosity. Thus, membrane surface chemistry and pore structures may need to be tailored for specific cultures to minimize the pore fouling. In the long term, membrane fouling is likely inevitable and the fouled membrane can be regenerated periodically. Effective regeneration or cleaning methods need to be further developed to achieve sustainable, long-time performance of membrane harvesting devices.

In parallel to the above research efforts, a cross-flow hollow-fiber membrane filter unit was loaned from Pall Corporation and tested at the cultivation site at Pecos, Texas. We performed seven groups of harvesting runs with the cultures cultivated over a period of time from December 2012 to early April 2013. The membrane harvester performed well for concentrations of cultures at solid loading, ranging from 0.3 to 1.8 g/L to 50 to 60 g/L. We observed no measurable algae leakage through the membrane. A higher degree of concentration may be possible, but the pumping system may need improvement. Recognizing that different membrane modules (or products) can operate very differently, testing results of Pall's filter at Pecos represents the state-of-the-art performance.

In conclusion, membrane filtration was shown to be a viable harvesting technique, but further improvements to filtration flux and fouling resistance are desirable to reduce the membrane cost and energy consumption.

Low-cost, Low-energy Methods of Dewatering Microalgal Cultures: A Comparison of Electrolytic Methods

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Microalgae are typically grown to a concentration of 1 to 2 grams of ash-free dry weight (afdw) per liter. Regardless of the technology used to extract the oil from the algae cell or convert to a bio-oil, the microalgae needs to be concentrated by two or more orders of magnitude prior to entering the next step in the process. Dewatering can prove to be a large portion of the production cost as a large amount of water is removed to increase concentration by one order of magnitude. For example, going from 0.1%–1% afdw requires that 900 mL/L of water be removed while retaining the solids in the remaining 100 mL of solution. Thus there is a need for a low-cost, low-energy method of dewatering microalgae solutions. Many options are available for dewatering a dilute culture of microalgae. One of the more interesting and promising techniques relies on electrophoresis effects, the interaction between microalgae surface charge and electrolytic processes. These processes require small amounts of energy and have the potential to scale up to commercial volumes. The goal of this research is to

evaluate three electrophoresis techniques for biomass recovery and energy consumption in harvesting microalgae from the growth media.

We examined two electrolytic methods in the laboratory for their efficiency of concentrating NAABB species of microalgae from dilute solutions: electrolytic coagulation and electrolytic flocculation. Electrolytic coagulation uses reactive metallic electrodes to produce positively charged ions that induce coagulation of the negatively charged microalgae cells. This removes the algae cells from the solution. Electro-flocculation utilizes inert electrodes and moves negatively charged algal cells to the positively charged anode. No ions are released into the solution. Once the cells reach the anode, the negative charge is dropped from the microalgae and they are able to form flocs, which settle and can be recovered.

Our commercial partner in this research was Kaselco, based in Shiner, Texas. Kaselco donated significant portions of the rental costs and technical support for the field tests, has off-the-shelf equipment in the range desired, and was familiar with research in the area. The Kaselco process is an electrocoagulation (EC) system available in modules that range from 2.5 gpm to 1200 gpm.

Both of the electrolytic harvesting methods tested have the potential for scale-up to commercial volumes. The goal of this project was to demonstrate performance of electrocoagulation and electro-flocculation at laboratory scale (100 mL to 2.2 L) and at pilot scale (100 L/h to 1000 L/h).

The objectives of this research were to (1) demonstrate the efficacy of electrocoagulation as a harvesting method for algae at lab and pilot scale and (2) demonstrate the efficacy of electro-flocculation as a harvesting method for algae at lab and pilot scale. We performed a series of experiments at both lab and pilot scale to address these objectives.

We found electrocoagulation to offer reduced capital and operating costs compared to harvesting with a centrifuge. Operating cost for electrocoagulation was approximately 5% of the operating cost of the centrifuge. We obtained the greatest recovery of algae with no pretreatment processes. Aluminum was the preferred electrode based on laboratory studies yielding the greatest recovery at the least amount of energy input. Additionally, mineral tolerances in cattle feed for aluminum were higher (2x to 10x) compared with other electrode materials and aluminum had cost advantages over nickel.

We performed two field tests of electrocoagulation at pilot scale during 2011 and 2012 at the Texas A&M AgriLife Research Experiment Station in Pecos, Texas. The goal of these tests was to evaluate a commercial electrocoagulation system for application to harvesting algae for biofuels production. Demonstrations during 2011 determined that the equipment, originally intended for wastewater treatment and much lower concentrations of contaminants, could effectively remove algae from a dilute solution (approximately 0.1% total volatile solids). Lipid-bearing algae could be harvested with equal facility as non-lipid–bearing algae, but algae concentration was not as great as noted in the laboratory, with the final concentration of the sediment at 3–4% afdw versus 6–8% afdw seen in

the lab. Consequently, a second stage of dewatering may be required at commercial scale depending on the lipid extraction method employed.

Stainless-steel electrodes were determined to perform better during pilot scale electrocoagulation experiments versus the aluminum electrodes used during the first year and found to be the best in the laboratory studies. However, separation with either electrode during the second year of pilot studies was not as good as in the first year. We attribute this to differences in the water source for the algae media, as all other factors were identical. A significant amount of sediment was generated from the Pecos well water during electrocoagulation and we thought it contributed to the high ash content noted in harvested algae biomass. Lastly, an electrocoagulation system was operated at a much higher flow rate (15 gpm) and a complete 4550 g batch of algae was harvested.

The electro-flocculation experiments at lab scale proved the technology to be viable and on a par with electrocoagulation in terms of energy consumption and biomass recovery. The performance curves for the range of culture densities did not vary significantly. The lack of variation may offer opportunities for flexibility in design of the harvesting system as a whole when considering growth/retention time of an algal culture. An advantage of using electro-flocculation technology was that no electrode material was consumed and thus no mineral was deposited to the biomass. Electro-flocculation was not successfully implemented in the field because the voltage rectifier did not have sufficient controls to deliver the low levels of current necessary to operate in that mode.

Electrocoagulation and electro-flocculation appear to offer significant advantages over centrifuge harvesting, particularly with regard to reduced energy costs and the opportunity to automate and reduce labor costs. Centrifuges, even automated ones, require a trained operator attending them, whereas the electrocoagulation equipment was designed to be fully automated in typical wastewater treatment facilities. There is a need for additional understanding of the interaction of water chemistry with the electrolytic processes to better explain the observations during the second-year field studies. There is also an opportunity to implement a field-scale electro-flocculation system if a more precisely controlled rectifier were available. Electro-flocculation eliminates ion residue in the harvested biomass and would be a preferred method since the amount of separation and energy consumption were on par with electrocoagulation.

Sequestration of High-value Algal Produced Molecules

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Acquiring all possible value out of microalgal oil is imperative to make it an economically viable feedstock for biofuel. Current technologies utilize food-based oils as the feedstock for base-catalyzed conversion of triacylglycerides (TAGs) to fatty acid methyl esters (FAMEs or biodiesel). These feedstocks are ideal because they contain > 99% TAGs. However, microalgal oil contains a greater variety of lipid and hydrocarbon molecules, some of which are considered high-value or value-added. As such, these biomolecules would contribute to the economics of

the microalgal biofuel production if they could be separated from fuel precursors and distributed to the optimal industry (i.e., the industry that gives the naturally produced molecule of interest the greatest value). Current separation and purification techniques for naturally produced lipids, hydrocarbons, and organic acids are challenging and energy-intensive, with extraction and distillation using organic solvents or supercritical fluids the most common techniques. We developed a high-surface-area, porous silica-based nanoparticle material for the selective sequestration and removal of these high-value and value-added molecules from microalgal oil.

Utilizing our strong synthetic background along with the ability to control the functionalization of these materials, we have synthesized and characterized a series of nanomaterials that independently target free fatty acids (FFAs), polyunsaturated free fatty acids (PUFAs), tocopherol (vitamin E), and microalgal cells from growth media as a magnetic dewatering technology. We found that pores with an average diameter of 10 nm were optimal to capture FFAs when compared to nanomaterials with smaller pore sizes. Decorating the pore surface with primary amine functional groups selectively targeted FFAs, restricting other molecules normally found in microalgal oil from entering the pores and adsorbing to these porous nanomaterials. By rinsing these nanomaterials with organic solvents we were able to desorb the FFAs, leaving a solution of highly pure, naturally produced FFAs. Following this discovery, we analyzed the purified FFAs to determine if targeting and removing the high-value FFAs (Omega-3/-6) is possible by adjusting some of the properties of the sequestration materials. Through extensive experimentation, we determined that smaller pore sizes capture PUFAs selectively while limiting the diffusion of saturated and mono-unsaturated FFAs into the pores. We attribute this to the folding of these organic acids in the microalgal oil. Because all double bonds in naturally produced fatty acids are *cis*, PUFAs will fold into tighter arrangements making the size of Omega-3/-6 the smallest among the different saturations.

Tocopherol is naturally produced by many microalgae strains in concentrations up to 5 wt% lipids. Naturally synthesized tocopherol has considerable advantages over industrially synthesized including higher nutritional value and improved environmental means. By functionalizing the pore surface with a unique organic moiety that reacts noncovalently with functionality on the tocopherol molecules we were able to synthesize and characterize a sequestration nanomaterial that selectively targeted vitamin E. We demonstrated that over 70% of the tocopherol in a simulated microalgal oil solution could be captured and removed while only capturing 15% of the FFAs and 4% of the terpenes in the same solution. An advancement that we made in this technology was to add magnetic functionality to these materials. This allowed us to separate the sequestration nanomaterials, after the tocopherol molecules were captured, from the microalgal oil by simply utilizing a magnet. We demonstrated, for the first time, that separation of these magnetic, high-surface-area porous nanomaterials is possible by including magnetic functionality to the synthesis.

Finally, we showed that these nanomaterials can be synthesized in a way that they can be utilized in a biorefinery fashion. To be specific, we synthesized and characterized a nanomaterial that had iron oxide and primary amine

functionality that was used to dewater microalgae cells from growth media, followed by extraction of the internal molecules by ionic liquids, which were captured and sequestered in the internal pore structure of the nanomaterials. First, these nanomaterials were placed in a suspension of viable microalgae cells and, after a certain incubation period, the cells and nanomaterials were removed from the suspension by simply placing a magnet on the side of the growth vessel. Utilizing another magnet, the cell-nanomaterial agglomerations were physically removed from the aqueous media and placed in ionic liquids. At room temperature, the hydrolyzing action of the ionic liquid lysed the cell walls; the lipids and hydrocarbons of interest were released from the interior of the cells. Using immiscible organic solvents, the lipids and hydrocarbons transferred to the top layer where the FFAs were captured in the interior pore framework of the nanomaterials. Desorption of the FFAs occurred upon solvent wash of the nanomaterials.

These nanotechnologies not only offer a new method to target and remove molecules of interest from the alphabet soup of molecules that are produced by microalgae, but will likely open the minds of researchers to other applications for these materials. The relative ease and speed at which these materials can be specifically functionalized along with the biocompatibility and nontoxic nature of these materials make them ideal platforms for exploration in biorefinery and biofuel research.

A Platform for Fuel-grade Products from Algae Using Wet Fractionation

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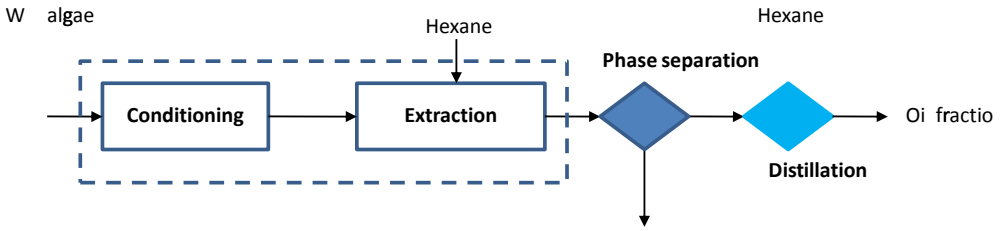
Whereas more traditional processes require algal cell drying and cell lysis, Valicor Renewables' AlgaFrac™ process is a wet extraction platform. Input algae biomass of 10–20% solids content can be rapidly processed into lipids, residual biomass, and aqueous streams. Valicor Renewables wet extraction platform (Figure 1) is cost- and energy-effective, recovering over 80% of the net available energy.



Valicor's goal was to provide toll processing of wet algal biomass extraction to satisfy this need. To this end, Valicor conducted a series of bench-scale extraction tests to optimize oil yields for various algae strains and harvests. Findings from bench-scale fractionation were used to define optimal conditions for toll processing of large quantities of algae. We processed NAABB-generated algal biomass in our pilot plant to produce requisite quantities of oil and LEA fractions. We also dried the LEA when necessary in our pilot plant for distribution to NAABB team members. We supplied the oil and LEA fractions to downstream partners for their tasks.

The species processed are listed below:

- *Nannochloropsis salina*
- *Nannochloropsis* sp.
- *Nannochloris oculata*
- *Desmid* sp.
- *Chlorella* sp.

These alga strains were extracted at the bench and pilot scale. We obtained good mass balance closure and consistent oil yields, indicating robustness of the Valicor Renewables process. A typical mass balance is shown below (Figure 2).

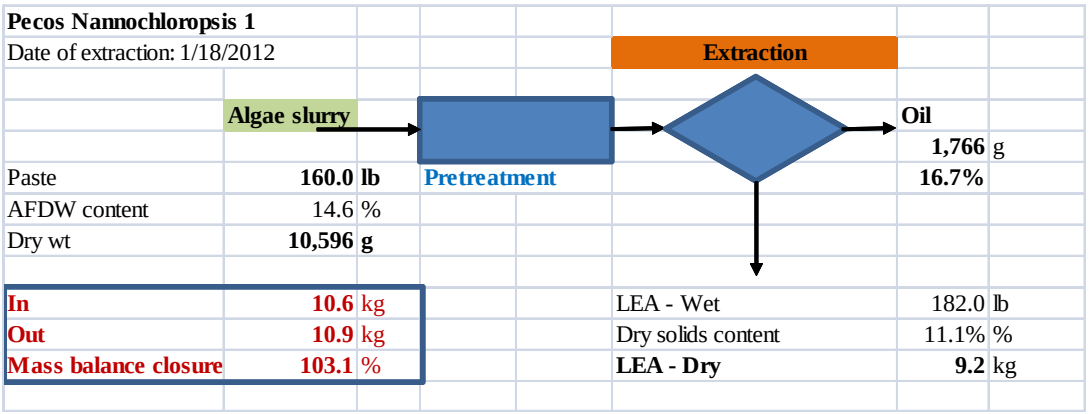


Figure 2. Mass balance for Pecos Nannochloropsis *extraction.*

A pilot-scale dryer was installed at the Dexter facility for the express purpose of LEA drying. Figure 3 depicts the two different ways dry LEA was produced: (1) dry LEA with dissolved sugars, etc., and (2) dry LEA after dewatering. Several kilograms of LEA from various extractions were provided to NAABB team members.

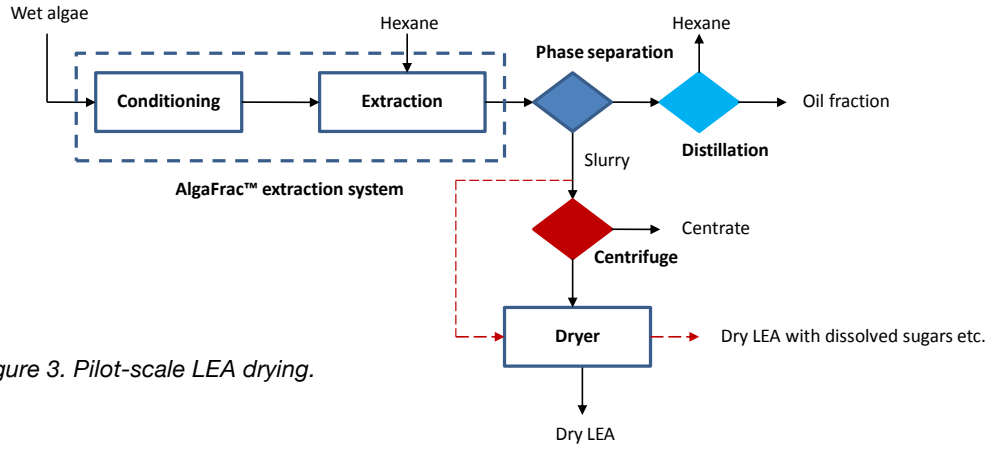
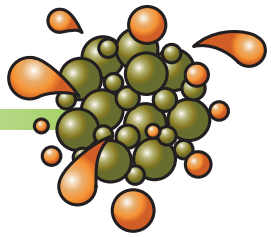


Figure 3. Pilot-scale LEA drying.

In conclusion, Valicor Renewables' extraction technology—AlgaFrac™—was successfully applied to various NAABB algae species. Large quantities of oil and LEA—both dry LEA with dissolved sugars, etc., and dry LEA after dewatering—were provided to NAABB team members. The process is scalable with no apparent showstoppers.



Analysis of Algal Oils for Contaminants: Metals, Chlorine, Nitrogen, Sulfur, and Acidity

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The major goal of this task was to gain information on the quality of algal oils that were produced by extraction of algal biomass by National Alliance for Advanced Biofuels and Bioproducts (NAABB) consortium members using a variety of extraction procedures and methods. The emphasis was to measure major properties and contaminant levels that might impact the subsequent conversion of these algal oils to hydrocarbon fuels. The goal was to obtain as many samples as possible from different NAABB members and analyze these oils for key parameters: total acid number, metals, chloride, nitrogen, sulfur, and phosphorous. These contaminants can have a number of deleterious effects on subsequent processing steps when converting oils to fuels, such as catalyst poisoning or corrosion within process equipment and vessels.

Chloride can lead to stress corrosion cracking in reactors operating at the high temperatures and pressures required for the conversion of algal oils to hydrocarbons by hydrotreatment. Nitrogen is a more difficult hetero-atom to remove from a hydrocarbon backbone than oxygen; therefore, significantly more severe and thus costly hydrotreatment conditions are required to remove nitrogen from algal oils. Nitrogen sulfur and oxygen must be removed from the algal oils for the final fuel products to meet the specifications for contaminant levels. Both the ASTM D7566 specification and the Department of Defense MIL-DTL-83133H specification for renewable jet fuels specify nitrogen in the fuel at ≤ 2 ppm and sulfur at ≤ 15 ppm. These same specifications require individual metal contaminants to be ≤ 0.1 ppm per metal. Alkali and alkaline earth metals can neutralize active sites of the catalysts and also poison catalysts by blocking or narrowing the pores on the catalyst surface, hindering the transport of reactants and products to the active sites of the catalyst. Phosphorous can also lead to formation of metal phosphates, interfering with the catalytic capability of these metals on the catalyst surface.

The amount of contamination within the oils provided by NAABB members varied widely. Highly refined triglyceride oils and distilled fatty acid methyl ester (FAME) oils were suitable for direct conversion to hydrocarbon fuels without any pretreatment, but most oils received were crude oil extracts with high levels of inorganic contamination that require significant pretreatment to be successfully converted to jet or diesel fuels. The refined triglyceride oils and distilled FAMEs were characterized by having very low acid numbers, very low metals, and very low nitrogen and sulfur levels. The crude oils, on the other hand, had high concentrations of these contaminants. The oil with the highest level of contamination was the hydrothermally liquefied algal bio-oil. Since this was

liquefied whole biomass and it was not extracted oil, much of the intracellular and cellular bound metals and nitrogen-containing compounds were present in this crude oil.

Therefore, refining of crude algal oils will be a key step in the commercialization of algal oil feedstocks for renewable jet and renewable diesel production.

Characterization of Physical and Chemical Properties of Algal Esters and Biofuels

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Microalgae lipids, lipid-extracted algae (LEA), and whole algal biomass can be readily converted into a variety of biofuels including fatty acid methyl esters (i.e., biodiesel) via transesterification, alkanes, or alcohols. In contrast to paraffinic fuels whose properties can be tailored for a specific application, the properties of algal methyl ester biodiesel are directly related to the fatty acid composition of the algal lipids. Several microalgae species that are suitable for large-scale cultivation, such as those in the genus *Nannochloropsis*, produce lipids that contain long-chain polyunsaturated fatty acids (LC-PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These constituents have high value as coproducts but are problematic in biodiesel properties such as ignition quality, oxidative stability, and other fuel properties.

A major objective of this project was to examine the effect of varying levels of EPA and DHA on algal methyl ester fuel properties. We measured oxidative stability, cetane number, density, viscosity, bulk modulus, cloud point, and cold filter plugging point for algal methyl esters produced from various microalgae feedstocks. We also measured these characteristics for model algal methyl ester compounds formulated to match the fatty acid composition of *Nannochloropsis* sp., *Nannochloropsis oculata*, and *Isochrysis galbana* that had been subjected to varying levels of removal of EPA and DHA.

The experimental results showed that the oxidative stability induction period varies exponentially with the bisallylic position equivalents (BAPE) value of the algal methyl esters. The results of this work also suggest that approximately 50% of the EPA and DHA would need to be removed from the *N. oculata* methyl ester to meet the 3-hour ASTM oxidative stability specification and approximately 80% would need to be removed to meet the EN 6-hour specification. For *I. galbana*, removal of 100% of the EPA and DHA was insufficient for passing either the ASTM or EN oxidative stability requirements. We also found the effect of EPA and DHA on ignition quality was substantial. Specifically, we found that nearly 100% of the EPA and DHA must be removed from the algal methyl esters considered herein to comply with the ASTM D6571 standard for a minimum cetane number of 47 for biodiesel. Other fuel properties such as viscosity and density were well within specifications for all the algal methyl ester formulations tested herein.

We found that a fuel additive containing tert-Butylhydroquinone (TBHQ) was effective in increasing the oxidative stability of the algal methyl ester model compounds. Specifically, we found that adding only 0.03% of TBHQ is sufficient for *Nannochloropsis* sp. methyl esters to pass the ASTM specification even if no EPA or DHA were removed. To pass the EN specification, 0.06% of TBHQ must be added to *Nannochloropsis* sp. methyl ester if no EPA or DHA were to be removed.

In addition to the model algal methyl ester compounds, we also tested three real algal methyl esters to determine their oxidative stability, ignition quality, viscosity, density, speed of sound, and cold flow properties. All three of the real algal methyl esters performed consistently with the model algal methyl ester formulation results. An algal methyl ester provided by Eldorado Biofuels containing no EPA or DHA passed both the ASTM and EN standards for oxidative stability without the addition of any fuel additives. Conversely, an algal methyl ester sample provided by Inventure, which consisted of nearly 50% DHA, exhibited very poor oxidative stability. A Solix BioSystems *Nannochloropsis salina* methyl ester had an oxidative stability induction period that passed the ASTM standard, but did not pass the EN standard.

We also found that the cloud point and cold filter plugging point were sensitive to the level of EPA and DHA present in the algal methyl esters since the balance of the fatty acid profile for algal methyl esters consists of a very high percentage of fully saturated methyl esters. After removal of all of the EPA and DHA from the algal methyl esters considered herein, the remaining product could contain up to 50% saturated methyl esters, which is similar to the saturated content of beef tallow methyl ester, a fuel that is known to have poor cold flow properties.

Ultrahigh Resolution FT-ICR Mass Spectral Analysis of Microalgal Lipid Feedstock, Biofuels, and HTL Bio-oils

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We applied direct-infusion Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometry (MS) for comprehensive characterization of microalgal lipid extracts, produced ester fuel, and hydrothermal liquefaction products. High mass accuracy and mass resolving power enable unambiguous determination of elemental composition for up to several thousand individual compounds present in typical algal lipid extracts and approximately 10,000 individual compounds in hydrothermal liquefaction (HTL) bio-oils. These data highlight trends in compound class distribution as well as abundance change for individual compounds to enable comparison of lipid extraction techniques, algal species, and/or growth conditions. Similarly, FT-ICR/MS analysis of produced microalgal fuels facilitates observation of compounds that are not detected by typical gas chromatographic (GC) fatty acid methyl ester analysis and describes conversion efficiency for individual lipids and lipid classes.

With this approach, we have described the neutral, polar, and membrane lipid components for several microalgal biofuel candidate strains. Polar lipids in particular are an important component of these mixtures because they contain both acyl-hydrocarbons, which may be converted to biofuel, and hetero-atom functionalities, which often present challenges to conversion process optimization. Additionally, FT-ICR/MS analysis has shown that most algal strains, regardless of production mechanism, do not show lipid-class–specific accumulation of the economically valuable polyunsaturated fatty acids (PUFA) to a degree that would facilitate selective harvest of those materials. One early hypothesis was that certain lipid classes would preferentially contain PUFA moieties and would therefore represent an opportunity for selective extraction of these valuable coproducts. Except for a few instances that involve lipid classes of low overall abundance, this was not the case.

We have identified the distribution of acyl unsaturation and carbon chain lengths for betaine lipids, sulfoquinovosyl diacylglycerols (SQDGs), glycerolipids, and sterol lipids, among many other lipid classes for several candidate strains. We have also identified a novel class of sulfate lipids in several marine species and have recently reported those findings. We have recently completed data collection and analysis for a full-growth time-course of *Nannochloropsis salina CCMP1776* through the transition from growth to stationary phase. There we observe lipid remodeling with preservation of bound fatty acyl content and *de novo* synthesis of monounsaturated and saturated fatty acids to generate energy storage lipids. We also observe increased PUFA content for some membrane lipid classes compared to storage/neutral lipids and find evidence that betaine lipids serve as analogs to phosphatidylcholine in the Kennedy pathway for lipid biosynthesis for photobioreactor-grown *N. salina*. This effort includes lipidomic (FT-ICR/MS), metabolomic (GC-MS), and proteomic (MS) components and is in the final stage of manuscript preparation.

We note that at this point in the early development of the algal biofuel industry no ester fuel conversion/catalyst regime has been well proven. Our FT-ICR/MS analysis of converted algal feedstock (base-catalyzed conversion, which has been the most commonly pursued conversion approach among our industrial collaborators) shows that SQDG and other sulfur-containing lipids do not convert efficiently. Additionally, acyl sterol glucosides are converted to sterol glucosides and the resultant fuel can contain a significant quantity of these compounds as well as sterol esters. Also, we observe residual C_{40} carotenoids in biofuel mixtures that are produced from acylated and glycosylated forms of those molecules. Residual unconverted lipids, free sterols, and peroxidation of methyl esters are primary fuel stability/performance challenges that would need to be addressed for scale-up of algal biodiesel production.

Alternatively, the generation of bio-oils by hydrothermal processes offers yield advantages over the lipid-to-biodiesel approach; the hydrocarbon products may be acceptable for direct input to traditional refinery operations if hydrotreatment or other upgrading processes can mitigate the abundant hetero-atom–containing molecules, particularly nitrogenous compounds. With both positive- and negative-ion mode FT-ICR/MS, we identify over 9500 elemental compositions in HTL oil produced from *N. salina*. Elemental compositions are sorted to illustrate broad

distributions of aromaticity and alkylation for a multitude of chemical classes based on observation of individual constituent molecules. Initial positive-ion mode FT-ICR/MS analysis of *N. salina* HTL material indicates generally high molecular nitrogen content observed as overlapping distributions of N_1–N_5 compounds and corresponding versions of those molecules with incorporation of 1 to 4 oxygen atoms. Few sulfur or phosphorous compounds are observed. The negative ion mode FT-ICR mass spectrum shows high quantities of free fatty acids (FFAs), di-acids, oxidized free fatty acids, long-chain saturated FFAs, and N_nO_o compounds. The strength of this qualitative approach lies in comparative analysis of process-related samples. We apply such comparisons to a variety of sample types to enable thorough evaluation of process chemistry and evaluation of upgrading options.

We have provided FT-ICR/MS analyses to numerous NAABB partner groups including Nirmala Khandan at New Mexico State University (evaluation of various cultivation-system configurations and CO_2 amendment), Juergen Polle at Brooklyn College (evaluation of lipid profiles for novel strains), Quingdao Institute (variable lipidomics for *Nannochloropsis OZ-1* growth time course for variable light cultures), New Mexico State University PBR effort (growth and nutrient amendment optimization and lipid profiling), and feedstock analysis for NAABB partners Cellana, Pacific Northwest National Laboratory, Terrabon, etc. We have also acted as the primary provider for lipid content/FAME analyses for the fuel conversion and oil supply team (numerous samples from Pecos facility, SRS, Cellana, Albemarle, etc.). Through those efforts, we have described the lipidome for several biofuel candidate microalgae including *N. salina*, *Scenedesmus obliquus,* and *Chlorella sorokiniana*. We have provided lipidomic analysis for the *DOE101* strain as well; however, an incomplete sample set has limited the interpretation of that data. We have provided compositional analysis of direct conversion biodiesel products of *N. salina*, distilled/processed biofuels, and supercritical fluid-extracted lipids.

Understanding the Relationships Between Algal Cultivation Strategies and Downstream Processing of Fatty Acids

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This subtask has two parts. The first is physical chemical property estimation by performing boiling point temperature curve experiments and obtaining fatty acid profile information to provide input for Aspen Plus simulation models. The second is determining the relationships between pond productivity, nutrients, extraction, and downstream processing. This characterization information is critical for two reasons: (1) process simulation models available prior to NAABB used surrogate chemical physical property data for soy oil or other vegetable oils instead of algal oil, hence the model output was less accurate, and (2) a systems approach that integrates biology, cultivation, and processing is required to make significant advances in the large-scale production of biofuels and bioproducts using algal biomass and therefore to decrease cost and enable a sustainable industry.

We characterized boiling-point temperature curves using Solix oil as well as the refined oil from Eldorado. UOP completed more -detailed characterization of all oils and their distillation curves are available to NAABB. We obtained lipid profiles for *Nannochloropsis gaditana* as a function of nitrogen source, growth phase, and water quality; *Nannochloropsis salina* as a function of nutrient source, growth phase, temperature, reactor type, water quality, water recycle strategy, extraction method, and drying method; *Chlorella protothecoides UTEX250* as a function of media composition; and *Chlorella* sp. *DOE1412* as a function of media composition and reactor strategy. We also determined the relative proportions of carbohydrate, protein, and lipid.

As the NAABB project progressed, understanding the links between cultivation and conversion became more important. In particular, the lipid profile is directly linked to the energy required to convert algal oil to biodiesel or jet fuel with lower chained fatty acids being more ideal (C10 to C18 range). The media (nutrients, water source, and metals) used and the cultivation method are directly linked to the amount of ash in the biomass. The ash content affects all processes from dewatering to extraction to conversion. The ash composition affects catalyst behavior as well as the nutritional content if the biomass or lipid-extracted biomass is to be used as feed. The following relationships were found.

N. gaditana

- Nitrate is the preferred nitrogen source to increase lipid productivity; however, small amounts of urea can be used to increase total biomass without decreasing lipid productivity.
- Lipid profile is primarily C16 and C18 compounds that are easily converted to jet fuel and biodiesel.
- The lipid profile does not significantly change as a function of growth phase.

N. salina

- Nitrate is the preferred nitrogen source and lipid productivity increases significantly under nitrogen starvation conditions.
- Lipid productivity in the laboratory can be as high 50%; however, in the field the maximum is 20% in traditional raceways and the Aquaculture Raceway Integrated Design (ARID) system.
- Temperature affects the lipid profile: lower temperature (15°C) results in a higher amount of C20 compounds that are better for health purposes like omega compounds but harder to convert to fuels; mid to higher temperatures (25°C to 30°C) result in higher amounts of C16 and C18 compounds. The temperature range for cultivation is more limited; low temperatures result in very low growth rates and cell death occurs about 32°C.
- Reactor strategy affects lipid profile, especially during winter cultivation. For example, cultivation in traditional raceways that are subject to low ambient temperatures (10°C) leads to more C20 compounds compared to cultivation in laboratory flasks and the ARID system.

- As water is reused, high biomass productivity can be maintained for up to 9 cycles of reusing 90% of the water or spent media. As the spent media are recycled, nitrate concentration increases after 4 cycles and the type of organic material in the water becomes more humic in nature.
- Cultivation of this species is highly resistant to metals and hence it is very feasible to cultivate this species in wastewater.
- Laboratory-scale extraction of the lipids is affected by solvent, drying method, and apparatus (microwave versus Soxhlet versus test tube). The most efficient and reproducible method is to dry in an oven and extract using a microwave.

C. protothecoides UTEX250

- Autotrophic growth was optimized as a function of nutrients on the laboratory scale.
- This strain is not feasible for open pond cultivation; it dies rapidly.

Chlorella* sp. *DOE1412

- Lipid profile contains mostly C16–C18 compounds, but many double bonds.
- Strain grows well on low-cost media ($0.50/1000 L) with low ash content in PBRs (5%).
- Strain grows well over a large temperature range (5°C through 40°C).
- Lipid and biomass productivity in ARID to be determined.

Catalytic Processing and Conversion of Algae-derived Oil

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This report summarizes the findings from the Catalytic Processing and Conversion of Algal Lipids to Fuels project that we carried out under the Fuel Conversion subtask of the NAABB consortium. The project began with the goal of performing high-throughput catalyst screening to discover catalysts capable of converting algal-derived lipids to hydrocarbons. The preferred catalyst would remove oxygen from the lipids through the formation of CO or CO_2. Thus, long-chain hydrocarbons suitable as a direct fuel blend stock or refinery feedstock could be formed without the need for hydrogen as a coreactant to reduce the oxygen content present in the lipids.

Several tests focused on algal-derived lipids extracted from an unidentified alga by Inventure, Inc. Based on the nature of the extraction performed by Inventure, the lipid extract was received as a mixture of fatty acid methyl esters (FAMEs). High-throughput catalyst screening was initiated using the lipid as feedstock but challenges were encountered immediately. The oil had a significant concentration of entrained solids, which could be removed by centrifuging. However, the decanted oil still contained high concentrations of inorganic

materials such as sodium (Na) and sulfur (S). Na and S are known to poison the state-of-the-art precious metal deoxygenation catalysts that were being screened. To remove the inorganic impurities, we investigated purification of the algal-derived lipids through treatment with Grace Davison Trisyl Silica®. The Trisyl Silica® is used commercially for the removal of phospholipids from edible oils. Processing with the Trisyl Silica® was useful for removing essentially all of the Na and about 30–50% of the S. However, about 50–60 ppm of S was likely in a form not readily absorbed by the Trisyl Silica® and remained in the oil.

Next, we fractionally distilled a sample of the centrifuge- and silica-treated Inventure lipid-derived FAMEs. We analyzed a sample collected when vapor temperatures were between 194°C and 232°C at 14 torr via gas chromatography using flame ionization detection (GC-FID). The fractionally distilled sample primarily consisted of methyl linolenate (C_{18}:3) and methyl docosoahexaenoate (C_{22}:6) FAMEs. We chose this fraction as a feedstock for high-throughput catalyst deoxygenation screening as a "worst-case scenario" feed because previous experience revealed deoxygenation catalysts are less active with unsaturated feedstocks. Of the 17 catalysts screened for deoxygenation activity, none produced more than 15 wt% deoxygenated alkanes after 4 hours at 300°C. Furthermore, a significant portion of the products could not be identified by GC-FID. We hypothesized the unaccounted products were high-molecular-weight material formed by bimolecular reaction of two FAMEs along points of unsaturation on the acyl chain. The high molecular-weight-material was likely not volatile enough for analysis on the GC-FID and was therefore not catalogued as a product. While none of the catalysts tested had significant activity, two catalysts did have slightly higher activity than the other catalysts. The second-best catalyst was chosen due to the availability of the catalyst on an engineered (e.g., tableted) support for scale-up testing in a laboratory-scale (20 cm^3) fixed-bed reactor.

Laboratory fixed-bed reactor testing commenced with silica treatment and distillation of the Inventure-extracted lipids. We collected a single fraction of distilled FAMEs that consisted of 61 wt% of the original mixture. We subjected the distilled lipid to continuous flow testing with the catalyst identified during high-throughput screening. We observed low yields of 0.25–0.30 g alkane/g feed at 300°C and 80 psig with a 5 SCCM nitrogen purge. Changing the purge from nitrogen to hydrogen resulted in a slight increase in the yield of alkanes to about 0.35 g alkane/g feed. Analysis of the alkane product distribution suggested that the increase in yield was due to saturation of the C_{22}:6 FAME as the yield of C_{21} alkane increased. Regardless of purge gas, about 55–60% of the products were not identified via GC-FID. The likely culprit for the low carbon balance was again thought to be bimolecular reaction of the FAMEs at points of unsaturation on the acyl chains, which produced high-boiling-point (i.e., non-GC-able) material.

The challenges associated with the inorganic impurities and the unsaturated nature of the algal-derived lipids shifted attention from relatively sensitive precious metal catalysts toward more robust deoxygenation processes. We investigated hydrotreating catalysts, which are used to upgrade bio-oil. In contrast to earlier studies, we employed excess H_2 at high pressure (1000-1500 psig) to saturate acyl chains and reduce oxygen present. Testing commenced with the Inventure-extracted lipids. However, to achieve high yields

and to challenge the catalyst, the oil was only decanted off from settled solids but was otherwise untreated. We performed parametric studies with two hydrotreating catalysts: a base metal catalyst and a sulfided CoMo catalyst. At 350°C and 1500 psig, both catalysts produced a significantly upgraded product. The sulfided CoMo catalyst did appear to have slightly higher activity as no unconverted FAME was identified. Analysis of the liquid products suggested that the mechanism of deoxygenation differed for each catalyst. The base-metal catalyst produced primarily odd-numbered hydrocarbons, which suggested a deoxygenation mechanism whereby CO or CO_2 was ejected from the molecule followed by reduction of the CO_x to methane. In contrast, the sulfided CoMo catalyst produced primarily even numbered alkanes, suggesting the oxygen present in the molecules was reduced to water. Interestingly, the extra H_2 required for the reduction of the CO_x with the base-metal catalyst increased the total hydrogen consumption to a greater level than the stoichiometric amount required to reduce the oxygen with the CoMo catalyst to water. Distillation and analysis of the liquid products revealed that both catalysts produced products with about 55 wt% of material in the jet and/or diesel range.

Hydrotreating tests with a sample of lipid extracted by Valicor Renewables from a Cellana-grown *Nannochloropsis salina* alga were less successful than the Inventure-extracted lipid upgrading. We selected the sulfided CoMo catalyst from the earlier test due to the relatively high activity and low H_2 consumption compared to the base metal catalyst. Unfortunately, the thermal instability of the Valicor-extracted lipid caused the reactor to plug after only a few hours on stream. We collected one sample at quasi-steady state. The single collected sample was promising as the upgrading consumed only 0.023 g H_2/g feed. Furthermore, about 85 wt% of the upgraded lipid distilled in the same range as diesel fuel. Future testing with the Valicor extracted lipid is warranted but a reactor configuration similar to that used for fast pyrolysis bio-oil upgrading should be employed. Fast pyrolysis bio-oil must first be stabilized at a lower temperature prior to higher-temperature hydrotreating to avoid reactor plugging.

We used data produced from upgrading the Inventure-extracted lipid via hydrotreating with the sulfided CoMo catalyst to produce a techno-economic model that estimated the cost of converting the lipid to finished fuel. At a scale of 1 billion gallons/year, the cost of upgrading the lipid to finished diesel fuel was determined to be $0.53/gallon when the cost of the lipid was not taken into account. The cost of the lipid was the largest variable in the model; no true market estimate of lipid cost could be determined. However, when we considered the NAABB target of $2.00/gal as the price for the lipid, the techno-economic analysis suggested that diesel fuel could be produced for $2.61/gal, which would be cost competitive with fuel at current market prices.

Conversion of Algal Triacylglycerides to Carbon Chain Extended Hydrocarbons Using the Kolbe Electrolysis Reaction

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The efficient conversion of algal lipids into high-energy-density fuels is of interest to the aviation industry and the U.S. Department of Defense. To convert these lipids for specified uses the chemical and engineering problem is two-fold: (1) removing the oxygen atoms from the oil and (2) reforming the resulting high-molecular-weight hydrocarbon to a lower-molecular-weight hydrocarbon (increased carbon branching, which lowers the operating temperatures of the fuels) and to some aromatic structures. From this second step must come a distillable product having properties indistinguishable from JP-8 jet fuel and other high-energy-density fuels.

Below we outline our routes to a method that should give rise to hydrocarbons that can feed directly into existing infrastructure for the production of high-energy-density fuels (Figure 1).

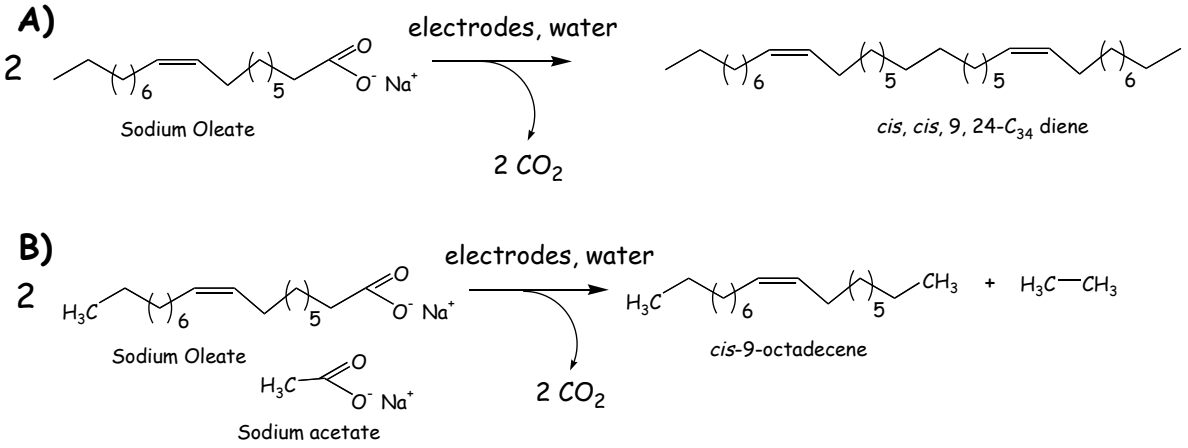

Figure 1. The Kolbe Reaction.

Kolbe electrosynthesis

The Kolbe Reaction, first described in 1849, was developed at the very dawn of organic chemistry. A simplified description is that a low voltage is applied to a water solution of a carboxylate salt, carbon dioxide is evolved, and two of the resulting organic radicals couple. Thus, the oxygens are removed (as CO_2) and a much longer hydrocarbon chain is produced. Other features in the original hydrocarbon chain, such as *cis* double bonds, are generally unaffected. This chemically simple process has been studied for usefulness by many investigators over the last 150 years. Useful applications, both industrial and laboratory-scale, have been elaborated.

Our work in this area has been to develop a Kolbe-based method of converting triglycerides from algal lipids to fuel. To free the long-chain carboxylates from the plant oil, either a stoichiometric hydrolysis using NaOH or Na_2CO_3, or a catalytic process using sulfuric acid can be used. Low-cost sodium bicarbonate is then used to generate the necessary sodium salts of the acids. Some work on the general area of Kolbe decarboxylative coupling of long-chain fatty acid salts has been published. Guryar developed an electro-synthesis of octadecane from caproic acid (the C10 acid). A 90% yield was "readily achieved" and the author speculates on a continuous process of hydrocarbon production based on his work. Sumera and Sadain electrolyzed mixtures of potassium salts of coconut-oil derived fatty acids and potassium acetate, a "crossed" Kolbe Reaction. A high energy density (47.56 MJ/kg) diesel fuel, requiring no further reforming, was produced.

Our current results indicate that production of hydrocarbons from this process occurs in 90–100% yields, the existence of one, two, or three double bonds in the carboxylic acid does not cause complications, and many of the operating parameters have been understood. In addition, using mixed systems has also been successful. Reaction of acetic acid with the lipids has given rise to good yields of the hydrocarbon.

In addition, the crude TAGs have been used in this process successfully. Reaction with the sodium hydroxide cleaved the triester. The electrolysis then provided for the coupling of the carboxylates to give rise to the extended-carbon-chain hydrocarbon. This represents a direct conversion of TAGs to hydrocarbons obviating the need for a hydrolysis step or conversion of the acid to the carboxylate.

Characterizing this conversion process is a promising step in developing sustainable, economical biofuels from algae-derived lipids. Future research will involve experiments to attain greater understanding of the reaction parameters (e.g., scale, solvent, concentration, pH, and temperature). Specifically, we will focus on discovering (1) if formic acid (potentially obtained from carbon dioxide) can be used as a donor in the Kolbe process, (2) whether raw-algal-oil fatty acids can be used directly in this process, and (3) whether low-temperature catalytic processes can be used directly on the triglycerides (algal oil) to produce hydrocarbons.

Conversion of Lipids to Biodiesel, Gasoline, and Jet Fuel Using Decarboxylation

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The one-year scope of Diversified Energy Corporation's (DEC's) effort was to develop the Centia™ technology for the conversion of algal oil to jet fuel and other fuel products. This development included small-scale algal oil conversion, technical risk reduction, and techno-economic evaluation. To accomplish this DEC worked closely with the research teams at North Carolina State University (NCSU) to improve the design for the overall Centia™ fungible biofuels system and model it with the available recent data.

The effort included successful liter-quantity batch hydrolysis and decarboxylation of algal oil from Eldorado Biofuels to produce renewable diesel. This represents a scale-up of the decarboxylation reactor by a factor of 500. Additionally, fed-batch deoxygenation has been achieved at 10 mL/min at steady state on fatty acids from algae, camelina, and canola.This is an important milestone toward continuous reactive distillation, which will be the design for commercial-scale deployment of the technology.

Improvement in the yield of the hydro-isomerization reaction was achieved, boosting useable fuels output from 40% to 86% of the undecane input. Successful aromatics production was similarly realized, increased from less than 10% to 49% single-pass yield.

Types of pretreatment of the algal feedstock to remove catalyst poisons were evaluated, including chemical, mechanical, and enzymatic approaches. Initial testing showed that the water hydrolysis reactor (already employed in the process layout) was successful in decreasing phosphorus content by 90%. Additional testing with dilute acid hydrolysis further reduced the contaminants, but has not yet been optimized to protect fatty acid yield.

The process flow diagrams and complete techno-economic evaluation created through this research period provided initial guidance to the Sustainability Team at NAABB. The results of the initial economic evaluation, using parametric modeling, was that a 10 MMGY Centia biorefinery (producing jet fuel and gasoline) would cost approximately $1.55 per gallon of capacity and run at an operating expense of $0.77/gallon. However, these results depend on achieving superior catalyst performance and lifetime, performance that has not yet been achieved, even at lab scale.

Generation of Algal-based Hydrocarbon Fuels for Characterization and Testing

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As part of the NAABB consortium, UOP, a Honeywell company, has been tasked with converting algal oils produced by NAABB partners into fungible transportation fuels such as Green Jet Fuel™ using the UOP Renewable Jet Fuel™ process and Green Diesel™ using the UOP/ENI Ecofining™ process. These processes are based upon UOP's more than 90 years experience and expertise in the petrochemical refining industry and hydrotreating technology. Hydrotreatment utilizes hydrogen at elevated temperatures and pressures in the presence of suitable catalysts to remove hetero-atoms (such as oxygen, nitrogen, and sulfur) and halides (such as chlorine) from organic molecules to produce a purely hydrocarbon fuel.

UOP received a number of algal-derived oils from NAABB partners for evaluation as feedstocks for both the Ecofining™ and Renewable Jet Fuel™ processes to produce drop-in hydrocarbon fuels. Our analysis of these oils

indicated that the level of contaminants such as metals, phosphorous, nitrogen, chlorine, and sulfur varied widely depending upon methods of both cultivation and oil extraction. Some oils were obvious candidates for conversion to hydrocarbon fuels whereas other algal oils presented challenges for processing without additional pretreatment. Pretreating the algal oils may include processes such as degumming, which is commonly used in pretreating vegetable oils. Pretreating algal oils not only removes catalyst poisons, it also provides a mechanism by which valuable nutrients, such as phosphorous and nitrogen, and divalent cations, such as calcium and magnesium, can be recycled back to the cultivation ponds from the extracted oils, especially if pretreatment is done near the source of cultivation.

UOP successfully converted a number of algal oils supplied by NAABB members to hydrocarbon fuels. One of these oils was a fatty acid methyl ester (FAME) oil produced by a NAABB member using a proprietary process to convert whole algal biomass to FAME oil and byproducts. Other oils were crude triacylglycerol oils extracted from algae grown in outdoor ponds. We also received a bio-oil produced by the hydrothermal liquefaction of whole algal biomass and processed it to jet fuel. All the crude algal oils required pretreatment to remove metals and phosphorous before conversion to fuels. We used a two-step conversion process to produce jet and diesel fuels with the required freeze and cold flow properties. The first step involved removal of oxygen, nitrogen, and sulfur. This step produced straight-chain normal (n-) alkanes from the fatty acid component of the algal oil. The second step involved the cracking and isomerization of these straight-chain alkanes into a mixture of highly branched (iso-) alkanes. We also cracked some of the larger-molecular-weight paraffins into smaller-molecular-weight paraffins, which increased the amount of material that was in the jet fuel boiling range. This flexibility allows the yield of jet and diesel product to be tuned toward either fuel, depending upon the current market value of jet fuel versus diesel fuel. We then fractionated the treated product by distillation into naphtha, synthetic paraffinic kerosene (SPK) jet fuel, and diesel fractions. Along with naphtha, some light fuel gas is also produced. Both of these products have value as fuels, and the naphtha fraction can be a feedstock for a reforming unit for gasoline or polymer grade olefins.

The SPK fraction from the algal oils met the recently published ASTM D7566 specifications for bio-SPK jet fuel component for density, freeze point, flash point, and distillation profile. Some of the feedstocks that contained high levels of metals exceeded the specified metal content in the D7566 standard for biologically derived aviation turbine fuel, highlighting the need to remove metals from crude algal oils before processing to final fuels.

Conversion of High-lipid Algal-oil and Algal-oil-surrogate Feedstocks to Biodiesel Utilizing Albemarle's Solid Catalyst Technology

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NAABB activities at Albemarle involve the development of an effective process to convert high-lipid algal oils to fatty acid methyl esters (FAMEs) or biodiesel utilizing our proprietary solid T300 catalyst. Catalyst operations are demonstrated at laboratory, bench-top, and pilot scale, showing flexibility of the solid catalyst in the transesterification of refined and less refined (or opportunity) feeds such as algae. We also investigated pretreatment and posttreatment system technologies surrounding the transesterification system with Albemarle's solid catalysts for the development of a comprehensive system. As these coupled systems are optimized, the processes are simulated, demonstrated, and ultimately progressively integrated into a pilot plant, capable of batch or continuous operations.

Activities at Albemarle support the NAABB Conversion Team in demonstrating the conversion of lipid algal oil to a drop-in biodiesel fuel. Our primary task is demonstrating the conversion of crude algal and algal-oil surrogates to FAME and glycerol using our T300 catalyst. We were able to perform trials on five different algal oil feedstocks from Eldorado, Solix, and SRS (Valicor), which came to us in varying quality. Upon receipt, we analyzed the oils and subsequently pretreated them prior to transesterification. We ran degumming experiments on some feedstocks to determine if metals and phosphorus reduction provided improved processing advantages. Through these experiments we discovered that T300 is tolerant of moderate levels of metals and phosphorus and degumming was not necessary to utilize T300 in transesterification. For most feedstocks, however, free fatty acid (FFA) reduction technologies were necessary to reduce the FFA to the approximate 1% FFA target range suitable for transesterification with T300. Glycerolysis proved to be particularly effective in the FFA reduction of algal oil and surrogate feedstocks. Glycerolysis provides greater flexibility to treat higher FFA feedstocks than other pretreatment methods, serves as a way to dry feedstocks, and eliminates the need for methanol rectification following pretreatment. The glycerin generated from the T300 transesterification process is of technical grade (nominally 95%) purity, so it is well suited for use in glycerolysis pretreatment without further purification. Glycerolysis reduced algal oil feeds from 35% to 1.9% FFA and surrogate feeds with very high FFA (up to 93%) by similar amounts. Albemarle designed and simulated these pretreatment systems to support the T300 solid-catalyst transesterification system and determine the limits of application of T300 technology.

Extensive transesterification trials with the T300 solid catalyst were performed on algal oil and less refined feedstocks (surrogate feeds). As the industry shifts to these less refined feedstocks to allow for higher profit margins, these technology developments become increasingly important. We monitored reaction kinetics and catalyst recyclability in these trials as parameters were optimized. Catalyst handling and recycling data were essential in these trials, as the R&D trials provided guidelines for scaling up these technologies in pilot operations.

Albemarle ran various process configurations—from batch to continuous operations and from one-reactor systems to two—defining the optimal conditions and comparing to conventional systems along the way.

Biodiesel posttreatment purification likewise was crucial to meet increasingly strict fuel standards. Albemarle developed posttreatment procedures suitable for removing calcium and other contaminants and further optimized these processes as scaled up in pilot operations.

The overall T300 process proved successful with algal oil and less refined surrogate feedstocks. Third-party economic analyses confirmed that implementing T300 can have an OPEX advantage of up to 12 cents per gallon of biodiesel and a CAPEX advantage of up to 24% for greenfield operations. Market analysis also indicated that the T300-generated glycerin can provide an additional coproduct advantage of 6–15 cents per pound.

Going forward, the challenges for algal oil in biodiesel production will be to establish consistency in feedstock quality and to capture the processing and system economics data for the full process. Details of the extraction and purification performed before conversion will need to be further understood to determine if the overall system is economically viable.

Direct Conversion of Wet Algae to Crude Biodiesel Under Supercritical Conditions

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This study demonstrated a single-step process for direct liquefaction and conversion of wet algal biomass to biodiesel under supercritical methanol (SCM) conditions with a noncatalytic transesterification approach in the presence of nitrogen. This process enables simultaneous extraction of lipids from wet algal biomass and efficiently converts/transesterifies them into fatty acid methyl esters (FAMEs), preserving the proteins and other valuable components in the lipid-extracted algae (LEA). The process conditions are milder than those required for pyrolysis and prevent the formation of byproducts.

The major elements and their approximate composition (wt%) found in algae were carbon, 70.43%; oxygen, 22.19%; sodium, 0.74%; magnesium, 1.16%; phosphorous, 2.42%; chlorine, 0.36%; potassium, 1.32%; calcium, 0.94%; and sulfur, 0.34%. The total lipid content in algal biomass was determined to be 50–55% (percent of dry biomass) by the Folch method. The lipid composition report reveals that algal biomass contains a major proportion of triglyceride (TAG) percentage followed by polar lipids, hydrocarbons, free fatty acids (FFAs), and various other molecules. The major fatty acid composition (percent as methyl esters) in the algal lipid was reported as tetradecanoic acid, C14:0 (4.32%); hexadecanoic acid, C16:0 (38.25%); hexadecanoic acid, C16:1n7 (35.34%); *cis*-9-octadecanoic acid, C18:1n9c (7.4%); *cis*-9,12-octadecadienoic acid, C18:2n6c (3.05%); *cis*-5,8,11,14,17-eicosapentenoic acid, C20:5n3 (6.8%). We calculated the saturated and unsaturated fatty acids to be 45.81% and 54.19%, respectively. We found the moisture content in the wet algal biomass was around 80+% (by TGA analysis).

We designed the experimental conditions for the SCM process as follows: wet algae to methanol ratios (weight/volume), 1:4–1:12; reaction times, 10–30 min; reaction temperatures, 240–260°C. We maintained the reaction pressure around 1200 psi for all experiments. To process the wet algae, we used a PARR 4593 micro reactor coupled with a 4843 controller. We used a response surface methodology (RSM) to analyze the influence of these three process variables on the FAME content.

Triglyceride and fatty acids contribute a higher percentage in the total algae lipid recovered and converted to crude FAMEs. Sulfur-, nitrogen-, and phosphorous-containing compounds in the algal lipid (mainly in the phospholipids and glycolipids) end up in the water soluble fractions following transesterification. In addition, some sugars, acids, and highly oxygenated compounds that would be soluble in the aqueous phase are separated from organic fractions. Based on the experimental analysis and RSM study, the optimal conditions for this process are reported to be wet algae/methanol (weight/volume) ratio of around 1:9, reaction temperature of about 255°C, and time of around 25 minutes.

The maximum crude algal biodiesel yield obtained at the optimal conditions for SCM process was about 40% (based on dry algal weight) and about 84% (based on total lipid content). The reaction temperature above optimal conditions was unsuitable for transesterification of the algal biomass due to slow decomposition of polyunsaturated fatty acids (isomerization) and/or autoxidation of the reactive intermediates (organic substrates). Unsaturated fatty acids in algal biomass lead to the formation of hydroperoxides, which are thermally unstable.

We estimated the total energy input (thermal and electrical) and energy ratio (energy output in biodiesel/total energy input) of the process to be 23.15 MJ/kg (6.43 kwh/kg algae) and 1.77, respectively. Biodiesel production from the wet algae eliminates the need for a costly and energy intensive drying process, which accounts for most of the total energy consumption of 84%.

From the gas chromatography–mass spectrometry (GC-MS), we observed that the algal biodiesel contains olefins, fatty alcohols, aldehydes, sterols, and vitamins in minor quantities. The jet fuel range of hydrocarbons, such as decane, dodecane, tetradecane, and hexadecane, were also detected from the analysis. A low percentage of methyl esters with a carbon chain of more than 18 carbons guarantees a low viscosity for the biodiesel. Palmitic acid, C16:0, and palmitoleic acid, C16:1, methyl esters contribute around 70–75% of total fatty acid methyl esters. The strong methyl ester peaks around 1740–1745 cm^{-1} (C=O ester) and 1160–1170 cm^{-1} (C-O ester) are clearly present in the spectra for wet algal biodiesel. The onset temperature for volatilization of crude algal biodiesel by thermogravimetric analysis was recorded around 140–145°C under nitrogen environment. The sample weight loss (thermal degradation) of 10%, 50%, and 90% to the initial weight was recorded at the temperatures of 195°C, 362°C, and 430°C, respectively. We recorded the onset temperature of oxidative decomposition for wet FAME at 115–125 °C. The oxidative degradation temperatures (oxygen environment) corresponding to the sample weight loss of 10%, 50%, and 80% to the initial weight was noted at 112°C, 210°C, and 358°C, respectively. It has been observed that the oxidation temperature of a biodiesel is inversely proportional to the amount of bisallylic hydrogens.

The preliminary report for fresh algae and LEA is summarized as crude protein, 36.6 and 34.3%; acid detergent fiber, 24.5 and 22.9%; neutral detergent fiber, 39.6 and 36.6%; total digestible nutrients, 87.4 and 68.4%; fat, 3.92 and 2.69%, respectively. The major metal contents detected in LEA are calcium, 1.69%; sodium, 1.39%; aluminum, 583 ppm; barium, 7.57 ppm; chromium, 45.1 ppm; iron, 4540 ppm; and manganese, 234 ppm. More in-depth studies are needed to verify these preliminary results.

Some of the high value bioproducts that can be extracted and analyzed by GC-MS from *Nannochloropsis salina* microalgae are phycobiliproteins carotenoids (e.g., indole, naphthalene); polyunsaturated fatty acids (e.g., eicosapentaenoic acid, arachidonic acid); and vitamins (e.g., ascorbic acid). We observed from mass spectrometry data that wet algal biodiesel retains some natural antioxidants present in algae such as sterols, tocopherols, tocotrienols, and oxalic acids that could help to enhance fuel properties. The preliminary report of micro-elemental (carbon, hydrogen, nitrogen, oxygen, and sulfur) analysis for algal biodiesel is summarized as carbon, 78.15%; hydrogen, 11.93%; nitrogen, 0.05%; oxygen, 9.91%; and sulfur, 0.02%. The report also indicates very low ash (inorganic) in the sample. We determined all these elemental analyses by standard ASTM methods. The density, kinematic viscosity higher heating values (HHV), and pour point of algal biodiesel were calculated as 0.85–0.87, 4–5.2 mm^2/s, 41–43 MJ/kg, and -12–-10°C, respectively.

The process improvements to supercritical methanol transesterification can be possible by the addition of cosolvents to reduce the severity of the reaction parameters and amount of solvent used in the process. The recovery and reuse of unreacted alcohol and value-added use of glycerol separated from biodiesel is also essential. The energy recovery and use are important issues that need more attention. This noncatalytic transesterification route greatly simplifies the downstream processes and leads to substantial cost savings in algal biodiesel production. In addition, this single-step process favors the energy requirements for biodiesel production by eliminating the needs for drying and extraction of algal biomass. This promising technology, if developed into a flow system coupled with power cogeneration or LEA employment to recycle CO_2, water, and nutrients and to use waste heat, has the potential to address the scale-up limitations for commercializing algal biodiesel production. This single-step process can potentially be an energy-efficient and economical route for algal biodiesel production.

Hydrothermal Processing of Whole Algae and LEA for Gaseous and Liquid Fuel Production

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Algae have been identified as a high-growth biomass with great potential for fuel production to reduce petroleum imports and reduce greenhouse gas emissions. The standard view of algae utilization has involved growing oil-producing algae under stressed conditions that maximize vegetable oil production. By this process extractable vegetable oil can be recovered and a lipid-extracted algae (LEA) byproduct is left.

The utilization of the LEA for energy production is one opportunity investigated by the NAABB consortium. Hydrothermal conversion (processing in hot, pressurized liquid water) is a valid option that has been investigated and developed for use with high-moisture content algae streams. Specifically, we have investigated bench-scale testing of catalytic hydrothermal gasification (CHG) for conversion of LEA to produce a fuel gas product for energy recovery and a clean aqueous byproduct for nutrient recycle in a continuous-flow reactor system. In addition, it is possible to use hydrothermal conditions to process whole algae, thereby recovering the produced vegetable oil but also converting a significant portion of the biomass (LEA) to an oil. The bio-oil product thus produced can be further upgraded to hydrocarbon liquid fuels by catalytic hydroprocessing. We also investigated both of these steps on the bench scale within the expanded scope of this project.

Algae and LEA are potential biomass feedstocks whose high-moisture characteristic makes them reasonable feedstocks for hydrothermal processing. Hydrothermal processing can be used to convert the organic material into either a liquid product (hydrothermal liquefaction, or HTL) or a fuel-gas product (CHG). While the initial effort in this project was the gasification of the LEA for energy recovery, further consideration led to gasification of whole algae as well as liquefaction of whole algae.

Previous R&D into biomass conversion has shown that hydrothermal process technology (HTL, CHG, or both) may be applied to algae, but a lack of available data required further process development and analysis. Specifically, algae and LEA needed to be characterized so we could better understand the pumping qualities of the potential feedstock; its usefulness based on relative amounts of carbon, hydrogen, and oxygen; and the potential effects of trace components, such as nitrogen, sulfur, and minerals. Although there was limited literature data on these processes, essentially all of it was performed in simple batch reactors, which did not represent process data needed for a techno-economic assessment or scale-up design. Hydrothermal processing tests in a continuous-flow reactor were needed to generate the required data for modeling/techno-economic analysis to determine the economic feasibility. The application of CHG to algae, LEA, and even the byproduct aqueous stream from HTL of algae also has a useful optional component of nutrient recycle using the aqueous and mineral precipitate byproducts from the process. This benefit needed to be tested.

With adequate understanding of hydrothermal processing of algae feedstocks it would be possible to close the loop in algae-based biofuels. Growth and harvesting of algae are only a partial answer to their utilization for fuels production. Hydrothermal processing provides an energy-efficient method for recovering the organic portion from the dilute algae stream. Some concentration of the algae stream into the range of 10–20 wt% dry solids from the as-grown concentration of 0.1 wt% would provide a useful feedstock for hydrothermal processing without the complete dewatering of the algae, required in some cases for oil extraction. Further, hydrothermal processing (HTL and CHG) provides a means to recover the bulk of the organic content without producing contaminated wastewater streams. Finally, the byproduct water stream from the combined hydrothermal processing of both HTL and CHG should be a useful nutrient recycle means for soluble minerals and nitrogen.

Through the use of a metal catalyst, we accomplished gasification of wet algae slurries with high levels of carbon conversion to gas at relatively low temperature (350°C) in a bench-scale, continuous-flow reactor system. In a pressurized-water environment (20 MPa), we achieved near-total conversion of the organic structure of the algae to gases in the presence of a supported ruthenium metal catalyst. The process was essentially steam reforming, as there was no added oxidizer or reagent other than water. In addition, the gas produced was a medium-heating-value gas due to the synthesis of high levels of methane as dictated by thermodynamic equilibrium. We removed algae trace components by processing steps so that they did not cause processing difficulties in the fixed-catalyst-bed tubular reactor system. As a result, the algae feedstocks, even those with high ash contents, were reliably processed without plugging the feeding systems or the fixed-catalyst bed. We obtained high conversions even with high slurry concentrations. Consistent catalyst operation in these short-term tests suggested good stability and minimal poisoning effects. We noted high methane content in the product gas with significant carbon dioxide captured in the aqueous byproduct in combination with alkali constituents and the ammonia byproduct derived from proteins in the algae. We found high conversion of algae to gas products with low levels of byproduct water contamination and low to moderate loss of carbon in the mineral separation step. This project demonstrated several alternative catalyst formulations. Although we showed several carbon supports to be unstable in the hydrothermal system, we identified one particular carbon granule, a high-surface area silicon carbide product, and an innovative nanocarbon formulation as useful supports in hydrothermal processing. An important process development that we demonstrated in the continuous-flow reactor was the separation of sulfate from the process stream as insoluble calcium sulfate. This separation facilitates subsequent processing of either the liquefaction or the gasification product.

Although we envisioned scale-up of the technology if we obtained useful outcomes of the bench-scale tests, we have moved the development only as far as preliminary design of a moderately sized (1 ton per day of algae slurry) scaled-up reactor system.

Similar to our work in the gasification tests, we also completed bench-scale HTL tests with algae and LEA. We achieved high yields of a gravity separable bio-oil product in a bench-scale, continuous-flow reactor system. Using the CHG process described above, we performed subsequent process tests to convert the organic materials lost in the aqueous byproduct into a useful fuel-gas byproduct. This innovative combination of hydrothermal processes is a patentable development. We also demonstrated at the bench-scale the conversion of the algae HTL bio-oil to liquid hydrocarbon fuels by catalytic hydrotreatment of the bio-oil in a continuous-flow fixed-bed reactor.

Efficient Scalable Production of Algae Biofuels Using Hydrothermal Processing

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When the NAABB project started, the general view of algae-based fuel production was that algae would be grown to produce lipids (oil), which would be extracted from harvested algae. It was expected that the algal lipids would be primarily triglycerides, which could be transesterified into biodiesel. The algae biomass left after the oil was extracted could be used for animal feed or as a source of other byproducts. The entire process would be examined and modeled to ensure sustainability. All of these steps had been demonstrated at small scales, but substantial work remained to determine if they could be scaled to commercial volumes. It was also anticipated at the outset that the project could lead to new approaches that did not necessarily follow this preliminary approach.

The initial task identified for hydrothermal processing fit into this structure as a method for producing energy from the algae biomass left after extracting the oil (the lipid-extracted algae, or LEA). The specific technology to be tested was catalytic hydrothermal gasification (CHG). This technology had been developed over a 30-year period at Pacific Northwest National Laboratory (PNNL) and had been successfully demonstrated as a way to convert wet feedstocks into usable fuels. Within NAABB, CHG was to convert the LEA to methane, which could be burned to make electricity and heat or could be cleaned and compressed to make renewable natural gas. Under the plan, PNNL and Genifuel would work together, with PNNL primarily doing research at bench scale and Genifuel building a demonstration machine at a larger scale and installing it at one of the algae-growing partners in NAABB.

While the original purpose of the task was to gasify LEA, as more algae became available we ran additional tests as part of the experimental work. Both Genifuel and PNNL observed that algae was an excellent feedstock and had many characteristics that fit well with the temperature and pressure used in the hydrothermal process. Specifically, algae is easy to make into a wet slurry feedstock, converts efficiently and completely, and gives good yields of methane gas from the CHG process. In addition, plant nutrients including the primary nutrients nitrogen, phosphorus, and potassium (NPK) are recovered. Minor plant nutrients such as iron, copper, zinc, boron, etc. are also recovered. The water from the process is recovered in a clean, sterile form, and even carbon dioxide can be recycled to the algae ponds. Thus the process allows almost complete recycle of plant nutrients and water, contributing strongly to sustainability.

PNNL had also performed substantial work outside of NAABB using a different form of hydrothermal processing to produce a liquid rather than a gas. This type of processing is called hydrothermal liquefaction (HTL). While HTL uses temperature and pressure similar to those in CHG, it does not use the catalyst. In the absence of the catalyst, biomass is converted to oil, while in the presence of the catalyst it is converted to gas. Because algae can be processed so successfully with

CHG, tests were performed to see if it could also be processed with HTL. These tests were also highly successful. While HTL was not specifically identified as a task in the original NAABB plan, we recognized that the liquefaction process offered significant value to the project. With this recognition, additional testing was scheduled by NAABB management.

The work on HTL of algae demonstrated a complete path from raw algae to finished fuels. In this path, algae is harvested and concentrated to about 15–25% solids in water. Both lower and higher concentrations are possible, but lower concentrations become less efficient and higher concentrations may become difficult to pump. This feedstock is made into a slurry and processed with HTL to recover liquid oil. The residual biomass is gasified with CHG to produce methane. The nutrients are recovered and recycled. A specialized hydrotreater upgrades the HTL bio-oil, and conventional refinery processes refine the resulting green crude. Overall, the primary products are jet fuel, diesel fuel, methane, and nutrients. Over 85% of the carbon in the feedstock can be recovered as fuel, and more than 99% of the organic matter is converted.

The HTL process actually performs two tasks in the original NAABB project structure. It is an extraction method, extracting oil from the algae, including triglycerides, membrane lipids, and free fatty acids. However, it is also a conversion method, because it converts a substantial amount of protein and carbohydrate in the biomass into oil. From high-lipid algae, HTL can recover total oil equal to 65% or more of the biomass. Even for low-lipid algae, more than 40% of the biomass can be converted to oil. This process enables oil production from a wide range of algae species, thereby providing substantial flexibility in what type of algae to grow.

When all of these steps are put together, hydrothermal processing is involved with extraction, biomass conversion, fuel conversion, valuable coproducts, and sustainability, extending across five of the six task areas of the original NAABB project structure.

As part of NAABB, Genifuel has extensively modeled the combined process using simulation techniques like Aspen Plus and a sophisticated techno-economic model developed by Genifuel. These models show great promise in scaling the process substantially and making it commercially viable.

During the program, Genifuel also notified NAABB of three patents or patent applications. These have resulted in one issued patent, with two still in process at the U.S. Patent and Trademark Office.

Finally, Genifuel had a specific task to provide a demonstration system for field installation at a site of one of the members of NAABB who was producing algae. This system would be larger than PNNL's systems. The member site chosen was at Reliance Industries, Ltd., near their refinery in Jamnagar, India. Originally this system was defined as a CHG system, in keeping with the plan for NAABB. When HTL was identified as an additional technology for processing algae, Reliance decided to add HTL capability to the reference system. The system as now defined can be configured for both HTL and CHG, though not simultaneously. Its capacity is 200 kilograms per day of dry feedstock, or 1 metric ton per day of wet feedstock at a concentration of 20% solids. It is planned for installation in India late in the program. While work is still needed to refine

various processes and develop more detailed data on some aspects of the technology, it appears highly probable that hydrothermal processing will be used in some form in the algae fuel industry of the future. This represents a very positive and significant result from the NAABB project.

HTL and CHG are highly effective in producing fuels from algae. The processes can be used alone or together to give liquid fuels, gas fuel, or both. Because hydrothermal processing recovers fuel not only from the lipid fraction, but also from other components of the feedstock, it gives higher fuel yields than processes that recover only lipids. This capability also means that algae growers do not need to focus exclusively on algae that produce high lipid content, providing much more flexibility in species selection and growing conditions. The combination of these factors makes hydrothermal processing an exceptionally promising method to advance algae as a viable path to large-scale production of biofuels.

Optimization of Operating Conditions for Bio-oil Production and Characterization of Pyrolysis Products from *Nannochloropsis oculata*

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The economic development of an industrial society relies heavily on the availability of energy sources. Oil is one of the fossil fuels that is considered cheap and easily transportable. However, dwindling oil production (about 6% to 7% per year), coupled with rapidly increasing oil usage (about 1% to 2% per year) threatens tremendous escalation in the price of oil in the near future. Moreover, the increasing awareness of the effects of greenhouse gas emissions from burning fossil fuels prompts investigation into carbon-neutral sources of energy. Thus alternative sources of oil should be evaluated to meet the rising needs.

Biomass pyrolysis is one of the technologies that are being explored as an alternative for the production of oil. Pyrolysis produces energy fuels with high fuel-to-feed ratio and the process can be easily adjusted to favor char, oil, or gas production. The bio-oil from pyrolysis of agricultural biomass is comparable to crude oil, which can be easily stored and transported, and it has low nitrogen and sulfur contents. It can be used for direct combustion or can be upgraded further to liquid transport fuels and biochemicals. One feedstock that is deemed to be a good source of bio-oil is microalgae. Compared to lignocellulosic materials, microalgae grows faster, has high photosynthetic efficiency, and has high productivity. It can be cultivated on salty water and does not require arable soils. The bio-oil produced from algae is more stable due to lower O/C ratio and has higher heating value compared to wood oil. Also, algae has high lipid content and it devolatilizes at lower temperatures than lignocellulosic materials. However, the right combination of operating factors that optimizes bio-oil yield from pyrolysis of algal biomass is not yet established.

This project, therefore, aimed to develop an efficient process for the production of high quality bio-oil from pyrolysis of algal biomass. It specifically aimed to determine the optimum conditions for maximum bio-oil yield from algae using pyrolysis. Several operating parameters such as temperature and pressure could affect the yield of bio-oil. Hence we evaluated the effects of temperature and pressure on product yields. From the results, we identified the combination of temperature and pressure that optimizes bio-oil yield. We also evaluated the physical properties (heating value, moisture content, etc.) and chemical composition of the products (bio-oil, biochar, and syngas) to establish their potential uses.

In Task 1 we investigated the extent of bio-oil production from pyrolysis of microalgae at different combinations of reaction parameters, particularly temperature and pressure. We also evaluated the individual effects and interaction of the selected reaction parameters (temperature and pressure) based on the yield of bio-oil and its coproducts (char and syngas). We established the optimum combination of temperature and pressure for maximum production of bio-oil from pyrolysis of microalgae using response surface analysis.

In Task 2, on the other hand, we focused on assessing the suitability of bio-oil produced from microalgae as an alternative for petroleum crude oil. We evaluated bio-oil properties by comparing them to petroleum crude oil characteristics (< 1% oxygen, < 0.1% moisture, and < 2 mg KOH/g acid value). Other parameters include ultimate analysis, chemical composition via gas chromatography–mass spectrometry (GC-MS), heating value, viscosity, flash points, pour point, distillation curve, and thermal stability. These parameters will determine the need for upgrading processes. In Task 3 we determined char and syngas compositions and properties to identify their potential usage. Char analysis included heating value, ultimate, and proximate analyses. We also determined the chemical composition and heating value of syngas.

Preliminary analysis of the microalgae feedstock *Nannochloropsis oculata* showed its favorable characteristics for bio-oil production since it has high volatile matter (81% wt), carbon (48% wt), and hydrogen (8% wt) contents. Consequently, the energy content of *N. oculata* was also high (24.7 MJ/kg). Pyrolysis of microalgae at different temperatures and pressures showed that operating conditions can be manipulated to favor biochar, bio-oil, or syngas production. Biochar was significantly affected by temperature ($p < 0.0001$) but not by pressure. Maximum biochar yield (56% wt) was observed at 400°C. Syngas yield, on the other hand, was significantly affected by temperature ($p < 0.0001$) and the interaction between temperature and pressure ($p = 0.0002$). Peak syngas production (20% v/w) was observed at 600°C and 100 psig. Bio-oil production was greatly dependent on both temperature ($p < 0.0001$) and pressure ($p = 0.0048$). Hence we optimized using Design Expert 8.0.7.1 to identify the combination of temperature and pressure that maximizes bio-oil yield. Numerical optimization and actual experiments showed that optimum temperature and pressure were 540°C and 0 psig, respectively. At 540°C and 0 psig, we attained maximum liquid product yield of about 43% wt (136 gal/ton algae). The liquid product consisted of the yellowish aqueous fraction (20% wt) and dark-brown organic fraction or the bio-oil (23% wt). Biochar and syngas yields were approximately equal to 32% wt (695 lb/ton algae) and 12% wt (3,267 ft^3/ton algae), respectively.

Characterization of the bio-oil obtained at optimum conditions (540°C, 0 psig) revealed that it could be a potential replacement for crude oil after further processing. The bio-oil from microalgae has high carbon (73% wt) and hydrogen (11% wt) contents, which contribute to its high energy content (36 MJ/kg). Some properties of the bio-oil were also determined including pour point (-6 ± 3°C), flash point (40°C), viscosity (10.29 ± 0.87 cSt), and density (840 kg/m^3). The bio-oil was also found to be a Newtonian liquid. Bio-oil can also be a good source of biochemicals and transport fuels since it contains a variety of organic compounds. GC-MS analysis of algal bio-oil showed that it contains alkanes (35.22%), alkenes (38.17%), alkynes (0.61%), alcohols (4.47%), acids (4.93%), benzenes, aromatic compounds (6.01%), esters (0.56%), amides (0.35%), and nitriles (9.68%).

The biochar from pyrolysis at 540°C and 0 psig consists of 50% carbon, 2% hydrogen, 8% nitrogen, 2% sulfur, and 31% oxygen by wt. After pyrolysis, the volatile matter in the biomass decreased from 81% wt to 22% wt. Consequently, the residual biomass (biochar) has lower energy content (20 MJ/kg) than the original feedstock. Syngas, on the other hand, consists of several combustible components including CH_4 (15%), CO (6%), H_2 (4%), C_2H_4 (3%), C_2H_6 (9%), C_3H_6 (3%), and C_3H_8 (4%). These gases contribute to its energy content, which was estimated to be equal to 21 MJ/m^3. These results indicate that biochar and syngas can also be used as alternative energy sources.

Bioconversion of Extracted Algal Biomass into Ethanol

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The algal biomass remaining after oil removal represents a significant energy resource. This lipid-extracted algae (LEA) biomass consists primarily of carbohydrates and proteins, both of which are readily used as growth substrates by bacteria. The goal of this project was to develop an engineered process to convert LEA to an additional fuel product, ethanol, which has greater fuel and economic value than biogas. Additional benefits of this conversion process over gasification/liquefaction processes include the ability to produce a wide range of fuel and non-fuel molecules using the same platform and the ability to convert primarily the carbohydrate fraction of LEA, leaving a protein product for use as an animal feed supplement.

A schematic of the conceptual process for converting LEA to ethanol is shown in Figure 1. Carbohydrates in LEA are converted to fermentable sugars by either acid hydrolysis or enzymatic hydrolysis, and, optionally, proteins are deconstructed to peptides. The products of those two deconstruction steps are fermented to ethanol. To achieve the project goal of developing this process, we conducted experimental and computational tasks to obtain data on the rates and yields of deconstructing LEA carbohydrates to fermentable sugars, determine any fermentation inhibitors produced during the carbohydrate deconstruction process, and evaluate the effects of process variables such as temperature and pH. We used process design methods to evaluate the tradeoffs among process options, such as yield versus reagent cost. In addition, we evaluated advanced bioprocessing alternatives, including simultaneous saccharification and fermentation and continuous-flow, immobilized processes.

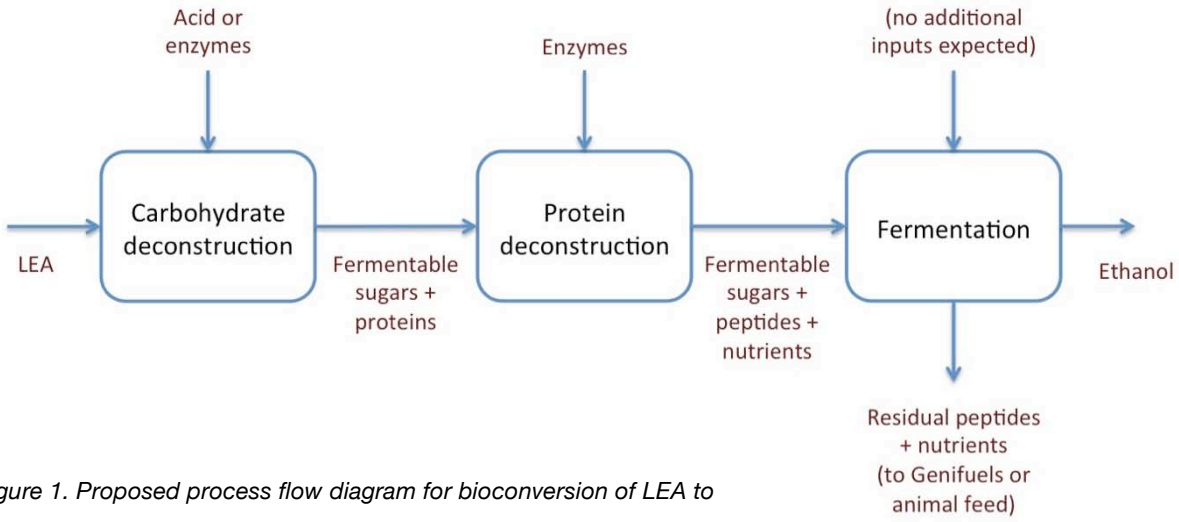

Figure 1. Proposed process flow diagram for bioconversion of LEA to

We tested four different LEA materials: Cellana diatom, Solix *Nannochloropsis salina*, SRS *Nannochloropsis*, and Cellana *N. salina*. We conducted the large majority of tests with the Solix *N. salina* LEA. The Cellana *N. salina* LEA also has potential as a feedstock for this process. However, the Cellana diatoms had minimal carbohydrate content, and the SRS *Nannochloropsis* LEA had properties that made it unsuitable as a feedstock.

We evaluated both acid and enzymatic hydrolysis methods for carbohydrate deconstruction. Tests of acid hydrolysis involved three acids under various temperature, concentration, and biomass loading conditions. Conditions of increasing severity released more sugars at higher rates but also produced more fermentation inhibitors. We tested mixtures of enzymes, some of which provided relatively high sugar yields and release rates, with no fermentation inhibitors.

In the fermentation tests, we used three strains of the yeast *Saccharomyces cerevisiae*, along with *Zymomonas mobilis*, an ethanologenic bacterium. The three yeast strains grew on both acid and enzyme hydrolysates, but *Z. mobilis* did not grow on acid hydrolysates. The microorganisms could grow on LEA hydrolysates without any additional nutrients. Ethanol yields for the yeasts were high on both types of hydrolysate and near the theoretical maximum. Incorporating a protein deconstruction stage resulted in a significant yield of ethanol production from LEA, indicating that additional useable carbon substrates were released.

We pursued alternatives to traditional batch processes with suspended cells and enzymes. Two that have particular promise are simultaneous saccharification and fermentation, in which the hydrolysis and fermentation conversions are performed in the same process vessel, reducing capital equipment costs. Preliminary results indicate that this is a viable option that should be explored further. Continuous-flow processes using immobilized cells and enzymes were also tested. Immobilized enzyme reactors allow the long-term use of enzymes and thus result in lower enzyme costs for the process. Immobilized cell reactors enable high reactor productivities because the cell concentrations are very high and because the process can be optimized for product formation rates without consideration of cell growth. Preliminary testing of both immobilized enzymatic

hydrolysis and continuous immobilized-cell fermentation of Solix *N. salina* LEA demonstrated that this approach has considerable merit, with volumetric productivities more than 100 times higher than the comparable batch process.

Preliminary process design analysis was performed using Aspen software. For the batch acid hydrolysis process using hydrochloric acid, we established a process design for conversion of 1000 million kg dry weight/year LEA. We then evaluated the influence of different versions of this process by comparing the economics of those options to a base case. The resulting design would have an ethanol production cost of approximately $2.00/gal. Similar analyses of the batch enzymatic hydrolysis process and the continuous-flow, immobilized biocatalyst process are in progress.

The hydrolysis-fermentation process developed in this project has the potential to provide considerable economic value to the production of lipid-based algal biofuels. The results not only indicate the feasibility of the process with two options for carbohydrate deconstruction, but they also suggest that the cost of ethanol produced is competitive with other sources. Furthermore, the preliminary results generated here demonstrate that advanced bioprocesses can significantly improve the process economics, resulting in lower ethanol production costs.

Conversion of LEA Biomass into Hydrocarbon Biofuels

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Processing lipid-extracted algae (LEA) is an important part of the algae economics that must be addressed to allow the recycle of important nutrients and micro-elements to the algae ponds or bioreactors. Without a process for handling the LEA, the algae production process could not reach the scales necessary to make a significant impact in the energy economy. The Terrabon process is a sustainable and efficient process for handling the leftover LEA biomass from the lipid extraction process. The Terrabon process uses a mixed culture of anaerobic microorganisms to convert all the components of the LEA into mixed carboxylic acids (e.g., acetic, propionic, butyric, etc.). These carboxylic acids are then recovered and chemically converted into ketones, alcohols, and hydrocarbons, such as gasoline and jet fuel. The Terrabon process recovers the nitrogen as ammonia and the phosphate, potassium, sodium, and other micronutrients as ash, which may then be recycled to the algae ponds or bioreactors. Figure 1 shows a process flow diagram of the conversion process.

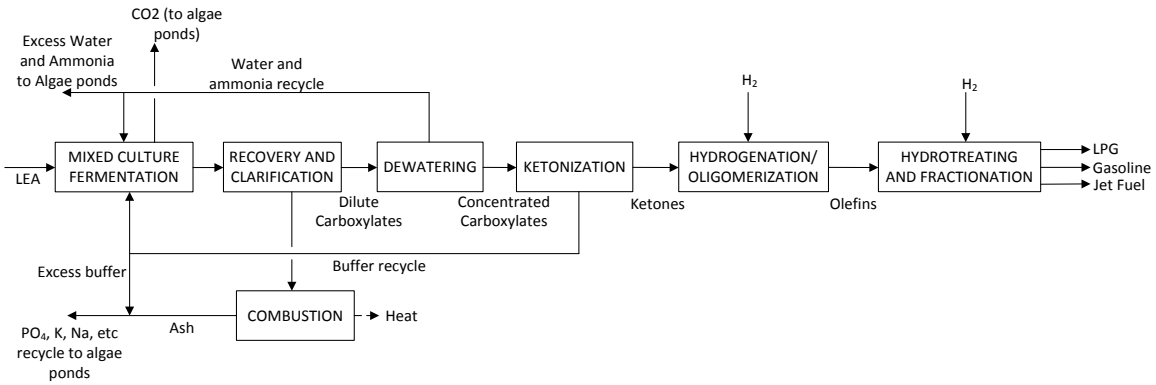

Figure 1. Process flow diagram of Terrabon process converting LEA into liquid hydrocarbon fuels.

This study tested different samples of LEA as a feedstock on the fermentation of the Terrabon process and assessed their compatibility. From the compositional analysis of the LEA it was clear that most LEA samples were not of good quality for any biological process due to their high ash content (> 30%). Only one LEA sample, the one from Solix, had low ash content (< 10%), which was adequate. In addition, during fermentation studies, it was observed that LEA had an intrinsic lower digestibility. We attributed this in some cases to certain components that were inhibitory to the microorganisms (e.g., leftover extraction solvents) as shown by the lower performance in the fermentations of LEA that were not properly extracted. We also attributed lower digestibility to innate recalcitrance of certain components (e.g., proteins) as demonstrated by the increased digestibility when we supplemented the fermentation with protease enzyme. Results also showed lime pretreatment of the LEA was an efficient method to increase digestibility to acceptable levels as compared to the surrogate feedstock, food waste. We can expect to obtain at least 75% of maximum theoretical yield of gasoline from LEA when performing such pretreatment. Future research should focus on optimizing this pretreatment. We also demonstrated that the organic acids produced in the fermentation were adequate for conversion to ketones and the ketones to hydrocarbons, such as gasoline and jet fuel. Analyses of such hydrocarbons produced showed that on-spec hydrocarbon fuels can be produced using this process.

Economic analysis performed on a Terrabon plant processing LEA using the simulator UNISIM and based on the composition from the low-ash Solix LEA showed that the plant would be economical at a scale of > 250 dry ton/day (90,000 dry ton/year), which would result in an output capacity of over 5MM gal/yr. The CAPEX for the first plant would be $75MM, which would be equivalent to $15/annual gal, but larger plants (> 1000 dry ton/day) can have CAPEX < $4/annual gal. The expected OPEX would be about $1.58/gal ($0.56/kg).

Other products that can be obtained from this process are micro-elements such as potassium, phosphate, and ammonium (in the form of ammonium sulfate), which can then be recycled to the algae ponds or bioreactors. The recovery from the process of these elements should be the subject of future research.

In general, we demonstrated that the Terrabon process is well suited to process a feedstock like LEA with such varied composition. Other biological processes would have a hard time converting components such as proteins and fats and would limit themselves to processing carbohydrates. Conversely, LEA is a good feedstock for the Terrabon process as long as the ash content can be managed. Nonetheless, lime pretreatment might be required to allow adequate digestibility of the LEA because of certain recalcitrance of some of the components in the LEA.

Production of Isocyanates from Algal Protein

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The scope of our effort was to develop an add-on to the existing algal biofuel process to use the proteinaceous material to produce ethanolamines. This development included small-scale conversion of amino acids derived from algae, parametric studies with several different catalysts, and an economic evaluation for scale-up to a continuous version of the aforementioned process.

This effort includes a liter-quantity supercritical methanol reaction to free the algae from lipids, extraction of lipid derivatives, pretreatment of proteinaceous material to avoid catalyst poisoning, reduction to ethanolamines, and fractionation to separate the final mix of amino alcohols. Existing technologies for producing amino alcohols rely on reduction reagents such as $NaBH_4$ and NaH to decarbonylate amino acids, which has proven to be economically inferior to the work completed in this effort.

Element 1/2: Perform control testing of amino acids in supercritical methanol reactions; identify resulting products from control testing

The main objective of this task was to determine thermal and chemical stability of amino acids in the supercritical methanol environment. If the amino acids' methylated derivatives could prove to be more stable, then they could stand a chance at surviving the onslaught of high temperature and pressure.

Tests were conducted mainly with lysine and glycine. They are known to decompose above 220°C in a dry, oxidative environment, but in supercritical methanol, we made mixtures of methylated derivatives, as well as dimers and trimers. We sent samples to The Scripps Institute for analysis and through their services attained a general idea of composition.

Element 3/4: Perform controlled testing of simple peptides in supercritical methanol reactions; identify resulting products from control testing

We tested short-chain peptides (C2-C10) in the same manner as the amino acids. The added variable studied here is the kinetics of hydrolysis of supercritical methanol and how that affects the kinetics of methylation.

Analysis revealed results very similar to those we saw with amino acids. Chain length of peptides, as well as their individual monomeric makeup, did not seem to play a part in reaction kinetics, at least within the parameters tested in this effort. We found only monomeric compounds post reaction, so all peptide bonds were successfully cleaved. Not as many dimers and trimers were made (probably due to the presence of different amino acids preventing such), but we still saw a mixed degree of methylation.

Element 5/6: Perform control testing of proteins in supercritical methanol reactions; identify resulting products

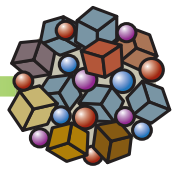
We subjected several different protein sources (bovine serum, LEA, and soy protein isolate) to supercritical methanol reactions. The testing of the hydrolysis power of methanol on a complex protein structure would prove quite interesting, mainly on the premise of whether it could totally break the large unit into its individual amino acids.

While the post-treatment smells differed somewhat from each other (release of NH_3, production of sulfurous amino acids), the results were almost identical to amino acid and peptide studies. We saw short-chain peptide survivors of the reaction, however, showing that the kinetics of hydrolysis of supercritical methanol are slower than those for methylation. These protein studies helped guide our changing parameters to maximize hydrolysis of peptide bonds while minimizing degradation products.

Element 7/8/9: Reduction of amino acid, peptide, and protein esters to ethanolamines

Before advancing to the catalytic reduction step, we took care to ensure the raw material did not contain catalyst poisons. We did several simple tests and concluded that as long as the chloride and fatty acid content is negligible, then the catalyst can do its job just fine.

Since we concluded that proteins and peptides did not form their respective esters, but rather broke into individual units, this study focuses solely on the reduction of amino acid esters to ethanolamines. Control reduction of pure amino acids was conducted first to arrive at a small window of testable parameters for the algae-derived material.



Micro-algae, Micro-algae Coproducts, and/or Micro-algae Oil as Feed-grade Ingredients for Mariculture of Shrimp and Fish

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As aquaculture continues to expand, protein sources have become more costly and less available. In fact, the increased use of fish meal and fish oil is not only associated with the tremendous increase in fish and shrimp aquaculture but also the continued used of high levels of fish meal and fish oil in shrimp and fish feeds. This has resulted in a major threat to the sustainability of the natural fisheries. Concomitant with the increase in fish meal prices there has also been a significant increase in the price of defatted and dehulled soybean meal used in aquaculture feeds. Over 40% of shrimp feeds consist of fish meal and soybean meal.

Simultaneously, lipid-extracted algal (LEA) meals (coproducts of biodiesel production) are becoming increasingly available as alternative sources of energy are investigated. Further, the nutrient profile of LEA indicates a high potential for replacing soybean meal (current price of $300 to $450/mt) and/or fish meal (current price of $1400 to $2000/mt). By integrating LEA into aquaculture diets and replacing the costly soybean meal and fish meal, not only could feed prices be lowered but also potentially increase feed nutrient quality. Further, because LEA would be replacing valuable soybean and fish meals the potential value of LEA could be up to $2.00/kg. This potential high value of LEA would offer a very significant additional revenue stream to biodiesel production. Thus in this project we conducted a series of nutritional investigations with red drum (*Sciaenops ocellatus*) as a model marine fish species and the major commercial shrimp species, *Litopenaeus vannamei*, to assess the potential nutritional and economic value of LEA as a protein feedstuff to replace soybean and/or fish meal in aquaculture diets.

We conducted three separate comparative feeding trials, each of seven weeks duration, to evaluate five different algal meals as partial replacements for fishmeal and soy protein concentrate in diets for juvenile red drum. Apparent digestibility coefficients (ADCs) for crude protein and energy in various LEA also were determined with red drum and hybrid striped bass (*Morone chrysops* x *M.saxatilis*). In the first trial, whole algae meal and LEA derived from *Navicula* sp. could replace 5% and 10% of the crude protein in the reference diet without significantly $p > 0.05$) reducing weight gain, feed efficiency, hepatosomatic index, or protein and energy retention of the fish. Algal inclusion significantly affected the ADCs of the various dietary treatments for dry matter, crude protein, and energy. A second feeding trial evaluated LEA derived from *Chlorella* sp. processed at high temperatures replacing 5%, 10%, 20%, and 25% of the crude protein in the reference diet. Weight gain, feed efficiency, survival, and protein efficiency ratio were significantly reduced at substitution levels of 20% and 25%. A third feeding

trial evaluated LEA derived from *Nannochloropsis salina* replacing 5%, 7.5%, 10%, and 15% of the crude protein in the reference diet. Weight gain, feed efficiency, survival, and protein efficiency ratio were significantly affected by some dietary treatments, with the 15% substitution level causing significant reductions in weight gain, feed efficiency, intraperitoneal fat ratio, and whole-body lipid. Based on the results of these experiments, replacement of up to 10% of crude protein from fishmeal and soy protein concentrate with LEA was possible without causing substantial reductions in fish performance, and the whole-algae product provided a more nutritious product than LEA. Red drum and hybrid striped bass showed similar responses in their ability to digest crude protein and energy from the various algal products, although ADCs varied greatly among the different products and processing methods.

Six growth and survival experiments were conducted in which six different algae meals were evaluated as replacements for soybean and/or fish meal in shrimp diets. The first four experiments in which whole and lipid extracted *Navicula* species and processed LEA at low and high temperatures from *Chlorella* sp. were considered preliminary because of the low protein levels of less than 23% in the whole algae or LEA samples evaluated. Very promising results were obtained since these four experiments indicated that up to 13.33% for all four batches of whole algae or LEA could replace either soybean and/or fish meal on an equivalent protein basis with no significant reduction in growth and survival. However, the amount of soybean and/or fish meal that was replaced was very small because of the low protein level in the four batches of algae.

The last two growth and survival experiments were conducted using two batches (A and B) of LEA from *N. salina* produced at the same facility using the same production method but at different times of the year. The crude fat level in Batch A (2.64%) was lower than the crude fat level of Batch B (9.92%), while the crude protein levels were similar (41.6% for A and 44.9% for B). The results indicated that even a 5% replacement of soybean or a combination of soybean and fish meals by LEA Batch A significantly lowered the growth rate of *L. vannamei*. In contrast, the inclusion of 30% of Batch B could replace 70% of soybean meal and 25% of Batch B could replace 66.7% of fish meal and 33.3% of soybean meal in the feed without significantly reducing growth and survival. These data are very significant in that large inclusion levels of LEA could replace large levels of soybean and/or fish meals in shrimp diets. However, if improper production, harvesting, extraction, and/or drying methods were used LEA could not be used in shrimp feeds.

Using the data from the experiment in which Batch B LEA from *N. salina* was evaluated and current values of commercial defatted dehulled soybean meal and fish meal, a computer least-cost-formulation program estimation of the potential value for LEA was made. The computer analyses indicated that LEA with a 42% crude protein level could replace 41% and 50% of soybean meal with an approximate value of $0.40/kg and $0.35/kg LEA and 14% and 22% of fish meal with an approximate value of $0.60/kg and $0.40/kg. The computer analyses indicated that the value of LEA would be significantly increased to at least a value of $0.80/kg if the LEA crude protein level was above 50%.

We also conducted two additional feeding trials with juvenile red drum to investigate the effects of relatively high levels of iron and aluminum in the diet because LEA may become exposed to these minerals during the flocculation process. We formulated a basal diet from practical ingredients and analyzed it to contain 467 mg Fe/kg and 1940 mg Al/kg diet. Experimental diets were prepared by supplementing different levels of aluminum (1000, 2000, 4000, or 8000 mg Al/kg diet) or iron (1000, 2000, 4000, or 8000 mg Fe/kg diet) or a combination of 4000 mg Al/kg and 4000 mg Fe/kg to the basal diet; supplemental levels were confirmed by analysis.

We conducted the feeding trials in 38 L aquaria operated as a recirculating system for 7 weeks. We collected water samples at Week 1, 4, and 7 to measure iron and aluminum accumulation in the water. The elevated dietary levels of iron and aluminum did not result in any significant differences in weight gain, feed efficiency, hepatosomatic index, or survival of red drum. Whole-body levels of aluminum and iron were negligibly affected by the increased dietary levels of both minerals. Iron content of liver was most responsive to increases in dietary iron. Therefore, based on the results of this study, it does not appear that high levels of aluminum or iron that may be found in LEA will exert adverse effects on red drum.

We also conducted two other eight-week feeding trials to evaluate the effects of dietary ash on red drum because LEA may contain substantial amounts of ash. We fed juvenile red drum semipurified diets formulated to contain 35% crude protein and graded levels of ash from diatomaceous earth ranging from 0% to 30% of diet, which was substituted for cellulose. We analyzed the diets to contain crude ash at 8.5%, 12.4%, 16.7%, 25.5%, and 33.8% of dry weight. Fish were stocked into 110 L aquaria operated as a recirculating system with each diet assigned to three replicate aquaria containing 25 fish each. We monitored body weight by collectively weighing fish from each aquarium every week. At the end of both feeding trials, it was apparent that red drum did not respond negatively in terms of weight gain, feed efficiency, survival, or body composition to increased ash levels in the diet. Therefore, inclusion of algae byproducts in diets of red drum will not be limited due to their contribution of ash. Also, the gastrointestinal tract of red drum was not adversely affected by the addition of ash in the diets.

Flocculation with high levels of Al and Fe is used to harvest algae resulting in increased Al and Fe levels in the whole algae and LEA. These high Al and Fe levels could decrease growth and survival of and/or increase Al and Fe levels in tail muscle of the shrimp, *L. vannamei*, to levels that would be detrimental to human health. Two growth and survival trials were conducted in which the shrimp diets were supplemented with 200, 500, 1000, 2000 or 3000 mg Al or Fe/kg feed. Two more growth and survival trials were conducted in which the shrimp diets were supplemented with 670, 1330, 2000, 2702, 3370, or 4050 mg Al/kg or 1650, 3260, 4910, 6640, 8290, or 10,044 mg Fe/kg. There was no effect on survival for all dietary supplemental levels of Al and Fe evaluated. There was no effect on growth until the supplemental dietary level reached 670 mg Al/kg or 1245 mg Fe/kg. Thus the upper limit of dietary supplemental Al or Fe level in LEA would be 3350 mg Al and 6225 mg Fe/kg assuming a maximum inclusion

LEA level of 20%. There was no significant increase in tail muscle Al levels with diets containing supplemental levels up to 670 mg Al/kg diet. However, there was a significant increase in Al tail muscle level (15 mg Al/kg) with a supplemental dietary level of 3000 mg Al/kg. There was no significant increase in tail muscle Fe levels with diets containing supplemental levels up to 3000 mg Fe/kg. The levels of Al or Fe in the hepatopancreas were greater than the levels of Al or Fe in tail muscle for all supplemental dietary levels of Al or Fe evaluated. The preceding data indicate that Al and Fe levels in LEA will not be limiting.

Therefore, based on the various results of this project, LEA should be able to replace up to 10% of high-quality protein in the diet of red drum without adversely affecting fish performance or health. The relatively high levels of aluminum, iron, or ash that may be found in LEA did not show any negative effects on the fish. Thus LEA appears to have considerable potential to replace some portion of the expensive protein feedstuffs in aquaculture diets.

The data from this project also indicate that at least a 20% inclusion level of LEA could be used to replace the expensive soybean and/or fish meals in shrimp feed. Also, the data from the experiments in which high supplemental levels of Al or Fe were added to shrimp diets indicate that Al or Fe levels in LEA from using Al or Fe as a flocculant will not be limiting. Of significance is that the computer least-cost analyses using data obtained in this project indicate that the value of LEA used in shrimp feeds has a potential of significantly contributing to an additional revenue stream to biodiesel production. Finally, data were obtained indicating that proper production, harvest, extraction, and/or drying methods will be required to produce LEA that can be included into shrimp feeds replacing soybean and fish meals.

Utilization of Post-extraction Algal Residue as Feed and Fertilizer

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Algal biomass has been identified as a third-generation biofuel. Significant quantities of the coproduct, lipid-extracted algae (LEA), remain after lipid extraction. LEA has potential to be marketed as livestock feed. After extraction, LEA is concentrated in protein, suggesting it may replace protein supplements in grazing cattle diets. Market value of LEA will be driven by its ability to compete with commonly fed protein sources, such as cottonseed meal (CSM) and dried distillers' grains (DDG). Therefore, we conducted four experiments to determine the suitability of LEA as a protein supplement for grazing cattle.

Our objective for Experiment 1 was to quantify the nutritive value of LEA produced from a single batch of *Nannochloris oculata* subjected to different methods of harvesting (centrifugation or flocculation), drying (drum-, spray-, or belt-dried), and extraction (ethanol or hexane) to determine the effect of upstream operations on downstream products. These processes resulted in 10 unique samples for determinations of nutritive value. Crude protein and lipid content (acid-hydrolysis) of samples ranged from 19% to 36% and 2% to 10%,

respectively. All LEA samples contained concentrations of S (≥ 0.81%), which may limit inclusion rate in cattle diets. Flocculation increased levels of Fe and Al ≥ 289% relative to centrifuged samples, which would restrict the use of LEA. Flocculated and centrifuged LEA had Ca:P of 18:1 and 9:1, respectively, which would present formulation challenges. Excessive mineral concentrations have negative impacts on animal health, weight gain, and predisposition to disease. Additionally, excessive mineral excretion increases the environmental footprint of livestock production. Flocculation decreased S and Na concentrations by 37% and 77%, respectively, compared to centrifuged samples; however, each remained present in quantities that would impact ration formulation. Reducing mineral concentrations and increasing protein content in LEA will increase its value and utilization in beef cattle operations. Mineral imbalances can be partially attributed to cultivation, harvesting, and extraction protocols, which can be avoided or managed, reinforcing the need for upstream operations to be aware of the downstream impacts.

We designed Experiment 2 to determine the palatability of LEA blended with commercially available supplements. Twelve steers were used in a 12 × 12 Latin square experiment consisting of 12, 4-day periods to evaluate palatability of LEA-containing supplements in steers consuming Bermuda grass. Each period included 3 days when we fed steers a test supplement and a 1 day washout when we fed steers DDG. We formulated supplements with different carrier ingredients (DDG, CSM, or liquid supplement, LS) at varying levels of LEA inclusion. Intake and time required for consumption were recorded daily. We observed complete consumption ≥ 91% of the time for DDG- and CSM-based supplements, which was markedly lower (55%) when LEA was offered alone. However, the rate of consumption of LEA was similar ($p \leq 0.05$) to that of CSM-based supplements.

Our objective in Experiment 3 was to determine the optimal level of LEA supplementation to steers consuming low-quality forage and compare effects of supplementation on N metabolism and forage utilization versus a CSM control. Five steers arranged in a 5 × 5 Latin square had *ad libitum* access to oat straw. Treatments were infused ruminally once daily and included no supplemental protein control; LEA at 50, 100, and 150 mg N/kg BW; and CSM at 100 mg N/kg BW. Increased provision of LEA stimulated total digestible organic matter intake quadratically ($p = 0.01$) from 0.9 control to 1.6 kg/d (100 mg N/kg BW of LEA). Organic matter digestibility increased quadratically ($p < 0.01$) with supplementation, with maximization at 50 mg N/kg BW of LEA. At isonitrogenous levels of LEA and CSM, total digestible organic matter intake was similar ($p = 0.13$) as was organic matter digestibility ($p = 0.50$). Negative N balance was observed for all treatments except LEA provided at 100 or 150 mg of N/kg BW. Nitrogen balance was greatest (1.84 g N/d) at 100 mg N/kg BW of LEA. There were no significant differences between LEA and CSM supplementation in measurements of plasma urea-N, ammonia, or volatile fatty acid concentrations. Our observations suggest cattle provided LEA utilize forage in a similar manner to those provided supplemented CSM, indicating LEA has potential to replace CSM as a protein supplement in forage-based operations. Forage utilization is maximized when LEA is provided at 100 mg N/kg BW.

In Experiment 4, six steers were used in concurrent 3 × 3 Latin square experiments to determine the effect of LEA on nutrient utilization and mineral intake in steers consuming low-quality forage. Treatments included no supplemental protein control and isonitrogenous levels of LEA or CSM. Provision of isonitrogenous levels of LEA and CSM stimulated ($p \leq 0.05$) total digestible organic matter intake to a similar extent ($p = 0.98$) and organic matter and neutral detergent fiber digestion was similar for all treatments ($p \geq 0.23$). Supplemented steers retained and absorbed more N ($p \leq 0.02$) than CON. Supplementation of LEA resulted in a Ca:P ratio of 8:1, which was the only mineral imbalance observed. Overall, our results suggest LEA can be blended (up to 60%) with existing ingredients to create suitable protein supplements for grazing operations.

Market value and extent of incorporation of LEA in the beef industry will be heavily influenced by organic matter and protein content; thus efforts should be made to decrease the ash content, minimizing toxicity concerns, while increasing the protein fraction of LEA. Further research is necessary to fully understand the interactions and consequences of upstream processes and a method should be selected and streamlined for optimal oil yield and nutritive value of resultant coproducts. To minimize concerns of palatability and mineral toxicity, LEA will likely be blended with an existing feedstuff to create a supplement easily incorporated into existing operations. Our observations indicate LEA can be blended (up to 60%) with DDG or CSM to formulate a supplement that would provide acceptable levels of protein and minerals without adversely affecting palatability.

Evaluation of Algae Coproducts in Animal Feeds

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Microalgae are unicellular organisms that live and reproduce in many forms of water (saline, brackish, fresh, and waste waters). These algae use sunlight, CO_2, and added nutrients to produce carbohydrates, proteins, and lipids. The lipid fraction of the microalgae can be extracted and then converted through biofuel production, mainly for biodiesel. This makes fuel from microalgae suitable for transportation and aviation applications. Use of algal biofuels as a jet fuel is of interest to the U.S. Department of Defense, specifically the Air Force Research Laboratory. Chisti (2007) compared the oil yield of various oil-producing crops with that of microalgae to show that it out-produces corn, soybean, and canola, while using a fraction of the available cropland in the United States.

Microalgae are generally grown by two different methods: open-air raceways, also called "ponds," or closed photobioreactor systems. Each methodology has its benefits and complications. The open pond system is less costly to start up over the closed system; however, it shows susceptibility to contamination as microorganisms are introduced via weather systems. Closed photobioreactor systems, while being more costly in terms of infrastructure, allow for control of variation in temperature, contamination, pH, etc., that is not easily managed in outdoor ponds.

The remaining portions of microalgae not used in biofuel production—carbohydrates, protein, and unextracted lipids—offer potential as a coproduct that may be available in excess of the main oil product. This is due to differences in the lipid content of microalgal strains, which varies anywhere from 20% to 77%. The use of lipid-extracted algae (LEA) as a coproduct requires the evaluation of multiple industries to determine the suitability of LEA use. One such sector is the animal feed industry, where LEA may be used as a high-protein animal feed. Therefore, we conducted two experiments to determine the influence of LEA on organic matter (OM) digestibility, N digestibility and flow, and rumen fermentation when included in a forage or concentrate diet using continuous flow fermentation. Four dual-flow continuous flow fermenters (2700 ml) were used in 4 periods. Temperature (39°C), pH, solid (5%/h) and liquid (10%/h) dilution rates, and feed schedule were maintained constant. Each experimental period consisted of 6 d adaptation and 4 d sampling periods.

There were seven treatments consisting of six different samples of LEA and a soybean meal control (SOY). Samples of LEA were *Nannochloropsis* sp. or *Chlorella* sp. Diets for Experiment 1 were formulated to be 13.0% CP (DM basis) using either soybean meal (SBM) or LEA and to meet or exceed the requirements of a beef cow (450 kg) not pregnant and nonlactating. The forage portion consisted of sorghum-Sudan hay (6.4% CP and 46.2% TD; DM basis) and alfalfa (26.1% CP and 82.3% TDN; DM basis). Concentrate diets used in Experiment 2 met or exceeded the nutrient requirements of a growing steer (400 kg) and contained 85% fine ground corn and included 7% (DM basis) SBM or LEA.

Data were analyzed as a mixed-effects model considering the effect of each LEA compared to SBM. Orthogonal contrasts were used to determine the overall effect of LEA genus versus SOY. True OM digestibility was not influenced by LEA addition to forage diets ($p > 0.08$) but increased with *Chlorella* LEA addition to concentrate diets ($p < 0.01$) but not *Nannochloropsis* LEA. Degradation of N was greater for SOY with forage diets and LEA for concentrate diets ($p < 0.0001$). Total VFA was greatest for SOY in forage diets and increased when LEA was added to concentrate diets ($p < 0.0001$). Microbial efficiency (MOEFF) did not differ between SOY and LEA in forage diets ($p < 0.08$). In concentrate diets *Nannochloropsis* decreased MOEFF ($p < 0.01$). Microbial efficiency results for *Chlorella* were more variable than for *Nannochloropsis.* One *Chlorella* sp. increased MOEFF by 36% over SOY ($p < 0.05$) and the other *Chlorella* sp. decreased MOEEF by approximately 42% compared to SOY ($p < 0.01$).

Overall, the results from both experiments are promising for LEA as a protein feedstuff in ruminant diets. Further research is necessary to fully understand the interactions and consequences of upstream processes and what role algal strain plays in LEA quality.

Effect of Algal Meal on Blood Metabolites and Histology

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To further explore some of the potential uses of algae coproducts for animal nutrition, we designed a project to determine the effects of lipid-extracted algal meal as a component of finishing rations for ruminant and monogastric livestock. The main project consisted of two studies that were conducted at the Colorado State University Agriculture Research, Development, and Education Center (ARDEC) facilities. We conducted both studies for 28 days, with 8 animals per treatment. Both studies had similar diet nutritional profile to a diet of a traditional commercial finishing facility.

The first study used crossbred wethers (n = 40; initial BW = 45.3 kg + 3.5) in a randomized complete block design to evaluate the effects of titrated concentrations of algal meal as a protein supplement on live performance, live health status, and carcass characteristics. Wethers were randomly assigned to one of the five treatments and blocked by time that they began the trial (10 animals per block). Treatments included (1) a control diet with soybean meal and rice meal as protein supplementation sources (CON), (2) 5% of algae meal on a DM basis (5% AM), (3) 10% of algae meal on a DM basis (10% AM), (4) 15% of algae meal on a DM basis (15% AM), and (5) 20% of algae meal on a DM basis (20% AM). We formulated all diets to be isocaloric and isonitrogenous. We fed all wethers a high-concentrate finishing diet once daily in individual stalls. Wethers were individually weighed on Day -1, 0, 21, and 28. On Day 22, we transported wethers to metabolic crates for determination of nutrient digestibility and retention. On Day 28, we transported animals to a commercial abattoir for harvest.

The null hypothesis is that the algae meal may be included in finishing rations at varying concentrations, without altering the performance of finishing wethers. Initial average (45.4 kg) and final average (44.5 kg) BW, average daily gain (ADG) for the adjustment period (0.24 kg/d), ADG for metabolism period (-0.84 kg/d), DMI (1.38 kg/d), and gain-to-feed (0.187) were similar ($p > 0.55$) across treatments. Furthermore, hot carcass weight, subcutaneous adipose depth, longissimus muscle area, calculated YG, marbling score, dressing percentage, muscle percentage, body-wall thickness, leg circumference, flank streaking, quality grade, carcass conformation, and carcass length were also similar ($p > 0.27$) across treatments.

Research results suggest that feeding up to 20% of algae coproduct meal as a replacement protein source to finishing wethers is feasible with limited impact on performance and carcass characteristics as compared to the standard protein sources that have been used by the industry. Further research may be necessary to determine the response of different levels of supplementation of algal meal for sheep, effects on animals in a different physiological stage, or effects on other ruminants in the finishing diet on performance and carcass merit.

The second study used crossbred barrows (n = 40; initial BW = 42.3 kg + 3.4) in a randomized complete block design to evaluate the effect of different levels of protein supplementation on live performance, live health status, and carcass characteristics. Barrows were blocked by time of start on treatments and randomly assigned to one of the five treatments. Treatments included (1) a control diet with soybean meal as protein supplementation sources (CON); (2) 5% of algae meal on a DM basis (5% AM); (3) 10% of algae meal on a DM basis (10% AM); (4) 15% of algae meal on a DM basis (15% AM); and (5) 20% of algae meal on a DM basis (20% AM). We formulated all diets to be isocaloric and isonitrogenous. We fed all pigs a typical high-concentrate dried-whole-corn-based finishing diet once daily in individual pens at ARDEC. Pigs were individually weighed on Day -1, 0, 21, and 28. On Day 21, we transported pigs to the ARDEC metabolism facility for the metabolic phase of the study. A lumbar spinal tumor was discovered in one of the animals fed the 15% AM treatment and it had to be removed from the study and euthanized. On Day 28, barrows were transported to a commercial abattoir for harvest.

Initial and final BW, average daily gain (ADG) for feedlot period, ADG for metabolism period, and gain:feed were significantly different ($p < 0.01$) across treatments, with greater performance values encountered in the control group and lesser values in the 5%, 10%, 15% and 20% AM. Furthermore, hot carcass weight, unribbed carcass weight, ham, loin, belly, butt, shoulder, feet, total parts, carcass length, ham circumference, and loin eye area were also decreased ($p < 0.01$) as the concentration of AM in the ration increased above 15% of the ration DM. Last rib fat, last lumbar vertebrae fat, first rib fat, belly thickness, tenth rib fat, LE L, LE a*, LE b*, marbling score, and color score were similar ($p > 0.17$) across treatments. These data suggest that feeding up to 20% of algae coproduct meal to finishing pigs has a negative impact on performance and carcass characteristics as compared to the standard protein sources that have been used by the industry. Further research may be necessary to determine the response of different levels of supplementation of algal meal for pigs and other monogastric species, effects on animals in a different physiological stage, and maximal tolerable levels of algae coproduct supplementation.

Utilizing the algal meal from *Chlorella* sp. as a source of protein in growing and finishing livestock rations is a possibility when the algal meal is priced the same or less than dried distillers' grains. At the same market price, the cost per kilogram of protein can be very competitive. The algal meal could also be considered a potentially competitive source of energy compared to dried distillers' grains when fed to ruminants, but likely not for nonruminant livestock.

In summary, the utilization of lipid-extracted algal meal from *Chlorella* sp. may be a safe alternative feedstuff as a protein source for growing and finishing livestock animals. For further understanding of other algal characteristics, effects, and economics, additional research is needed especially in large-scale experiments.

Lipid Extracted Algae as a Fertilizer and Soil Amendment

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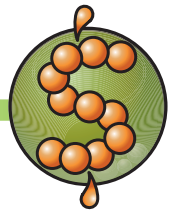
The potential exists to use lipid-extracted algae (LEA) produced from algae grown as a biofuel feedstock as a fertilizer and soil amendment. Typical commercial fertilizers are expensive to produce and/or mine. Agricultural coproducts, such as manure, poultry litter, and straw, are commonly used as renewable, organic alternatives to synthetic fertilizers. These alternative fertilizers are beneficial in that they provide carbon to the soil, which enhances microbial activity of the soil and enhances the release rate of minerals to better match that of plant uptake and utilization. The LEA can be sold from the extraction facility, which would provide an additional income source directly to the algae facility, or it can be sold with added value for further processing. Due to the agricultural equipment available for fertilizer and byproduct application to land, the LEA may be wet or dry to suit the application equipment available. The large area of cropland for application provides a sink for excess LEA or LEA that may have a lesser nitrogen concentration than that required for animal feeds or with components not desirable for other uses. Additionally, microminerals are the most expensive fertilizer to apply and are often deficient in soils. Tolerance levels of metals and micronutrients are greater for land use than for other potential coproducts and may be desirable, added-value uses of LEA as a soil amendment. Fertilizer required for algae production will compete with food production, and use of LEA as a fertilizer may replace some of the fertilizer needed for crop production. Therefore, application to the soil is a feasible and sustainable valuable coproduct.

The use of algae as a fertilizer is well documented in the scientific literature; however, there is little information regarding the use of LEA for soil fertility and crop production. Therefore, studies were needed to address key concerns regarding the use of LEA as a fertilizer and soil amendment. The key research objectives for this task were to (1) quantify chemical constituents and characteristics of LEA that are relevant to soil fertility and fertilization of crops; (2) determine the mineralization rates of carbon, nitrogen, phosphorus, and other key chemical components of LEA and the effect on soil microbes; (3) refine the application rate and assess the effects of LEA on crops; and (4) test field application and the effects of LEA application to soil carbon and structure.

Chemical composition of whole and lipid-extracted *Cyclotella* and *Nannochloropsis salina* are similar to the plant nutrients (N, P, K) provided from manure application. The sodium concentration is much greater than that of soil. Application of whole and lipid-extracted *Cyclotella* and LEA of *N. salina* to the soil increased both soil pH and electrical conductivity. Nearly half of the C of LEA mineralized after 280 days. LEA residue is labile and highly mineralizable compared to wheat straw, making it less likely to aid in C sequestration but a greater source of plant nutrients. Percent LEA N mineralized ranged from 30–55% for 5% and 1% LEA, respectively, at 56 days of incubation. After 56 days of

incubation, there is sufficient (350 mg N kg^{-1} soil) plant-available N for the 5% LEA amendment level and potential residue for plants in subsequent growing seasons. Yields of first cuttings of salt-susceptible and salt-tolerant crops were not affected by soil treatment with LEA. Rattoon crops of pearl millet and sorghum-Sudangrass were greater for 3.0% LEA and 1.5% LEA amendment level, respectively. The effect of treatments on growth during the rattoon crop and not the first cutting is likely due to the lag time of mineralization of amendment that is required before nutrients become plant-available. Greater SPAD measurements indicate a greater uptake of N by pearl millet and sorghum-sSdangrass from the 3.0% LEA-amended soil.

Addition of LEA has the potential to increase the pH of acidic soils, such as those common in the southeastern United States. Increased soil salinity may have negative effects on plant and soil microbial growth with repeated applications of LEA. Amendment of soil with LEA will not serve as a C sink; however, it is a readily available source of plant nutrients with potential for carryover to subsequent growing seasons. The LEA must mineralize prior to plant uptake and continues to mineralize for at least 280 days post application. Plants can use nutrients from LEA as a source of fertilizer, and the microminerals provided by LEA may serve as an economical source of these nutrients for agriculture. It is not recommended that LEA be applied annually to soils because of potential toxicity of sodium. Management practices are not yet refined based on this initial evaluation, but would likely warrant application every 3 to 4 years. It is suggested that further research to determine the effects of LEA fertilizer on crop production and soil health be conducted prior to recommending this practice on a large-scale basis.



Algal Oil Industry Model for Selected Sites

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The National Alliance for Advanced Biofuels and Bioproducts (NAABB) Consortium was formed to evaluate the potential of algal biofuels in our current energy scenario. The NAABB consortium team is made up of biologists, chemical and environmental engineers, economists, and others who have made significant contributions in algal biology, cultivation, harvesting/extraction, conversion to biofuels, economic/energy balance modeling, and sustainable resource management and who have developed new technologies. The purpose of this report is to present results of our study on the algal oil industry model for selected sites. Our goals are to identify environmental conditions (salinity, pH, temperature, nutrients) that enhance growth and lipid accumulation in *Nannochloropsis salina*, while limiting undesired organisms; compare productivity and stability of diverse algae assemblages (several species) to algae monocultures; and quantify and qualify invading organism of the New Mexico State University (and others) algae production systems.

Techno-economic assessment and life cycle analysis are used to quantify and qualify algae production systems. We took a different approach in understanding the current state of technology by applying the principles of production function and estimated a production function for *N. salina* using an unbalanced panel data set created by pooling data from several production facilities. Production function is one of the tools economists use to describe economic growth. Production function can be defined as an expression that relates an output of a production process to its input and their variables. Production function is used in both micro and macroeconomics to describe the availability of resources and their uses by a production process. The efficiency of allocation of resources aided by managerial and technological inputs can be estimated by production function. It does not estimate whether the process is efficiently managed.

Five different sites located in the southwestern United States provided the data to form an unbalanced panel of algae producers. They are coded as SAPPHIRE, CARLSBAD, PECOS, CORPUS (Christi) and NMSU. Mean, standard deviation, minimum, and maximum values are the variables used in this analysis. Some dummy variables were used because of confidentiality issues. Table 1 provides all the descriptive statistics.

Table 1. Descriptive statistics.

Variable	Units	Description	Obs	Mean	Std. Dev.	Min	Max
AFDWGM^{2}D	g/m^2/day	Ash free dry weight generated over growth period per day	288	4.7080	4.7680	-13.5470	39.2570
INITIAL	g/L	Initial ash free dry weight at the beginning of a growth period	288	0.4160	0.2400	0.0160	1.9330
PAR	µmol/(m^2 sec)	Average daily integrated PAR over the growth period	288	4.14E+07	1.17E+07	1.58E+07	6.18E+07
TEMPFLUX	F	Average min/max daily temperature fluctuation over the growth period	288	26.5830	7.3250	9.4330	39.6000
DAYS	#	Number of days in the growth period	288	18.0420	22.7340	3.0000	177.0000
AREA	m^2	Pond or raceway surface area size	284	146.3410	228.7000	2.7850	808.2710
PHFLUX	pH	Difference between the maximum and minimum pH in the growth period	218	1.0820	0.8930	0.0600	7.5000
SAPPHIRE	dummy	Dummy variable indicating growth at Sapphire location, Las Cruces, NM	288	0.163	0.37	0	1
CARLSBAD	dummy	Dummy variable indicating growth at Carlsbad, NM location	288	0.191	0.394	0	1
PECOS	dummy	Dummy variable indicating growth at Pecos, TX location	288	0.222	0.416	0	1
CORPUS	dummy	Dummy variable indicating growth at Corpus Christi, TX location	288	0.181	0.385	0	1
NMSU	dummy	Dummy variable indicating growth at the NMSU campus in Las Cruces, NM	288	0.243	0.43	0	1
WINTER	dummy	Dummy variable indicating growth occurred during winter months	288	0.16	0.367	0	1
SPRING	dummy	Dummy variable indicating growth occurred during spring months	288	0.181	0.385	0	1
SUMMER	dummy	Dummy variable indicating growth occurred during summer months	288	0.524	0.5	0	1
FALL	dummy	Dummy variable indicating growth occurred during fall months	288	0.135	0.343	0	1

A correlation matrix for the data set is produced. Largest positive correlations are found between PAR and TEMPFLUX (0.5135); INITIAL and TEMPFLUX (0.5255); and AREA and INITIAL (0.5635). Largest negative correlations in absolute terms are found between CORPUS and PAR (0.6073); CORPUS and TEMPFLUX (-0.8665); CORPUS and INITIAL (-0.5730); and WINTER and PAR (-0.6671).

Considerable variation among sites became apparent as we analyzed the data by site. Ash free dry weight production varies from an average of .987 g/m^2/d in CARLSBAD to an average of 8.632 g/m^2/d in nearby PECOS relatively. PAR

varies from 4.66E+07 w/m^2 to 2.78E+07 w/m^2 in CORPUS. Area varies from an average of 521.311 m^2 in CARLSBAD to 2.795 m^2 in CORPUS.
Initial density varies from an average of .709 g/L in CARLSBAD to an average of .276 g/L at SAPPHIRE. The site with the largest coefficient of variation for PAR is CORPUS (0.3446 μmol/m^2 sec) while the site with the smallest coefficient of variation for PAR is PECOS (0.0809 μmol/m^2 sec). SAPPHIRE has the largest coefficient of variation for fluctuations in pH (1.1780 pH); CORPUS had the least (0.7389 pH). Most observations (43.0%) were recorded in the spring. The least number of observations were recorded in the fall (10.5%).

We assume that the production function for *N. salina* can be represented by

$$Q_{it} = e^{\eta_i + \nu_{it}} F(X_{it})$$

where i = 1,2,..,5 is an index of locations, t is a time index, Q_{it} is output at time t for location i, X_{it} is a vector of factors that affect the algae production also indexed for time and location, is an unobservable firm-specific effect, and ν_{it} is a random component. In what follows, we assume that F(·) can be approximated as log-linear.

We denote the natural logarithm of Q_{it}, and X_{it} by q_{it} , and x_{it} , respectively. The logarithmic specification of the production function is

$$q_{it} = \varepsilon_X^Q x_{it} + \eta_i + \nu_{it} \quad (1)$$

Where ε_X^Q is a vector of production elasticities for the inputs included in x_{it}.

That is, a 1% increase in x_{it} causes an ε_X^Q percent increase in q_{it}. Table 2 presents the main results of this analysis, in which equation (1) is estimated using six different ways. Models vary either by the explanatory variables included or the estimation methodology. Model 1 is the simplest model, using ordinary least squares (OLS) and including only two variables: the natural log of PAR and TEMPFLUX. Model 2 is estimated using OLS and adds the natural log of area and the natural log of initial ash free dry weight. The coefficient on both is significant and negative. Model 3, also estimated using OLS, adds the log of number of days between harvests, which is significant and negative. Model 4 is estimated using Two State Least Squares (2SLS), which substitutes an OLS estimate of the endogenous variable for the actual variable, thereby eliminating the endogeneity.

Model 5 adds the natural log of PHFLUX, which is insignificant. Interestingly, however, the adjusted R^2 for Model 5 is considerably higher than for Models 1 through 4. Model 6 re-estimates Model 5 using fixed effects least squares (FELS), which control for location by including a dummy variable for each location. Carlsbad is the reference location. The results are similar to Model 5 except that the coefficient on the natural log of DAYS is no longer significant.

Looking across the six models, there is reasonable stability in the coefficients. All variables retain the same sign in each specification, with two exceptions. The coefficient on the natural log of TEMPFLUX becomes insignificant in the presence of the log of PHFLUX in Model 5 and Model 6. The coefficient on the log of PHFLUX is also insignificant. The general stability of the coefficients lends credence to the models presented.

Table 2. Fixed effects estimation of the pooled-time-series, cross-section model.[a]

Dep. Variable: (ln) AFDWGM2D	Model 1 OLS	Model 2 OLS	Model 3 OLS	Model 4 2SLS	Model 5 OLS	Model 6 FELS	Model 7 FELS
	b/se	b/se	b/se	b/se	b/se	b/se	b/se
CONS	2.778***	2.646***	2.801***	2.898***	1.327***	2.144***	3.395***
	(0.271)	(0.258)	(0.254)	(0.280)	(0.341)	(0.448)	(0.018)
(ln) PAR	0.076**	0.079***	0.077***	0.076**	0.143**	0.106**	
	(0.017)	(0.016)	(0.015)	(0.017)	(0.018)	(0.020)	
(ln) TEMPFLUX	-0.172***	-0.136***	-0.114***	-0.100**	0.011	-0.070	
	(0.014)	(0.017)	(0.017)	(0.022)	(0.033)	(0.043)	
(ln) AREA		-0.007**	-0.007**	-0.007*	-0.043***	-0.035***	
		(0.003)	(0.003)	(0.004)	(0.009)	(0.010)	
(ln) INITIAL		-0.091*	-0.246***	-0.343***	-0.189**	-0.159	
		(0.055)	(0.056)	(0.091)	(0.091)	(0.113)	
(ln) DAYS			-0.055***	-0.089***	-0.029**	-0.014	
			(0.010)	(0.027)	(0.013)	(0.013)	
(ln) PHFLUX					0.008	-0.029	
					(0.018)	(0.019)	
SAPPHIRE						0.017	0.108***
						(0.034)	(0.027)
CORPUS						-0.021	0.210***
						(0.070)	(0.020)
PECOS						0.089***	0.179***
						(0.028)	(0.030)
NMSU						0.000	0.059***
						(0.000)	(0.021)
SPRING							0.050***
							(0.013)
SUMMER							0.048***
							(0.016)
FALL							-0.001
							(0.018)
N	286	284	284	284	214	214	287
R-sq	0.1642	0.1972	0.2919	0.2547	0.4670	0.5015	0.3850
Adj. R-sq	0.1583	0.1857	0.2791	0.2413	0.4516	0.4795	0.3696
AIC	-418.4764	-425.6960	-459.3470		-361.2409	-369.5534	-469.5640
F	73.63	39.42	44.68	29.93	52.23	41.13	68.35
Overid (Chi-sq)[b]				0.4290			
Endog (Chi-sq)[c]				2.210			

Notes:

[a]Robust standard errors in parentheses.

[b]Overid (chi-sq) tests the validity of chosen instruments ([ln] PRECP and [ln] average harvest amount), with the null hypothesis that instruments are independent and valid.

[c]Endog (chi-sq) tests exogeneity of the questioned explanatory variable, with the null hypothesis that the variable is exogenous. The null is not rejected in both tests.

* $p < 0.10$, ** $p < 0.05$, *** $p < 0.01$.

Thus, for the first time, we have used production function analysis to assess productivity to determine the feasibility of production of biofuels from microalgae. We have created a pooled-time-series, cross-sectional data set taken from five different production locations and used this data set to estimate a production function for *N. salina*. We assume that the output of *N. salina* depends on the initial concentration of *N. salina*, PAR, temperature fluctuations, number of days in the production cycle, pond or raceway surface area, pH fluctuations, and location.

The raceway surface area (AREA) variable may be significant in determining scalability. Our estimated models display reasonable stability across specifications, although the explained variation is somewhat low. We find PAR to have a positive effect on output and temperature fluctuations (when significant), area, initial concentration, and days in the production cycle to have a negative relationship. Temperature fluctuation was insignificant in two specifications and pH fluctuations were not significant. We also found that location dummies were a significant determinant of production. These dummies serve as indicators for differences in managerial technique and technology used.

Economic Sustainability of Algae Farms in Regions Identified by the 2012 DOE Harmonization Study

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NAABB scientists have developed numerous technologies for improving production, cultivation, harvesting, and extraction of microalgae for biofuels. The purpose of this report is to present an economic and financial analysis of four key technological advances from NAABB scientists. Numerous other technologies could be analyzed, but for the present study one technological breakthrough was selected from each of four areas: algal biology, cultivation, harvesting, and extraction. We compared the technologies to the state-of-the-art technologies pre-NAABB.

For the economic and financial assessment we used a Monte Carlo farm-level model that simulates the monthly operations of an algae farm for 10 years. The economic model explicitly includes prices (inputs and outputs) and risks associated with algae production, harvesting, and extraction. The model used for the economic analysis was the Farm-level Algae Risk Model (FARM), which the authors developed for the NAABB project.

The scenarios analyzed included a base scenario, which represented technology prior to the NAABB project. A scenario to partition out the most promising harvesting technology was the same as the base but used electrocoagulation (EC) in place of centrifuges. The third scenario highlighted the best extraction process of hydrothermal liquefaction catalytic hydrothermal gasification (HTL-CHG) in place of a wet solvent extraction. The fourth scenario highlighted algal biology advances and used higher biomass production (164% more annual biomass than the base). Scenario 4 also included EC and HTL-CHG technologies. The fifth

scenario highlighted the new cultivation system, ARID, coupled with EC and HTL-CHG. The sixth scenario combined all of the NAABB technologies, EC, HTL-CHG, increased biomass production, and ARID.

We presented results for the six scenarios using key output variables in the FARM simulation model. By using Monte Carlo simulation techniques the model can estimate the probability of economic success for an algae farm and the risk about the average cost of production for algae lipid. The probability of economic success is the probability that the farm will generate a return on initial equity greater than the 10% discount rate. Total costs of production for lipid in the FARM model are the sum of all costs including dividends paid to investors who risk their investment in the farm's initial equity.

The initial analysis for each of the scenarios indicated the algae farm had a zero probability of economic success, so we conducted a sensitivity analysis using cuts in CAPEX and OPEX. The initial CAPEX and OPEX values for each scenario were reduced in 10% increments from 10% to 90% resulting in 100 combinations of cost reductions. Results of the FARM analyses are reported for the first combined reduction in CAPEX and OPEX that resulted in about a 50% chance of economic success, if possible.

The results in Table 1 summarize the probable economic outcomes for a large algae farm (4850 hectares with 9855 acre-feet of water) under the alternative technology assumptions. The first two lines in the table report the reductions in CAPEX and OPEX necessary to achieve the probability of success in row three. Under the base scenario a 90% reduction in both CAPEX and OPEX results in a zero probability of economic success and has a $15.73/gallon average cost of production for lipid. The EC harvesting system would reduce the total cost of lipid production to $12.55/gallon. Scenario 3 has a lipid production cost of $3.74/gallon if CAPEX is reduced 80% and CAPEX is reduced 70%. Even with the large reductions in CAPEX and OPEX, Scenario 3 only offers a 48% chance of economic success.

Combining EC, HTL-CHG, and improved biomass production in Scenario 4 along with 40% reductions in both CAPEX and OPEX results in a 57% chance of economic success and an average cost of lipid of $3.64/gallon. The 95% confidence interval on lipid costs is $2.93 to $4.35/gallon (Table 1).

Scenario 5 is an analysis of the NAABB cultivation system ARID. The scenario uses the EC and HTL-CHG technologies and the base biomass production level. Scenario 6 combined all four NAABB technologies. With a 30% reduction in CAPEX and a 40% reduction in OPEX, the ARID farm with improved biomass production, EC, and HTL-CHG has a $3.45/gallon average lipid cost with a 95% confidence interval at $2.74 to $4.16/gallon. The farm has a 57% probability of economic success.

Table 1. Summary of economic feasibility analysis for selected NAABB technologies on a 4,850-hectare algae farm.

	Scenario 1 Base	Scenario 2 EC	Scenario 3 HTL-CHG	Scenario 4 Biomass, EC, and HTL-CHG	Scenario 5 ARID, EC, and HTL-CHG	Scenario 6 ARID, Biomass, EC, and HTL-CHG
Reduction in CAPEX	90%	90%	80%	40%	70%	30%
Reduction in OPEX	90%	90%	70%	40%	70%	40%
P (Success)	0.0%	0.0%	48.0%	57.2%	75.8%	57.4%
Average Total Lipid Cost $/gal	15.73	12.55	3.74	3.64	3.52	3.45
Lower Confidence Interval TC $/gal	8.84	6.48	2.91	2.93	2.77	2.74
Upper Confidence Interval TC $/gal	22.97	19.10	4.58	4.35	4.25	4.16

The results of the economic assessment for NAABB technologies indicate that significant advancements have been made in reducing average costs of production. Even with 90% reductions in CAPEX and OPEX the pre-NAABB technologies had zero probability of economic success and lipid production costs greater than $15/gallon. For the two best scenarios (4 and 6), the probability of economic success is 57% after reducing CAPEX and OPEX by 40%. With NAABB technologies and 40% cuts in CAPEX and OPEX, cost of algal lipid averages about $3.50/gallon and has a 95% confidence interval in the range of $2.74 to $4.35/gallon.

Future microalgae to fuel research should focus on increasing biomass production without increasing costs of production, and reducing CAPEX for harvesting, extraction, and cultivation. For every 1% increase in biomass production net cash income increases 6%. A 1% reduction in CAPEX for cultivation, harvesting, and extraction increases net cash income 0.9%, 0.8%, and 0.7% respectively.

National and Global Economic Effects of Policies Establishing Large-scale Algal Fuel Production

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We introduce algae production into a general equilibrium model of the world economy with special emphasis on agriculture, biofuels, and bioenergy policy. Algal

activities are specified using information from other projects within the NAABB consortium. We consider the economic and food insecurity effects of encouraging 5 billion gallons per year (bgpy) of algal biofuel production and use. We consider five possible technological scenarios, four based on estimates of costs for current technology and one based on possible future technological improvements. For each configuration, we consider the institution of a blending mandate within the EPA's Renewable Fuel Standard (RFS2) framework and a volumetric blending tax credit similar to that which exists for biodiesel. All scenarios reflect updated labor and capital endowments corresponding to projections for the year 2030 and increased yields of agricultural production activities.

Under currently available technologies, algal biofuels are quite expensive to produce relative to competing biofuels. Under currently available technologies, introduction of government programs that would result in production and consumption of 5 bgpy of algal biofuels as a component of a total goal of 36 bgpy would incur substantial annual monetary expense relative to biofuel portfolios without an algal component. The incremental societal monetary cost of including algal biofuels in the portfolio using current technologies ranges from $5 billion to $400 billion per year.

If 5 bgpy of algal fuel use is achieved using an algal blending mandate, the monetary burden is borne primarily by consumers in the form of higher blended-fuel prices. If such use is achieved by a blending tax credit, the burden is borne primarily by taxpayers.

Very large improvements in algal production technology are needed to make the net societal monetary costs of a biofuels portfolio that includes 5 bgpy of algal biofuels similar to the costs of portfolios without algal fuels.

Under current technologies, forcing production and consumption of 5 bgpy of algal biofuels would tend to increase global food insecurity by between 4 million and 12 million people in most scenarios. The changes are mediated predominantly by reductions in the value of U.S. crude oil imports.

If very large improvements in algal production technology are realized, the changes in food insecurity caused by increasing algal biofuels production will be similar to those caused by increasing production of other biofuels.

Overall, the changes in global food insecurity caused by increasing any U.S. use of any biofuel are small compared to the improvements caused by growing global capital stocks and increases in agricultural productivity.

Introducing government programs encouraging increased use of algal fuels may produce benefits unrelated to net societal monetary costs and global food insecurity. Mandated blending of higher-cost biofuels would tend to reduce consumption of fossil fuels with high lifecycle greenhouse gas emissions, both through direct substitution and by reducing total quantities of blended fuel demand. Algal fuels also have the potential to improve energy security and rural economic well-being. The possibility of encouraging the technological development and use of algal biofuels should be considered in light of all of these issues and should certainly be revisited in the event that substantial improvements in algal production technology are realized.

SUSTAINABILITY

Enhanced Resource Assessment Using the Biomass Assessment Tool

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Commercial-scale algae-based biofuel production will consume large quantities of land, water, and nutrients. The quantities required are largely dependent on geographic location. Proximity to transportation and downstream refining infrastructure is also critical. Therefore, spatial modeling and assessment of resource and infrastructure requirements and availability is crucial to economic planning and assessing location-based commercial viability and sustainability. A key component of this assessment is determining the relative value between algae products and production (resource) costs. The overarching objective of this project, in support of NAABB program goals and objectives, is to provide comprehensive, multi-scale assessments of microalgae production potential at farm scale for open pond systems and to aggregate this information to regional and national scales using readily available but highly detailed information.

The research conducted under this project builds on the capabilities of the Biomass Assessment Tool (BAT), previously developed by the Pacific Northwest National Laboratory (PNNL). The BAT provides multi-scale screening, physics- and biophysical-based modeling, and other analyses using the best and most currently available climate, water, land, and infrastructure data, along with environmental constraints, biomass growth rates, and resource requirements. Previously, the BAT has been applied to assess site-specific sustainable algal feedstock production rates based on climatic conditions, land suitability, consumptive and nutrient water requirements, and access to downstream infrastructure across a range of local to national scales. In this project, we extended the BAT to consider saline water availability and transport (seawater, saline groundwater, and produced water), growth characteristics and resource requirements for alternative algae strains, and evaluation of multiple trade-offs for prioritized site selection and resource allocations.

Previous assessments of national biomass productivity potential using the BAT assumed water supply was unlimited. However, it was recognized that freshwater availability is a critical constraint and that geographic contrasts in climate result in marked differences in water use efficiency between sites. For example, results showed that the amount of makeup water needed to grow a unit of algae varies by three orders of magnitude across the U.S. Consumption rates are largest in the Southwestern U.S. due to the combination of high evaporation and low precipitation. Conversely, sites in the Eastern U.S. consume significantly less water because of lower evaporation rates combined with pond recharge by precipitation.

A key advantage of using microalgae for biofuel production is the ability of some algal strains to thrive in waters unsuitable for conventional crop irrigation, such as saline groundwater or seawater. However, while the monetary costs to provide freshwater are relatively small, the costs to provide alternative saline waters are potentially large. To more thoroughly explore and understand these water issues,

we expanded the BAT to combine location-specific estimates of algal biofuel production and water demand with water availability from a variety of sources. To accomplish this, we developed a GIS-based model to estimate the availability and costs of providing water from a diverse range of sources. The model uses water consumption and biofuel production rates in conjunction with the water supply model to assess the range of viable water options and the most cost-effective source for each site.

To assess site-specific seawater and saline groundwater costs, we developed a routing model using GIS-based cost-distance analysis. The model links each potential farm site to the location of the most cost-effective water source. We determine the most likely water source for each site using the following rules. If sufficient freshwater is available to meet feedstock production demand, it is selected as the water source for the site. Otherwise, the least expensive alternative water source is chosen as long as it meets the screening criterion (percent of biofuel value). If freshwater is not available and both saline sources do not meet the screening criterion, the site is eliminated. Once the water source is selected for each site, priority within the down selection is based on biofuel production value minus water cost.

Initial application of the alternative water source model produced a number of findings. First, we found that seawater is an option that can be potentially considered up to 20 km inland from the coast depending on local water demand and construction conditions. This is a much larger distance compared to results from past studies, but our costs compare well to those of real world pipeline projects. Because of decreased transport distances and often smaller salinity compared to seawater, saline groundwater is typically more economical even for coastal locations, an exception being the Texas Coast, where 3.86E+6 m^3 yr^{-1} (1.02 bgpy) of seawater-based production is possible. It is well understood that freshwater supplies are a challenge in the Southwest and alternative saline waters are a possible solution. However, the current analysis shows that a limited number of sites in the region (364 of 23,016 sites) have economical access to saline waters, and a total of 6.21E+7 m^3 yr^{-1} (16.4 bgpy) of potential biofuel production is lost due to water limitations. However, many sites are less than 10% above the 20% cost criterion, and therefore there is considerable uncertainty in the production potential for saline groundwaters. From a water resources perspective, the largest portion of the production potential occurs in the Texas Coast, Southern U.S., Florida peninsula, and the south Atlantic coasts, where inexpensive freshwater is used for the majority of production. However, because this is not a full techno-economic analysis, we made no judgment as to the economic viability of any region, and there are many issues requiring further exploration before definitive conclusions can be reached.

Algae grow by converting solar energy during photosynthesis to chemical energy storage in the form of oils and other biomass. Commercial-scale production of algal biofuels will require strict, site-specific alignment of critical resource requirements (suitable land and climate, sustainable water resources, nutrients, etc.) with the growth performance characteristics and media requirements of particular algae species. The BAT utilizes a biophysics-based growth model to simulate biomass production for a generic algae species. While the generic model was adequate for the initial regional and national scale assessments of algal

biofuel potential and resource requirements, more realistic, strain-specific estimates of biomass production are needed to compare region-to-region and season-to-season performance of promising strains and assess the optimum combinations of strains, regions, water sources, nutrient sources, etc.

Another objective of this project was to modify the BAT to provide biomass productivity estimates for specific microalgae strains. We enhanced the growth model by determining species-specific parameters to calculate light utilization efficiency and the water temperature correction. Building on work conducted under the NAABB Cultivation task, we conducted experiments to measure the specific growth rate as a function of temperature and light intensity for two algae strains, *Nannochloropsis salina* and *Chlorella* sp. Resultant analyses based on the enhanced growth model showed significant cross-species growth characteristics in terms of average annual growth, peak and minimum site-specific growth rates, and tolerance for extreme hot or cold temperatures.

The final thrust of this effort was development and demonstration of a trade-off analysis methodology to enable prioritized site selection based on multiple criteria. The methodology is designed to conduct comprehensive, detailed trade-off analyses to enable prioritization of resource allocations for microalgae biofuel production. Key factors considered include climate, water source and type, algal strain, alternative carbon dioxide and nutrient sources, land availability and preparation costs, and access to transportation and downstream processing infrastructure. For the multi-parameter analysis, the criterion for site selection was that the specific resource costs be less than the value of the biofuel the site could produce. For the resultant collection of sites, the annual production potential is 23.8 bgpy of biofuel. The most favorable sites are long the Gulf of Mexico and the south Atlantic coasts where productivity is high, water consumption is relatively low, freshwater is generally more available, and land is flat. Other observations include the following:

- CO_2 is relatively important in all regions, particularly in the areas of high productivity.
- Freshwater costs are relatively low, while saline groundwater costs are proportionally high in the West.
- Land costs show up as significant in the Central Valley of California and the Eastern seaboard.

Life Cycle Assessment of Selected Processes for the Production of Fuels from Algal Biomass

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The Michigan Tech/UOP LLC life-cycle assessment (LCA) work within the NAABB Sustainability team has focused on providing scenario analyses to complement the primary LCA activity being performed within the research consortium. Starting with the Harmonized Baseline LCA model developed by

Argonne National Laboratory for renewable diesel fuel production, this work evaluated several aspects of the algae fuels life cycle that are not subject to variations within the primary LCA modeling work. We studied the environmental impacts of cultivation infrastructure development, fertilizer choice, hydrogen sources, non-lipid algal fraction usage, allocation methodology, and conversion to other fuel products such as jet fuel. We incorporated data and guidance from NAABB researchers, peer-reviewed literature, government reports, prominent LCA databases, confidential UOP process data, and international organizations such as the Intergovernmental Panel on Climate Change (IPCC). The study results provide important insights for consideration when planning for the future of this developing industry.

Through a variety of means we assessed the impacts of developing an algae cultivation infrastructure. We used IPCC Tier 1 methodology to assess potential emissions resulting from conversion of Gulf Coast and U.S. Southwest grassland, cropland, and forestland in several management conditions. This assessment was combined with guidance from Pacific Northwest National Laboratory about promising sites for algae raceway development to estimate industry-wide greenhouse gas (GHG) emission impacts due to direct land-use change (dLUC). Impacts of including pond liners in algal raceway facilities were assessed by using the Ecoinvent database to tabulate impacts of polyethylene liner production. Land preparation (bulldozing, leveling, and removing soil) activities were estimated with the help of an industrial partner using proprietary construction modeling software to estimate machinery and fuel-use impacts. LUC impacts appear to be a larger infrastructure development concern than pond liner deployment or land preparation and could result in GHG emission equivalent to one or several important processing stages such as algae dewatering and drying, especially if previously forested lands are cleared.

Although the lipid-extracted algae (LEA) fraction of algal biomass may have economic value as an animal feed, displacing soybean meal, cottonseed meal, or fish meal, initial LCA results indicate exporting LEA off-site as a coproduct would result in a worsening environmental footprint for algae biofuels. LEA that is reused on site after anaerobic digestion has considerable value as a source of heat, power, and nutrients in the algae biofuels life cycle. In our initial analysis, the benefit from displacing an animal feed in the technosphere does not appear to outweigh the burdens associated with replacing these requirements that LEA currently satisfies, resulting in a GHG emissions profile for algal fuels that is worse than fossil fuels in some cases.

Potential benefits of utilizing algae cultivation as a method of removing N and P from nutrient-laden wastewaters were assessed by combining peer-reviewed literature in this field with UOP technical assessments of whole-cell pyrolysis-based fuel production involving mixed culture, low-oil algae biomass. The potential to replace current nutrient removal techniques used by wastewater treatment plants with algae cultivation systems would confer significant energy savings credits in the algae fuels life cycle, making the process an appealing pathway for algal fuels with a low GHG-emissions burden compared to fossil fuels. An initial assessment of the potential for scale up of this fuel production strategy indicates that a limited amount of algal biofuels could be created in this manner, owing to the constraints of available municipal wastewater, although

future work to explore the suitability of key industrial wastewaters may reveal promising synergies between industrial operations. Our report details the methods and LCA results for these scenarios and other important considerations in the algae fuels life cycle.

Design of a Process to Convert Algae Oil to Biodiesel

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The primary purpose of our work was to design and optimize a chemical process that converts algae or algae-extracted triglycerides to transportation-grade biofuels. We obtained thermophysical and transport properties from the National Institute of Standards and Technology databank, the literature, and group-contribution methods (the Joback method for pure-component properties and the universal functional-group activity coefficients [UNIFAC] for liquid activity coefficients); however, this limited our scope to processes that could be accurately modeled by the group contribution methods, excluding pseudocomponents and supercritical systems. A report from the University of Arizona provided the distributions of triglycerides (TG) in algae; we simplified this to compounds for which we had reliable thermophysical properties and normalized, yielding 4.6 wt% trimyristin, 81.79 wt% tripalmitin, 2.5 wt% tristearin, 10.34 wt% trioleate, and 0.7 wt% trilinoleate.

Because of the restrictions mentioned above, we focused our efforts on the catalytic conversion of TG to fatty acid methyl esters (FAMEs). Acid catalysis was discarded because it led to high residence times and high temperatures, and enzymatic catalysis was deemed infeasible because of the fragility and cost of the enzymes involved. Therefore, we decided to explore base-catalysis. Our initial efforts involved aqueous base-catalysis, using sodium hydroxide; however, the only kinetic parameters available were for vegetable oils. After discovering the T-300 solid-base catalyst by Albemarle Catilin, a NAABB partner, we collaborated with them. The advantages of the T-300 catalyst over caustic soda are that it is environmentally benign, provides faster reaction rates, and can be separated from the liquid product with much greater ease.

A sample of oil from Solix Biofuels revealed that it contained too many impurities to be handled by a base-catalyst without pretreatment. We performed glycerolysis to reduce the acid number, and we discussed but did not perform degumming. Afterward, we performed three transesterification experiments at different temperatures so we could analyze the effect of temperature on reaction rate. Using the data obtained from Albemarle Catilin, 24 kinetic constants (pre-exponential factors and activation energies) were regressed to represent the 12 glycerolysis reactions and another 12 kinetic constants to represent the 6 transesterification reactions. These constants are recorded in the full report and will also be published in the literature.

We developed and simulated a process with Aspen Plus v.7.3.1 to convert approximately 67,000 kg/h of algae lipid into FAME. The lipid is combined with recycled glycerol and sent to a heater, where its temperature is raised to 209˚C. The preheated feed is sent to the glycerolysis continuous-stirred-tank reactor (CSTR), which is under vacuum at 0.464 bar. The fatty-acids are reacted with glycerol (forming monoglycerides) until they comprise less than 3 wt%. A vacuum system removes vapor wastes, including water from the reactions and air that has leaked into the vessel. The liquid effluent is sent to a decanter, which separates the refined oil from glycerol, the aqueous phase.

The glycerol is recycled, while the lipid is mixed with excess methanol (8000 kg/h, containing T-300 catalyst). The mixture is heated and sent to a CSTR, where the purified lipids are converted to the FAME product and glycerol byproduct. The effluent is filtered to remove the catalyst (which is recycled to the reactor) before being sent to a decanter. The decanter separates the FAME (light phase) and glycerol (heavy phase) by gravity; methanol distributes itself between the two phases. The light phase is sent to a second CSTR for further conversion. Its effluent is subjected to the same separation techniques and sent to a distillation column, where the FAME (67,000 kg/h) is recovered from methanol. The glycerol effluents from the decanters are combined and sent to a distillation column, where nearly pure glycerol (5000 kg/h) is recovered from methanol. The methanol effluents are combined and recycled to the beginning of the transesterification section, while the glycerol is recycled to the glycerolysis and cultivation sections.

Using the Aspen Process Economic Evaluator (APEE) as well as quotes from industry and the literature, we computed cost estimates for the glycerolysis and transesterification process equipment. The total depreciable capital cost was approximately $20 million. Chemical reactors (10 for glycerolysis and 2 for transesterification) were the most significant capital expense, accounting for over $14 million. Adding the costs for contractor engineering, general and administrative overhead, and contingencies yielded a capital cost of approximately $27 million. Operating costs were divided between the utilities cost ($2.5 million), catalyst costs ($11.6 million), methanol costs ($18 million), lipid costs (discussed below), and labor costs ($2.1 million). We took ethanol and utilities costs from the literature while we took labor costs from APEE. Direct pricing information for the T-300 catalyst was not available; therefore, a range of lifetimes for the catalyst was provided by Albemarle Catilin and combined with pricing data for sodium methoxide (a competitive catalyst) to provide the estimate.

We performed a techno-economic profitability analysis using an investor's rate of return at 10%, a project life at 15 years, a tax rate at 35%, and 2012 dollars. We took the price of algae-extracted oils from four sources; the differences between them spanned half an order of magnitude. The model that provided the most favorable cost estimates ($3.02/gal of crude lipid) was the Pan Pacific cultivation model coupled with the most promising models from the NAABB harvesting and extraction reports. The Pan Pacific model is purely thermodynamic and, consequently, represents the best achievable cultivation cost for a photosynthetic efficiency of 4% and a cell oil-concentration of approximately 37 wt%.

The Sun et al. results are based upon estimates by the National Renewable Energy Laboratory, Sandia National Laboratories, New Mexico State University, and Seambiotic. They involve a mix of processes; however, no clear specifics are provided. Davis et al. use Aspen Plus for simulation of their flow sheet. They compare open-pond raceways and photobioreactors for cultivation and flocculation with chitosan and centrifugation for harvesting; then they use high-pressure homogenizers for extraction. Richardson et al. examined the Davis et al. best-case scenario and coupled it with a risk analysis, examining parameters often ignored in other models (e.g., pond crashes). Both the Sun and Davis articles focus heavily on technologies and processing methods that were available pre-NAABB, in contrast to the Pan Pacific model, which presents an optimistic case based on emerging discoveries. The Richardson et al. model presents the most pessimistic scenario for pre-NAABB technologies ($12.74/gal of crude oil).

In conclusion, after working with algae-oil in the Albemarle-Catilin labs, it is clear that the extraction processes employed thus far are inadequate when base-catalysis is used. The combined glycerolysis and transesterification processes, using the T-300 catalyst, are a cheap and efficient method of converting algae oils into transportation grade biodiesel, provided the feedstock is acceptable. The major hurdles for commercialization of an algae-to-biodiesel process lie upstream of conversion, primarily in the cultivation section. Although no algae-based process has yet been proposed that can compete with traditional petroleum diesel, the results of the Pan Pacific model suggest that such a process is theoretically possible for currently attainable biological parameters (like photosynthetic efficiency and oil content in the cell).

Life Cycle Assessment of Greenhouse Gas Emissions of Algal Biofuels and Analysis of Future Production Fertilizer Needs

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There are a variety of technologies available for producing biodiesel from microalgae. Open systems, including raceways or ponds, require less energy and are cheaper than closed systems. The lower energy requirement also corresponds to lower greenhouse gas (GHG) emissions. There are multiple technologies available for dewatering; some, like flocculation, require daily materials, while others do not. Centrifugation is efficient at capturing cells, but has a high energy use. Extraction methods use dry algae, therefore requiring energy-intensive dewatering methods. Wet extraction methods are not commercially available, but would help the algae-to-biodiesel production process become sustainable. Fuel conversion methods turn extracted lipids into biodiesel. The lipid-extracted algae contains byproducts that can be used for energy production or they can provide high-value coproducts.

There are three main foci of this project: (1) evaluate what has been found previously in open pond growth life-cycle assessments, (2) determine the sustainability of the nitrogen and phosphorus used in algae growth, and (3) find the GHG emissions of dewatering technologies based on NAABB data. For the first task, we put energy and material results of nine authors with 24 different growth scenarios into the same functional unit of 1 kg of biomass. We then ran results through the UOP Renewable Jet Fuel Process to show how algae compare to other feedstocks in biodiesel production. The GHG emissions for all scenarios ranged between 0.1–4.4 kg CO_2eq/kg biomass, with the fossil energy demand of 1–48 MJ/kg biomass. When compared to other feedstocks like corn, soybeans, jatropha, and camelina, the lowest end of algae ranked the lowest along with camelina at 0.1 CO_2 eq/kg algae. Some algae scenarios were also significantly higher than any other feedstock at 4.4 CO_2 eq/kg algae, with soybeans the next closest at 0.5 CO_2 eq/kg biomass.

Secondly, to evaluate the nutrient requirements for algae, we assumed a baseline of 5 billion gallons per year (bgpy) of biofuels. We found compositions of *Nannochloropsis* and *Chlorella* and compared them to the 2011/2012 world surplus values of both nitrogen and phosphorus. To produce the target amount of biofuels, *Chlorella* would use almost 20% of the surplus and *Nannochloropsis* almost 40% for nitrogen. The same trend is seen for phosphorus, with *Nannochloropsis* using almost all of the commercial fertilizers. We evaluated nutrient recycling scenarios (anaerobic digestion, hydrothermal liquefaction, and catalytic hydrothermal gasification) to see how they could decrease the commercial fertilizer requirement. Catalytic hydrothermal gasification had the largest recycling percentages of nitrogen and phosphorus at 95% and 90%, respectively. For alternative sources of nutrients, we evaluated concentrated animal feeding operations (CAFOs), animal slaughterhouses, aquaculture, spent coffee grounds, and chicken litter. Wastewater provided less than 4% of the nitrogen and 3% of the phosphorus for *Chlorella* if it was grown in the Gulf region. The source with the largest potential was CAFOs, with broiler hen waste providing more than three times the required amount of phosphorus for *Chlorella* in the Gulf region. Combining recycling options with other nutrient sources would lower the nutrient requirements even further.

Technology choices like hydrothermal liquefaction (HTL) and anaerobic digestion (AD) can recover nutrients from residual biomass that can be used for algae growth. HTL can recycle 84% of the nitrogen in *Chlorella* and 74% in *Nannochloropsis*. This brings the highest nitrogen requirement of *Nannochloropsis* down from 33% of the world surplus to 9%. We found no results for phosphorus recycle. Anaerobic digestion recycles 75% of the nitrogen and 50% of the phosphorus for both algal species. The phosphorus requirement for *Nannochloropsis* fell from 101% of the world surplus to 50%. Using additional sources of nutrients like wastewater or animal waste could lower the amount of world surplus fertilizers required even further.

Finally, we compared six different dewatering technologies, which include membrane filtration, a combination of flocculation and belt filtration, electrocoagulation, acoustic harvesting, flocculation with chitosan, and a combination of dissolved air flotation (DAF) and centrifugation. The first five came from NAABB data available in the 18-month harvesting and extraction

report. The final technology was a model available in the Algae Process Description (APD), which is a modeling tool used with the Greenhouse Gases, Regulated Emissions, and Energy Use in Transportation (GREET) Model to find the greenhouse gas emissions of an entire algae-to-biofuel process. We expressed results in terms of renewable diesel (RD) for comparison. Of the NAABB technologies, membrane filtration required the lowest amount of energy at 0.1 MJ/day and the combination of flocculation and belt filtration the highest at 0.99 MJ/day. When running all technologies through an entire process to produce renewable diesel, we found that membrane filtration had the lowest GHG emissions at 42,000 g CO_2 eq/MMBtu RD. DAF and centrifugation had the highest at 55,000 g CO_2eq/MMBtu RD. To determine if one technology is better than another, the sustainability of the materials used should also be evaluated. Membrane filtration uses a ceramic membrane, flocculation and belt filtration uses aluminum chloride, electrocoagulation uses aluminum for electrodes, and flocculation uses chitosan. There may not be enough of some of these materials to support 5 bgpy. Acoustic harvesting requires no additional materials, but there are concerns about the scale-up of this technology.

A Techno-economic Analysis of Biofuels from Algae: An Integrated Systems Approach

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This study investigated an integrated systems approach to developing a process-systems design for algal biofuel production. We used Aspen Plus to simulate key inputs from the NAABB to offer a holistic process-engineering and techno-economic evaluation of commercial, sustainable biofuel production. It highlighted solutions to previously intractable problems while pointing to critical areas for future investigation in the ongoing search for optimizing process design and process economics.

Developing an integrated process model for algal biofuel production requires completing a series of critical steps to overcome many barriers, such as the need to establish reliable thermodynamic data and reactor kinetics for such a complex bioprocess. The model used Aspen Plus v.7.3 with related economic evaluation packages. We also used the Aspen Simulator Workbook interface to couple Aspen to Microsoft Excel, permitting comprehensive analysis and data checking, thus making both the process and data more accessible to the broader NAABB community.

The system design involved creating a heuristic model within Aspen Plus in a novel way whereby insights could be obtained rather than a particular solution advanced, such as open ponds versus photobioreactors. With this model we could simulate the conditions and specific needs of the NAABB process and accommodate variables such as location, species, evaporation, and oil distribution. Once constructed, it allowed us to simulate a full system process or isolate unit operations for closer investigation. The results obtained from the integrated process system model include a fully converged mass and energy

balance together with data on water, carbon, glycerol, and biomass recycle; reactor design and system optimization; photosynthetic efficiency and evaporation; pH; reactor depth; organism stability; and process economics. We undertook a techno-economic analysis of the results. It involved two components—conventional engineering costing of the process followed by a more inclusive financial/venture capital model that incorporated real-world economic factors such as capital, taxation, depreciation, and other costs that must be considered when modeling the true cost of sustainable, cost-effective commercial-scale algal biofuels and biomass production.

The following section highlights results and conclusions emerging from our analysis of the vast quantity of data obtained during the NAABB study.

- The lack of reliable thermodynamic data has always been a major barrier to simulating biological processes such as algal biofuels. In particular we had to establish the enthalpies, ΔH, of the reactions (First Law) and the free energies, ΔG, (Second Law) to find thermodynamically "lost work" that could be recovered—a key to developing more sustainable processes. There were even substantial differences of opinion in standard databases as to whether the entropy was positive or negative, let alone agreement on its magnitude. Such difficulties continue to impose a major and unnecessary constraint on developing efficient and sustainable processes.
- The fractional stoichiometry coefficients of biological reactions are notoriously difficult to generate balanced equations. We found a solution using matrix algebra to create a 7 x 7 matrix to solve for these coefficients. It can be readily operated in Excel.
- The strength of having a fully converged mass and energy balance is that only then is it possible to obtain results for key variables such as productivity, lipid accumulation, or reactor depth, which Aspen is able to integrate from inputs. It also can provide additional unexpected results, for example, the number of generations that the organism needs to pass through to achieve desired output. Curiously, in this context chemical engineering is thus putting a constraint on the biology. We also found that the complex ionic species data and solver built into Aspen was vital for establishing the complex equilibria and ionic species required for balanced nutrition of the algae. It predicted, and thus avoided, the onset of massive precipitation, which would have been extremely harmful to machinery and would have made key nutrients biologically unavailable.
- We created a series of flow diagrams to establish the critical flows of individual components. These are particularly useful in tracking the flow of carbon, nitrogen, phosphorous, and especially water through the production process. Mass flow and energy flow, both enthalpy and exergy, proved to be critical diagnostic tools.
- The problem of water was always implicit, but from the simulator perspective it seemed to dominate the process. Some ways to reduce water consumption were identified. Most importantly, it focused attention on the critical need for recycle. While the recycle of nutrients is often mentioned, the critical case for water recycle has been less well made. If the water is recycled, nutrients will similarly follow.

- As part of the investigation for ways to reduce water, we undertook some light-penetration studies with NAABB member data using finite element analysis as part of our integrated approach. We established an effective optical depth of 1 cm. There are a number of very good reasons why operating a very shallow reactor could be difficult, but the balance between incoming solar energy and the latent heat of evaporation showed that there was a range of operating conditions possible at shallow depths. We established a design equation to assist in identifying operating ranges.
- We undertook a series of simulations to examine the impact of varying types of recycle on process economics. We found that recycling water had the most significant effect while recycling carbon similarly had a major impact. Recycling glycerol also helped bring the primary cost of production of a gallon of biodiesel to approximately $3.50/gallon.
- We took techno-economic analysis one stage further by executing a venture-capital style financial modeling process to take full account of depreciation, financing costs, the time value of money, and taxation. While these other factors did add substantially to the costs, they were also overshadowed by the tax shields of 35% applied to the major costs in the first 10 years of the project. It had the result of lowering the apparent cost by about $1/gal rather than raising it. A major study of taxation effects is recommended.

Development of an Integrated Assessment Framework for Analysis and Design of Algal Farm Systems

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The task presented to the Idaho National Laboratory (INL) was to support the Pacific Northwest National Laboratory (PNNL) in developing an integrated framework for assessing algal systems. As part of this work, the INL has been developing an Algae Logistics Model (ALM) that enables dynamic spatiotemporal assessments. The model is developed in a system-dynamics software package that enables assessments at a designated time-step and enables operational constraints to be considered. The model is composed of process modules allowing the system design to be reconfigurable. The model is also developed to interface with growth and resource assessment models to perform spatiotemporal cost and performance assessments.

INL has been working with PNNL on integrating their Biomass Assessment Tool (BAT) with INL's ALM within the Integrated Assessment Framework (IAF). The ALM and IAF work were funded separately by DOE and leveraged by the NAABB as an assessment tool. BAT is a growth and resource assessment model capable of predicting potential growth, nutrients, and resources through spatiotemporal assessments. Since the ALM was developed to interface with growth and resource assessment models, we have been working with PNNL to create the IAF to enable cost and performance assessment considering varying levels of productivity across the country. Much of the work this year was focused

on integration and coupling of the two models within the IAF. We have worked closely together to better understand each other's models and their capabilities. This has allowed us to coordinate our development so that our models complement one another and provide data to better parameterize our model.

With the release of the manuscript titled Renewable Diesel from Algal Lipids: An Integrated Baseline for Cost, Emissions, and Resource Potential from a Harmonized Model (ANL, NREL, PNNL, 2012), the ALM has been updated to reflect those changes, allowing us to work from the harmonized baseline design. We have submitted a publication discussing the structure and capability of the ALM to the *Algal Research* journal. In addition to this paper, we are working on another publication with PNNL that discusses the IAF and its ability to perform cost and performance assessments at the regional and national scale through an integrated model. A valuable analysis performed by IAF relates to scaling of downstream harvesting and processing equipment taking into consideration historical variation in algal productivities at given sites.

CO_2 Sequestration through Microalgae Production at Cellana's Kona Demonstration Facility (KDF)—Flue Gas Capture, Treatment, and Delivery for Large-scale Microalgae Production

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One advantage of microalgae production is its high efficiency in CO_2 fixation while producing renewable bioproducts. Flue gas released by combustion of fossil fuels in power plants is the primary contributor to excess CO_2 that has been linked to climate change. The success of producing microalgae with CO_2 from flue gas will not only help to alleviate the trend toward global warming, but also reduce the operational cost by replacing pure CO_2 with flue gas. To test the feasibility of utilizing flue gas as a source of CO_2 at an algae production facility, an engineering design was provided to safely capture and deliver flue gas from on-site diesel generators to open algae ponds. The following flue gas criteria were achieved:

- The system should not put backpressure on the generator exhaust piping. The diesel generators were limited to less than 1 psi backpressure.
- The system should reduce the soot in the flue gas.
- The flue gas temperature must be reduced to less than 200°C.
- The material must be acceptable for sulfuric acid that may form in the piping.
- Health and safety concerns must be addressed through gas monitoring and alarms.
- The process parameters must be easily monitored and recorded.

Cellana uses three diesel generators to produce the power required for the facility that is operated off the local electrical grid. The three generators typically operate as two duty and one standby. We directed the flue gas from downstream of the

diesel generator muffler system to the test pond. We installed isolation valves downstream of the generator tie-in to ensure the gas from one generator was directed at a time. We designed a blower to pull the gas through a process tank and pressurize the treated gas to deliver at the pond approximately 20 feet away from the gas collection area. The process tank incorporated a seawater spray system. This system was designed to cool the flue gas and reduce soot in the system. We successfully reduced the flue gas temperature from more than 200°C to below 40°C and the soot was reduced. However, the process was still affected by the remaining soot and we subsequently installed an air filter upstream of the blower. The spray system also slightly reduced the NO_X and SO_2. Material selection was set at each stage of the process. We selected the material to withstand high temperatures and the potential for acid to precipitate in the system. We posted signs alerting staff to hot surfaces and the potential for carbon monoxide, sulfur dioxide, and nitrogen dioxide to be released into the surrounding work area.

We installed ambient air quality monitoring equipment at the process tank and at the test pond. The air quality monitoring system was tied into the facility controls and was set to alarm and shut down the process if air quality was above OSHA requirements. Ambient air quality was monitored and showed no detectable CO, NO_2, or SO_2 during the flue gas operation.

We also used the facility control system to control the flue gas delivery based on test pond pH and temperature of the process tank. Alarms and automated controls incorporated into the design mitigated operator time and tracked system performance.

We successfully grew the *Nannochloropsis oceanica* strain *KA19* with treated flue gas from onsite diesel generators, with more than 100 kg of biomass produced in total. When growing on flue gas CO_2 and pure CO_2, *KA19* exhibited no difference in growth rate, nutrient uptake rate, and biomass productivity. The biomass harvested from culture grown on different sources of CO_2 showed no difference in biochemical composition, including content of lipids, protein and carbohydrate, and fatty acids profile. The contents of heavy metals, including arsenic, cadmium, and lead, were elevated slightly in the biomass from flue gas, but were all below the guidelines published by the International Union of Pure and Applied Chemistry for single cell protein. We observed dust in the test pond, leading to the installation of an air filter upstream of the blower. We will conduct future experiments to examine the effectiveness of the air filter.

Geochemistry of Algal Cultivation Media Derived from Recycled and Alternative Water Sources, Including Oil and Gas Produced Water, Brackish Groundwater, and Tertiary Treated Wastewater

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Sources of water other than freshwater are needed to support an algal biofuels cultivation industry. Both oil and gas produced waters and recycled media can provide valuable sources of water, salts, and nutrients to a cultivation process. The feasibility of using these sources is still poorly understood. Here we examined a state-of-the-art treatment process for oil and gas produced water, including organic and metals removal. We also tested multiple rounds of cultivation, including media, recycled media, and bulk algae, where recycled media was used as a significant component of total media volume. Treatment of produced water succeeded at removing all but trace levels of volatile and semivolatile organic compounds, which are toxic to algae and the environment. Adjusting pH and polymer flocculation also removed trace metals, some major cations, and divalent and transition metals including calcium, iron, manganese, zinc, chromium, and copper and the non-metal arsenic. Treatment converted some reduced sulfur to sulfate, indicating increasingly oxidizing conditions in the water during treatment. Feedstock quality of algae cultivated in produced water was found to be similar to algae cultivated in a freshwater-based media mix (testing conducted by New Mexico State University for Eldorado Biofuels, Figure 1).

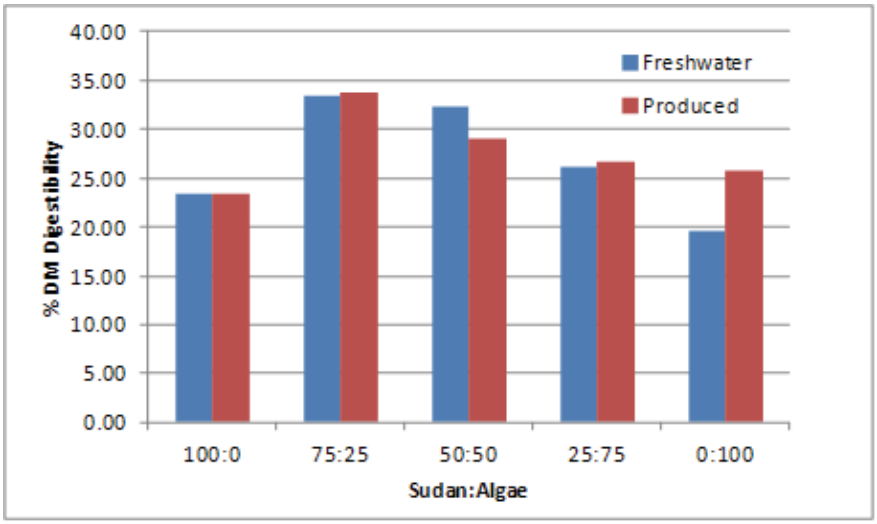

Figure 1. Digestibility studies of algae grown in freshwater and produced water media, Jal, NM. Information courtesy of Eldorado Biofuels.

Recycling did not produce increases in dissolved divalent and transition metals over time. Losses of metals via sorption to the algae were the likely sink for metals removal from the system, based on algae total metals analysis. Metal composition of the harvested algae correlated with the dissolved metals concentrations in the

media, also showing no significant increases. While salinity (predominantly NaCl) eventually increased over time across multiple cycles of cultivation and reuse, overall production metrics including lipid yield and ash-free dry weight were unaffected. Both produced water and recycled media show promise as potential sources of water, salts, and nutrients for algal biofuel cultivation.

Geochemical modeling

Two major limiting factors associated with algal cultivation are water and nutrient availability. Tertiary wastewater, oil and gas produced water, seawater, and brackish ground waters not only provide alternatives to freshwater; they may also contain much needed salts and nutrients, including nitrogen compounds, phosphate, and trace metals. We examined the quality of various alternative water sources and their potential use as a cultivation media mix solvent. Using Geochemist's Workbench to perform complex geochemical calculations, we examined the effects of mixing samples from various alternative water sources to create the commonly used medium f/2 minus silica (f/2-Si) When f/2-Si is mixed with pure water in the laboratory it tends to precipitate minerals. Geochemical modeling suggests that these minerals are predominately calcium carbonate and apatite ($Ca_5(PO_4)_3(F,Cl,OH)$. Mineral scale formation is undesirable because it can reduce nutrient availability to the algae, while the precipitated mineral matter can harm moving parts (e.g., pumps, centrifuges) in an algal cultivation system. Calcium (Ca), phosphorous (P), iron (Fe) and other elements already present in alternative water sources can increase this mineral formation which, in turn, can decrease nutrient solubility, thus making elements such as Fe and P less bioavailable to algae.

Taking solubility into consideration, we simulated the dissolution of the media mix into each alternative water (f/2-Si + alternative water). We next varied the quantities of added salts and nutrients to f/2-Si levels and then evaluated mineral precipitation, including Fe and P solubility. To further reduce mineral precipitation and increase Fe and P solubility, the amounts of Ca, magnesium, P, Fe and other elements were optimized in the model by decreasing the added amounts of each and by eliminating fluoride. These steps resulted in a significant reduction in apatite and calcium carbonate mineral formation in the modeled system. Geochemical modeling of alternative water sources, along with a thorough knowledge of initial water and desired media compositions are important to conserve nutrient use and prevent mineral precipitation.