Bioenergy Technologies Office (BETO) 2021 Project Peer Review

Integrated Low Cost and High Yield Microalgal Biofuel Intermediates Production

Algal Biomass Yield Phase 2 (ABY2) DE-EE0007691

03/10/2021

Advanced Algal Systems

Dr. Aubrey Davis, Dr. John Benemann, P.I.

MicroBio Engineering Inc.

Algal Biomass Yield Phase 2 (ABY2) Project Participants

- **U.S. Department of Energy**
  - Dan Fishman (project officer)
  - Evan Mueller (project monitor)

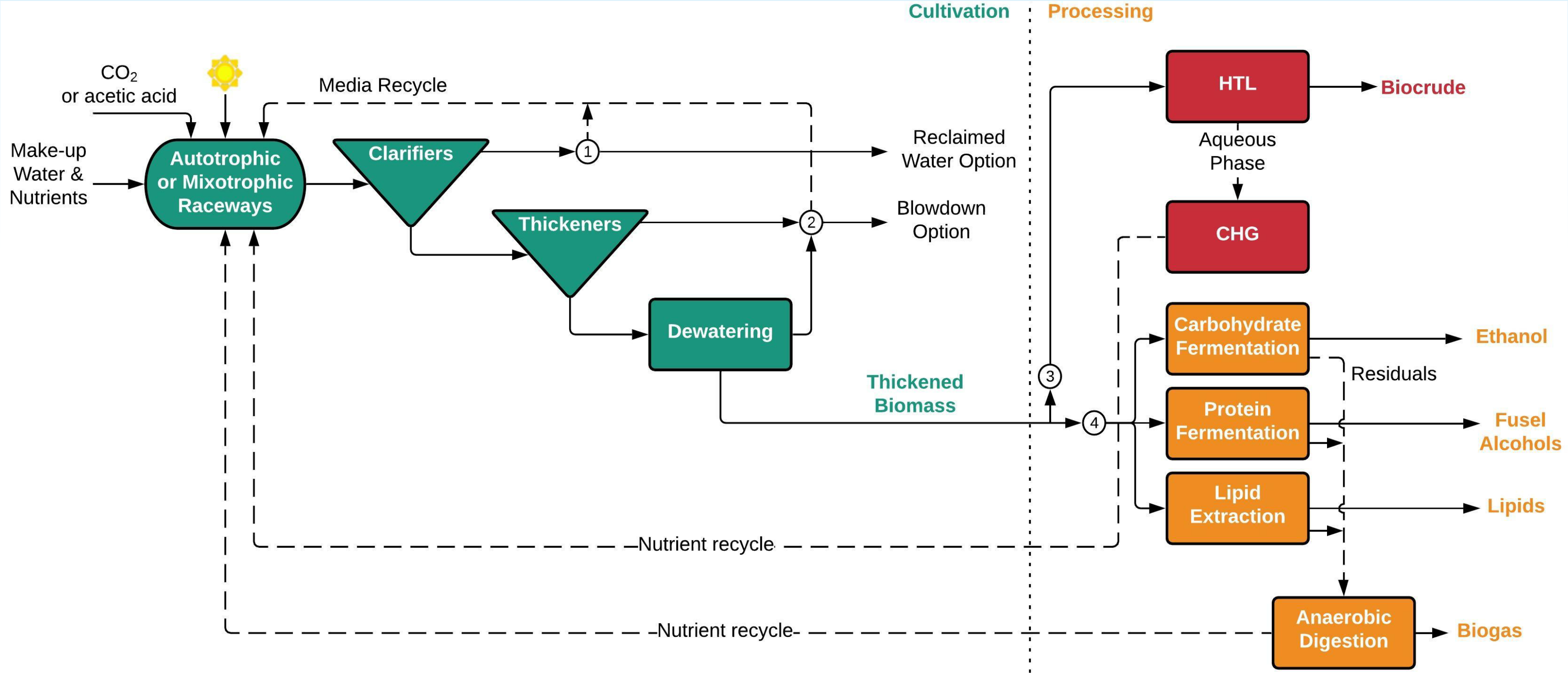
- **Project Team**
  - Todd Lane & Krissy Mahan, SNL
  - Ryan Davis and team, SNL
  - Mike LaMont, Steven Pflucker and team
  - Tryg Lundquist, Ruth Spierling, Staff and students at Cal Poly
  - Aubrey Davis, Cooper Gibson and MicroBio Engineering staff
  - John Benemann, MicroBio Engineering, P.I.

- **Other Participants**
  - Juergen Polle, Consultant

Project Overview

- Developed the capability for over 3,700 gal/ac-yr of biofuel intermediates through microalgal strain improvement, mixotrophic approaches and novel conversion technology.
- Demonstrated the annual productivity of improved strains and benefits of mixotrophic cultivation in outdoor raceways.
- Modeled the autotrophic and mixotrophic processes through TEA and LCA.

Full-scale process flow diagram



1 – Management

TASK 1: Verification
(Lead: MicroBio Engineering)

TASK 2: Project Management, Coordination
(Lead: MicroBio Engineering, Dr. John Benemann)

TASK 3: Outdoor Cultivation and Harvesting
(Lead: Cal Poly, Dr. Tryg Lundquist)

TASK 4: TEA/LCA Studies
(Lead: MicroBio Engineering, Dr. John Benemann)

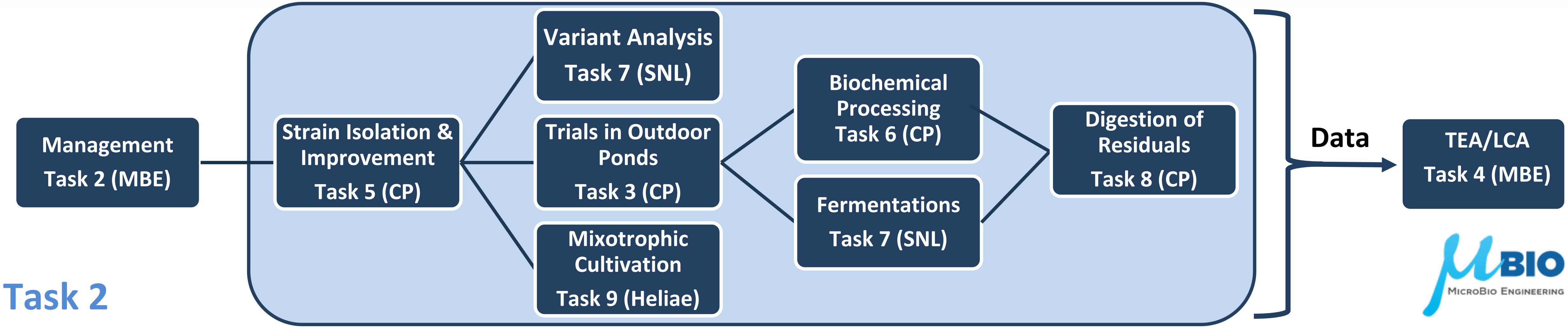
TASK 5. Strain Selection, Isolation, Characterization
(Lead: Cal Poly, Dr. Aubrey Davis)

TASK 6. Biomass Processing, Fermentations
(Lead: Cal Poly, Dr. Tryg Lundquist)

TASK 7. Biochemical Conversion and Variant Analysis
(Lead: SNL: Dr. Ryan Davis, Dr. Todd Lane)

TASK 8. Anaerobic Digestion Conversion
(Lead: Cal Poly, Ms. Ruth Spierling)

TASK 9. Mixotrophic Productivity & Management
(Lead: Heliae, Dr. Mike LaMont)



Task 2

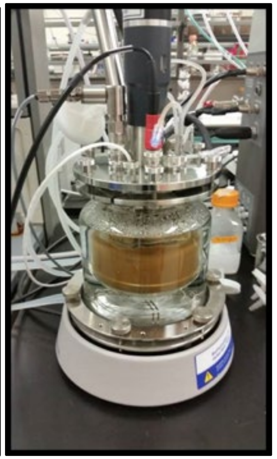
2 – Approach

This project targets critical barriers including productivity improvements, long-term cultivation outdoors, improvements in cultivation practices, nutrient recycling, and improvements in conversion technology.

Specifically, this project was to:

1. Develop more productive algal strains through Adaptive Laboratory Evolution (ALE).
2. Demonstrate improved strains in wastewater in outdoor raceway systems.
3. Leverage mixotrophy in algal cultivation to improve productivity.
4. Convert algal carbon to biofuel intermediates through extraction, fermentations and digestion of residues.
5. Validate economic and environmental performance through TEA/LCA.



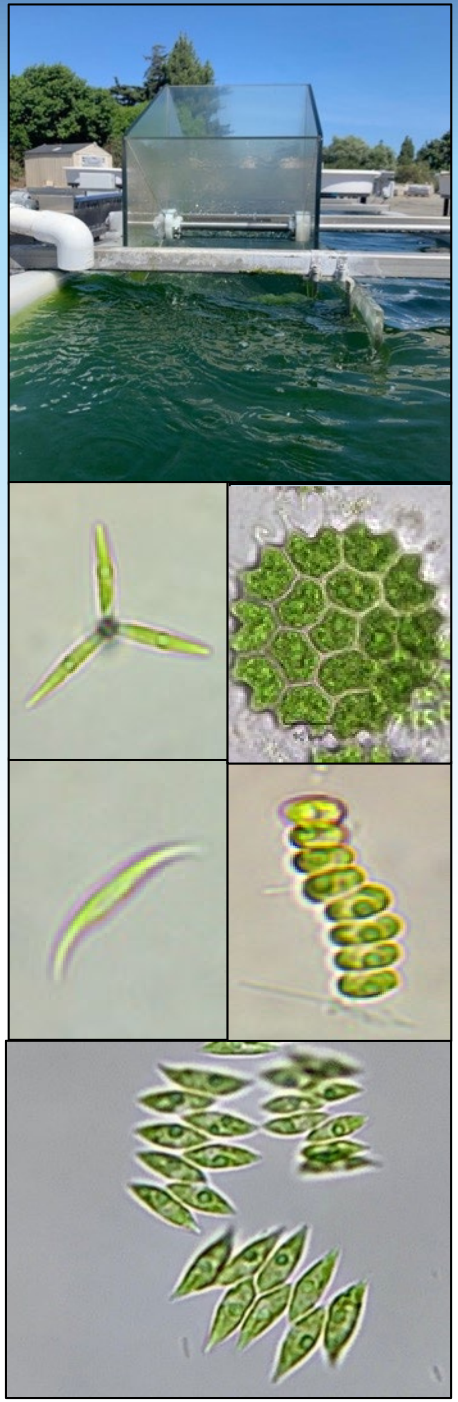




3 – Impact

Met the BETO MYPP goal of developing technologies that enable mature modeled annual average algae yields of 3,700 gallons /acre year and advanced progress in the field toward the next 5,000 gallons/acre year goal.

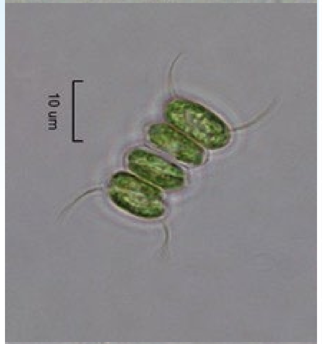
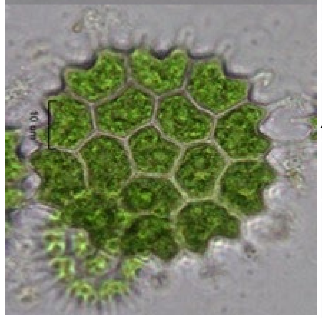
These advances make algae biofuels potentially commercially feasible, in particular for mixotrophic pathways.

Results have been presented at conferences and are being disseminated in peer reviewed journals and through the Joint Genome Institute PhycoCosm portal.

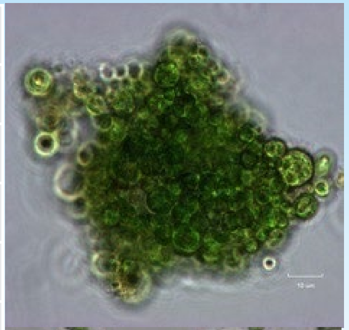
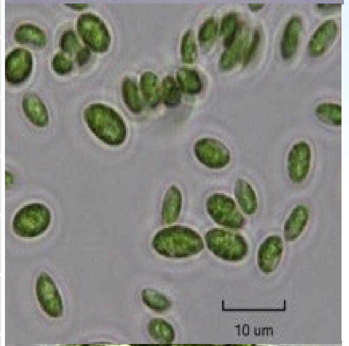


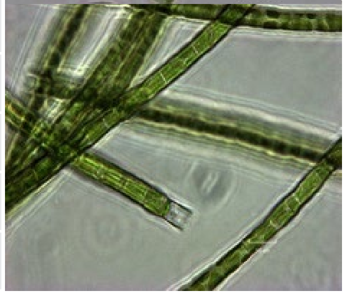
4 - Technical Accomplishments

Screened 15 strains and chose 3 for continued effort based on a list of criteria, including productivity, composition, mixotrophic capabilities and genome availability (chosen strains highlighted in blue).

Name	Number	Location
Scenedesmus obliquus	**UTEX 3031**	**Lab/Out**
Scenedesmus obliquus	UTEX 393	Lab
Scenedesmus obliquus	DOE 0111	Lab
Desmodesmus sp.	DOE 1051	Lab/Out
Desmodesmus sp.	DOE 1357	Lab
Desmodesmus armatus	**UTEX B 2533**	**Lab/Out**
Chlorella sorokiniana	DOE 1412	Lab/Out
Chlorella antarctica	UTEX 1959	Lab/Out
Coelastrella sp.	DOE 0202	Lab
Chlorococcum sp.	UTEX B P1	Lab/Out
Selenastrum capricornutum	UTEX 1648	Lab
Ankistrodesmus sp.	UTEX B 3015	Lab
Wastewater isolate	RWW3	Lab
Tribonema minus	**UTEX B ZZ1240**	**Lab/Out**
Pseudopediastrum boryanum	RWW8	Lab/Out

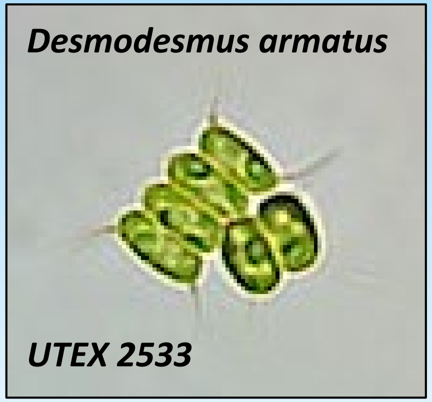





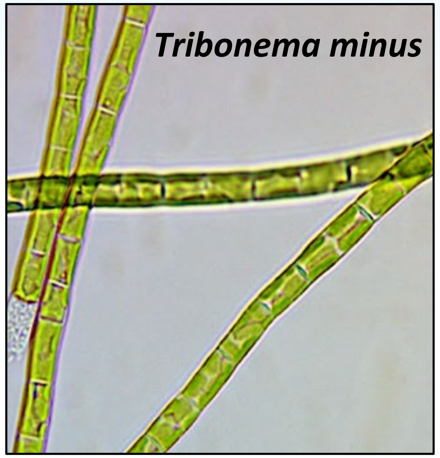
Task 5

Genomic Characterization of Promising Strains

Strains from UTEX



Wastewater Isolates



A genome sequence was available for *S. obliquus* (UTEX 3031) at the beginning of this project. The genome was re-sequenced in order to characterize genetic variants (following slides).

Additionally, bioprospecting efforts continued for the duration the project, and the genomes of promising strains were sequenced, assembled and will be made available through the JGI PhycoCosm portal.

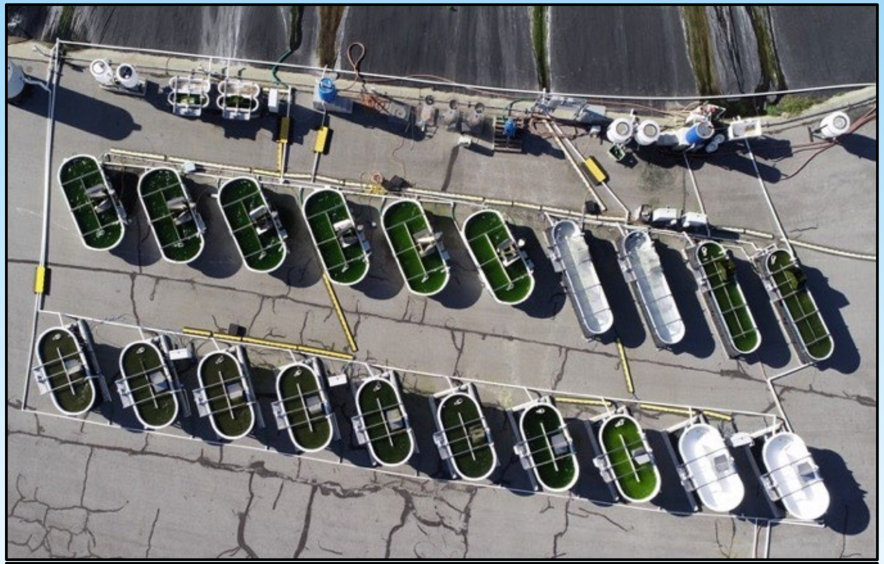
Species	Method	Status
T. minus (UTEX B ZZ1240) (wastewater isolate)	PacBio & Illumina	Assembled & Annotated
D. armatus (UTEX B 2533)	PacBio & Illumina	Assembled & Annotated
C. zebra (wastewater isolate)	PacBio & Illumina	Assembled

Task 5, 7

Sandia National Laboratories

MBIO MicroBio Engineering

Outdoor Cultivation at the Algae Field Station Operated by Cal Poly with MicroBio Engineering at the SLO Water Resource Reclamation Facility



Objectives to:

- Determine outdoor robustness of monocultures
- Establish annual averages for baseline productivity (g AFDW/m^2-day)
- Compare improved strain productivity to wild type
- Produce and process biomass for fermentation tasks

Cultivation Approach:

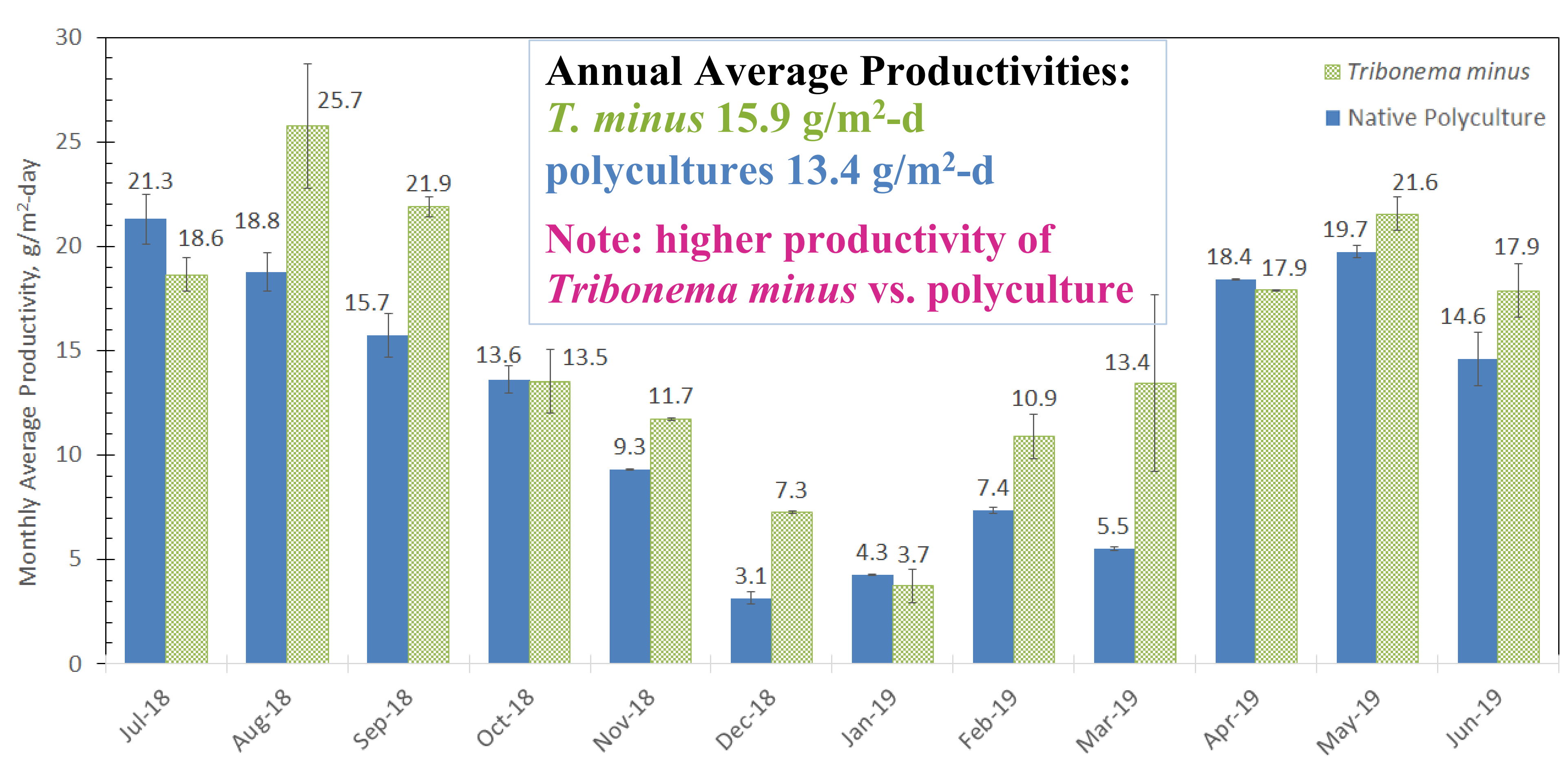
- Ponds operated in duplicate in reclaimed wastewater
- All productivity determined from pond effluent
- Diluted to a 2-4 day hydraulic residence time
- Where HRT=V(L)/Q (L/day)
- 30cm depth
- AFDW sampled 3x per week

CAL POLY

μBIO MicroBio Engineering

Task 3, 6

Tribonema minus isolated from wastewater polyculture demonstrated to be robust, productive and easy to harvest over a year of cultivation outdoors.

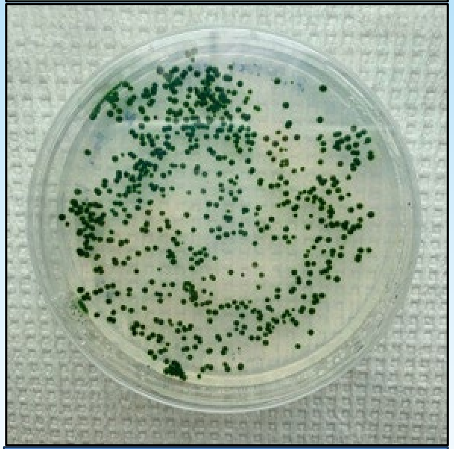


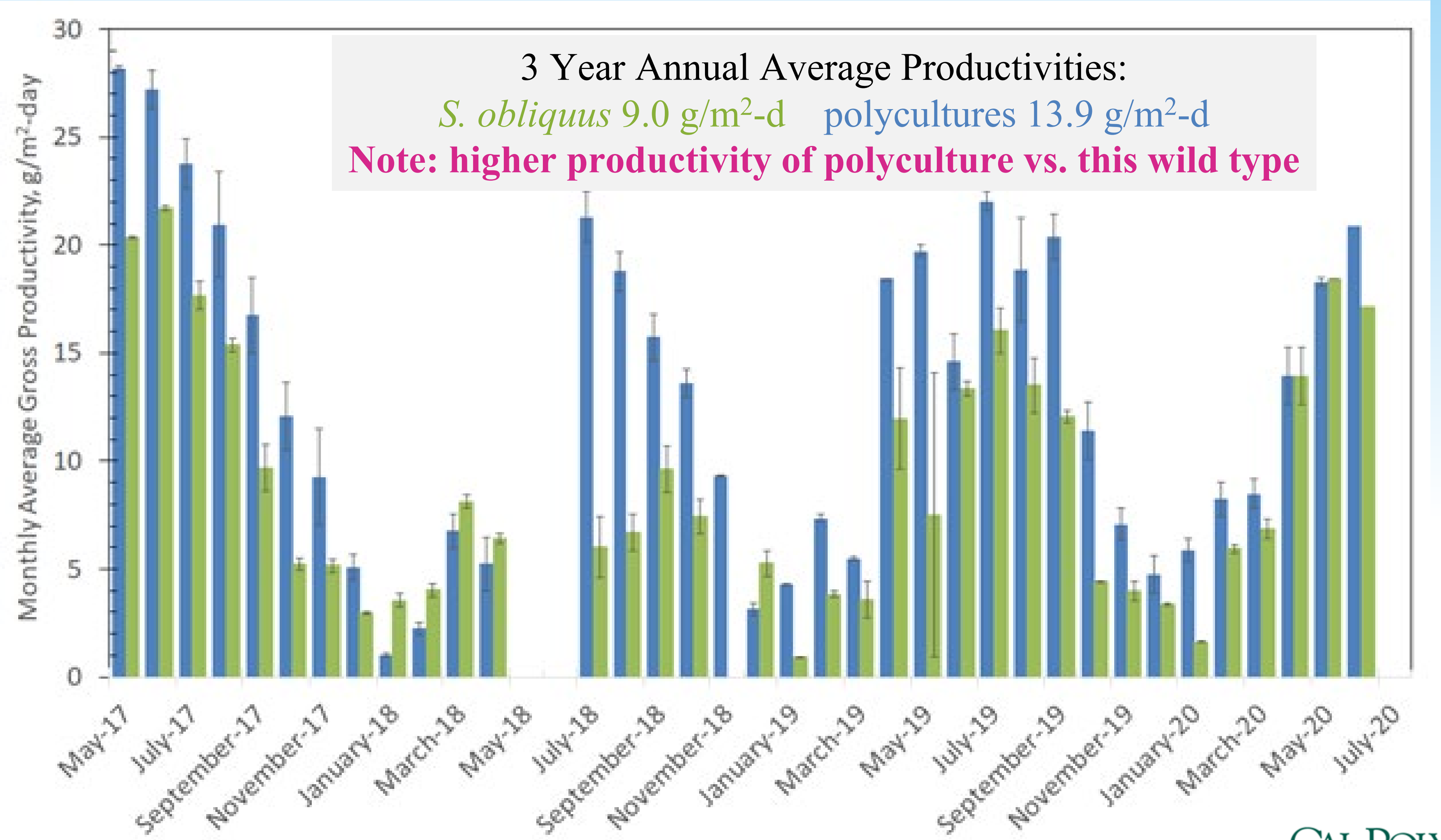
- All resources have been made publicly available
- Strain has been deposited with UTEX culture collection
- The genome is available through the JGI PhycoCosm portal

Task 3, 6

CAL POLY

Scenedesmus obliquus (UTEX 3031) productivity over three years of outdoor cultivation





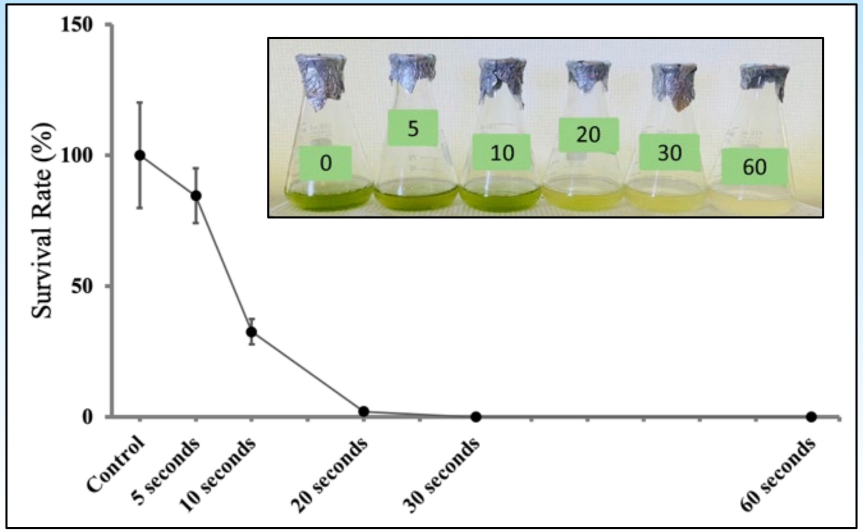
Task 3, 6

CAL POLY

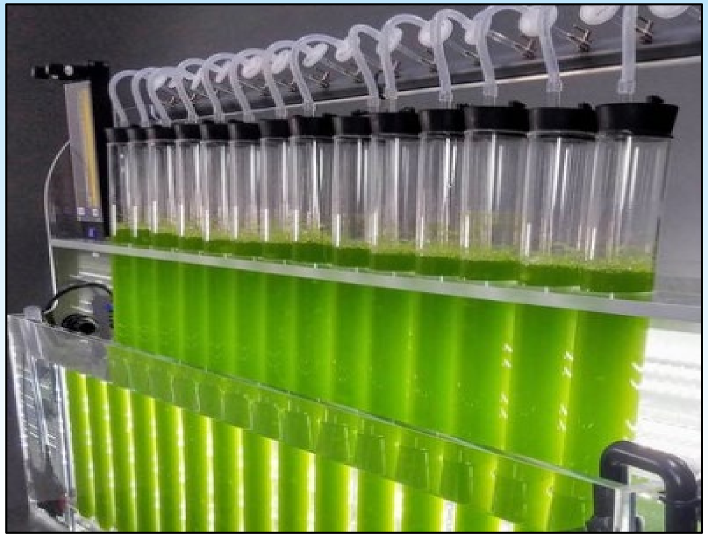
Improvement of *S. obliquus* Through Adaptive Laboratory Evolution

Non-GMO approach using random mutagenesis followed by selection for highly productive strains that grow rapidly in low density and high light conditions.

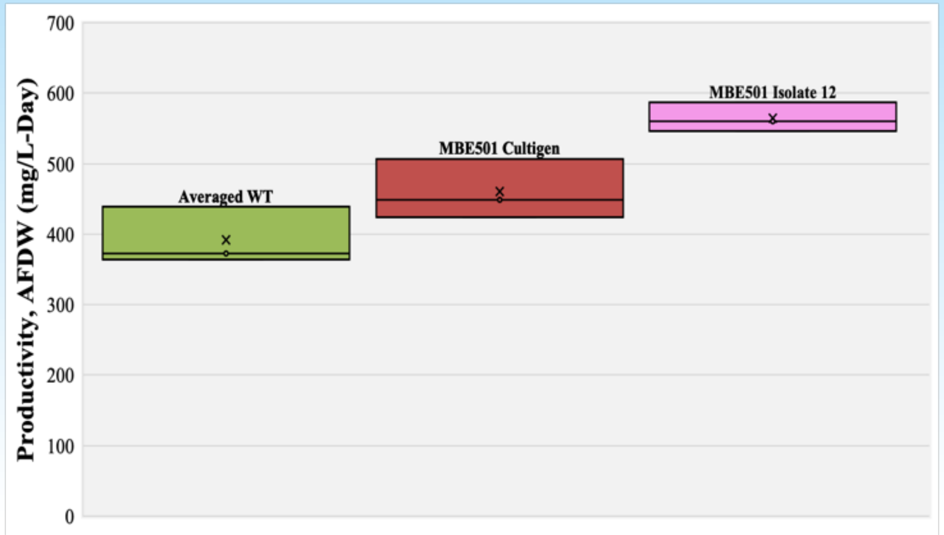
UV lethality curve



Selection in photobioreactors



Laboratory Screening



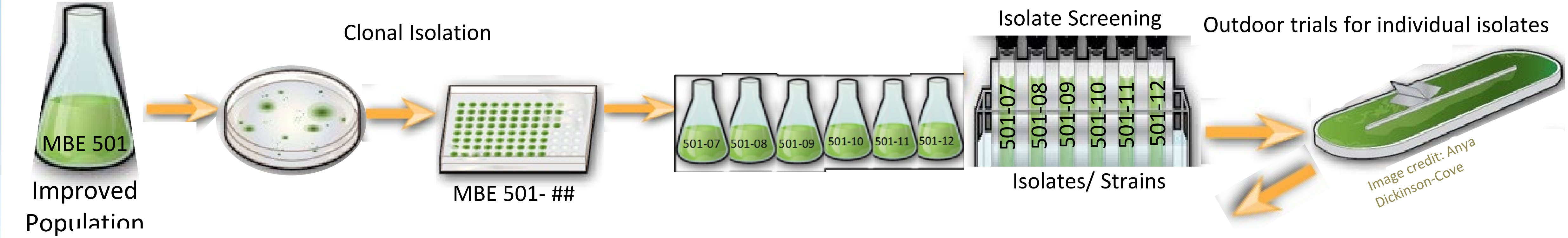
- Productivity improvements through Adaptive Laboratory Evolution were successful
- Indoor trials demonstrated improvements after 290 generations under selection
- MBE 501 population and the isolated clone MBE 501-12 were on average 17% and 44% more productive, respectively, than the wild type in the lab.
- Improved MBE 501 and MBE 501-12 were scaled-up and tested outdoors.

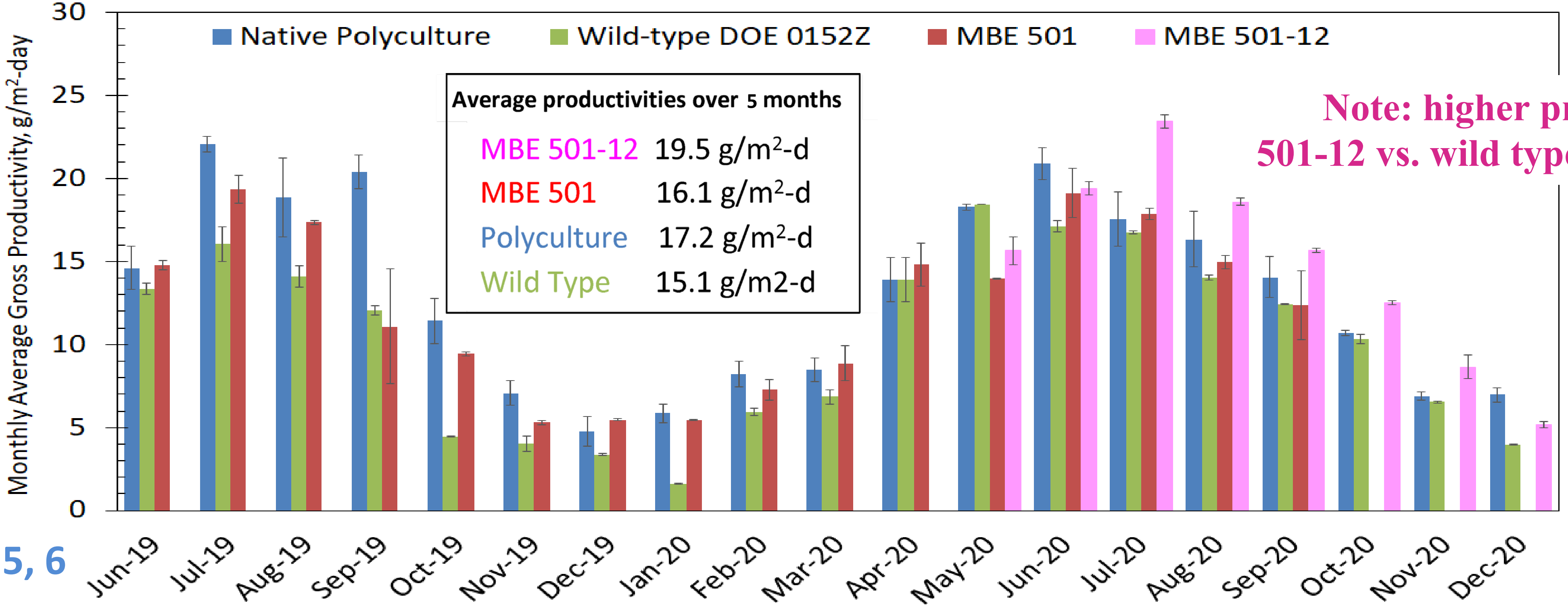
Task 5, 6

CAL POLY μBIO MicroBio Engineering

Outdoor Trials of Improved *S. obliquus* Strains in Reclaimed Wastewater

Improved MBE 501 & MBE 501-12 are more productive than wild type in long-term, outdoor trials.





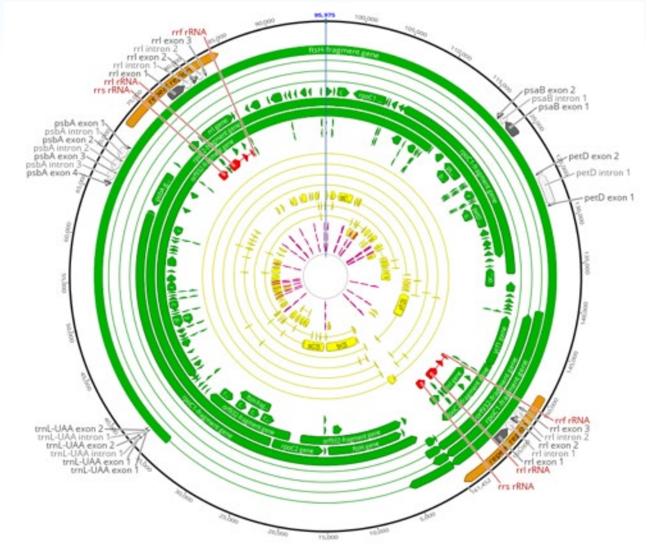
Note: higher productivity of 501-12 vs. wild type and polyculture

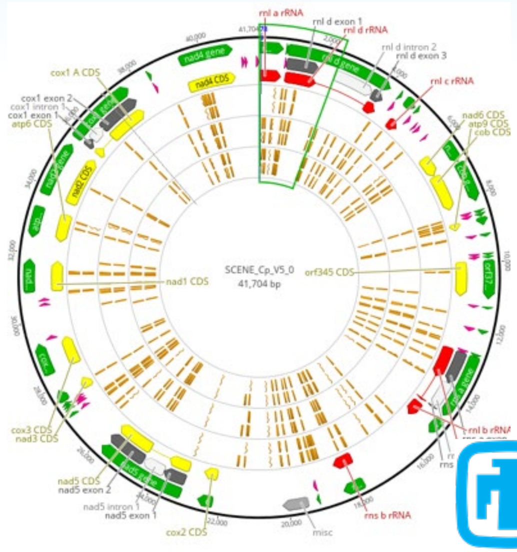
Task 3, 5, 6

Variant Analysis of Improved Phenotypes. Bioinformatics pipeline was developed for genetic characterization and variant analysis.

- Mutations identified in the MBE 501 selected population and the isolated clone MBE 501-12.
- More mutations present in the mixed population (genetically heterogeneous) than in a clonal isolate (single genotype).
- In 501-12, mutations found in interesting plastid encoded genes, including a tRNA and the *psaC* gene, which is essential for photochemical activity.

Variant Analysis using Freebayes v 1.3.2		
Genome	**MBE 501 Cultivar**	**MBE 501 12 Isolate**
Nuclear	61,608	24,442
Chloroplast	101	47
Mitochondria	100	19





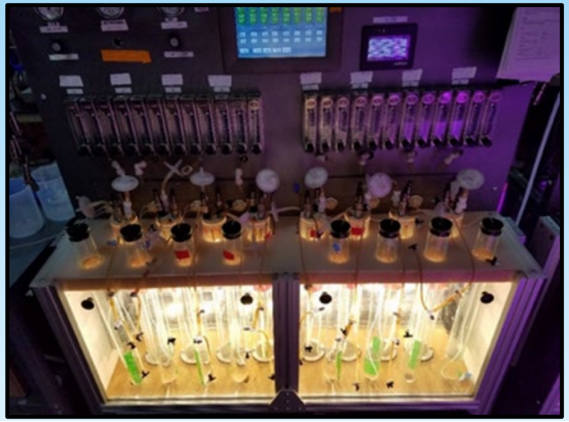
Task 7

Sandia National Laboratories

Task 9

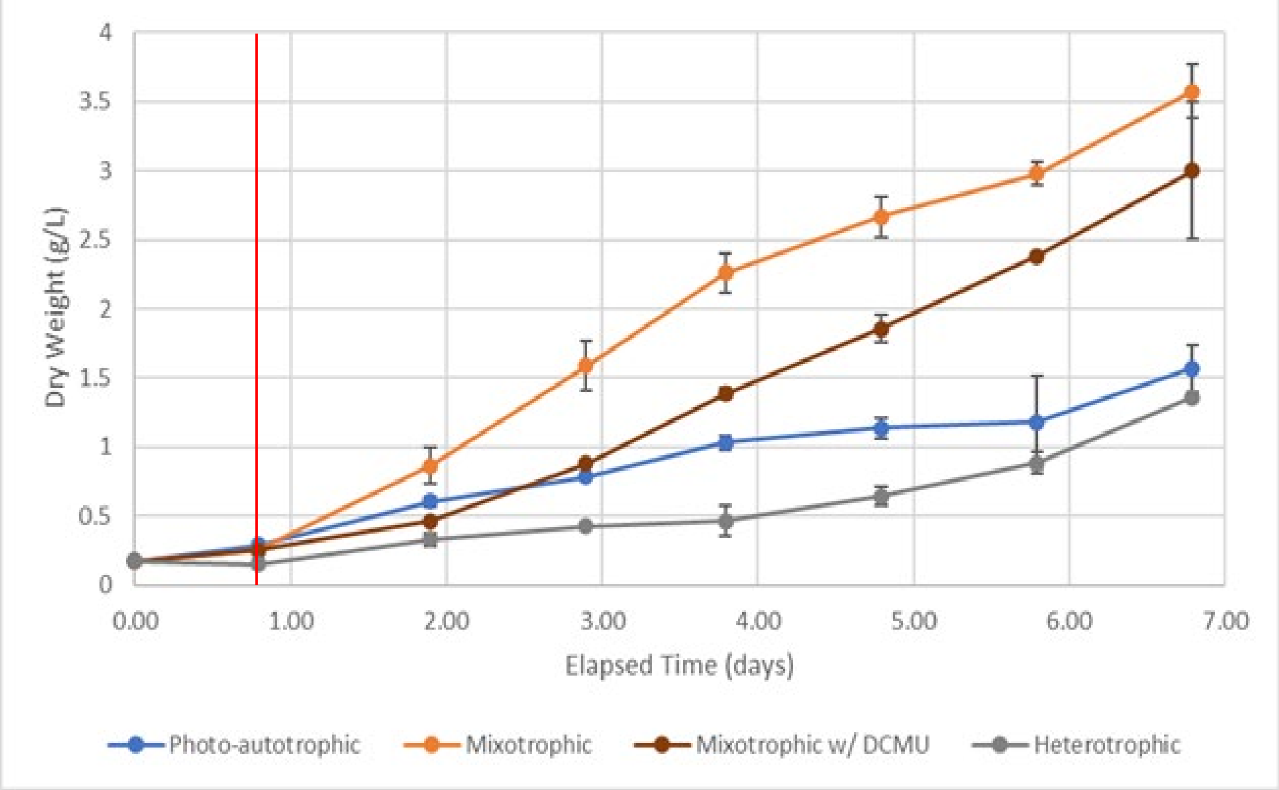
Mixotrophic Cultivation

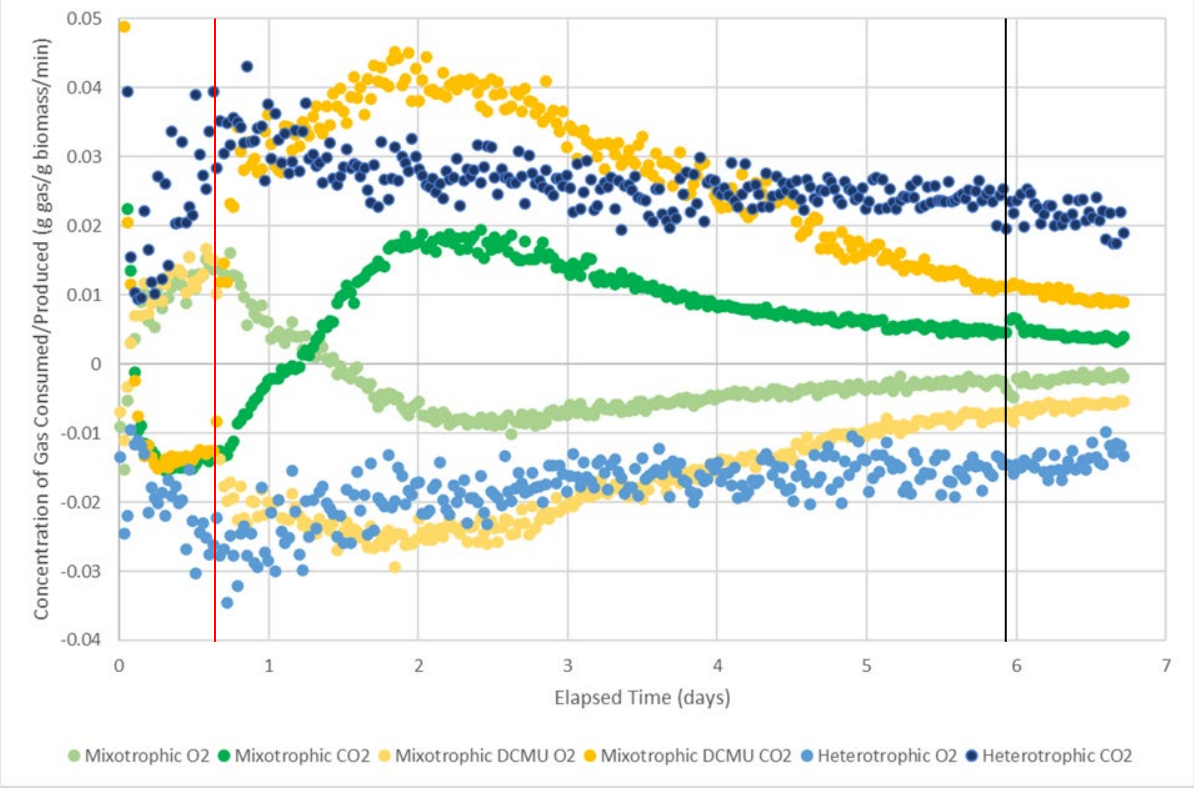
Indoor Trials conducted to characterize mixotrophy in *S. obliquus*.



Advanced Mixotrophic Platform (AMP)

- Glass bubble columns. LED lighting
- CO_2 or acetic acid titration capable
- Mass spec to analyze inlet and outlet O_2 and CO_2 concentration

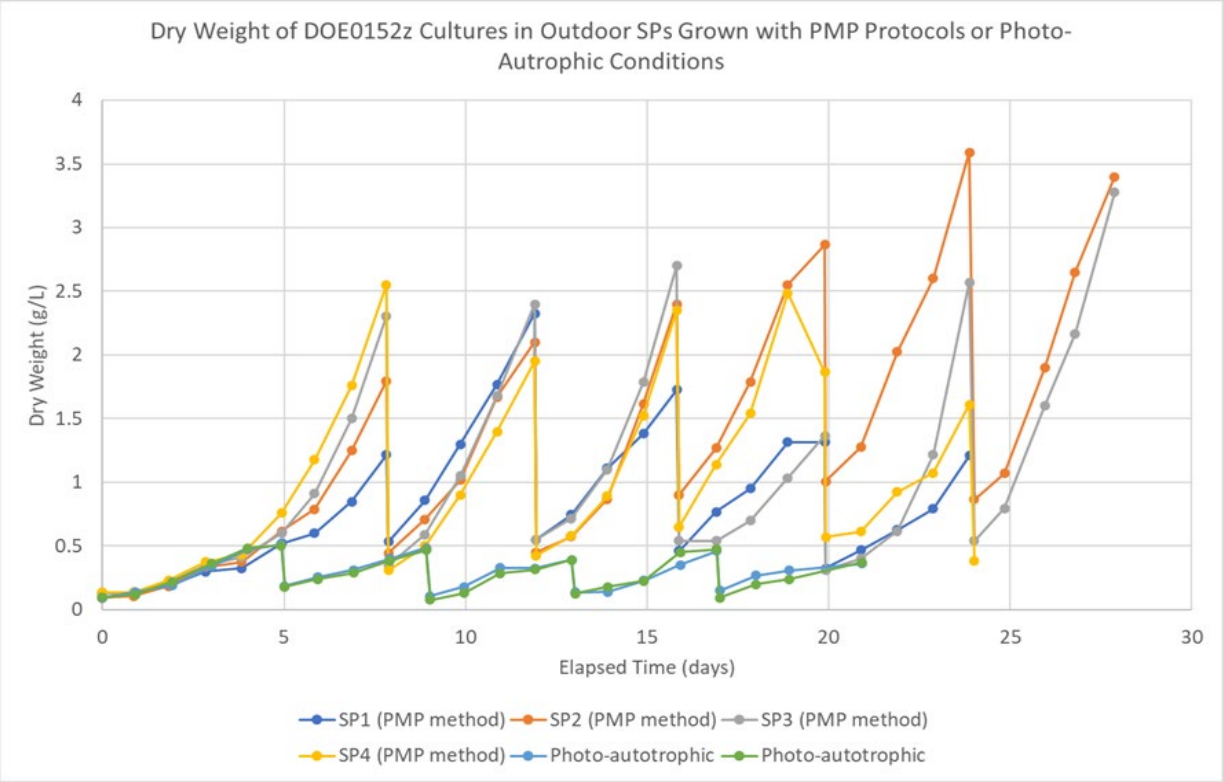


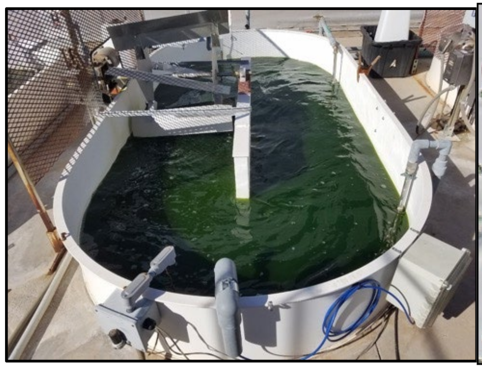


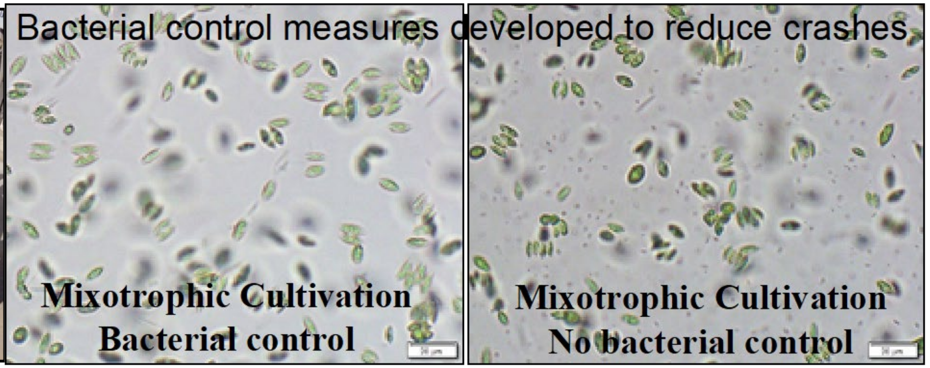
Mixotrophic cultivation was extremely successful and increased productivity in outdoor trials conducted with *S. obliquus* permitting the BETO milestone to be met.

<u>Cultivation in Small Ponds (SP)</u>

- Outdoor 5.6m^2 raceways
- pH control with either CO_2 or acetic acid titration
- BG-11 used to develop methods for stable outdoor mixotrophic cultures with acetate as a substrate
- Developed Photo-Mixo-Partial (PMP) method 4 days of photoautotrophic growth, then mixotrophic growth
- **Projected annual average of >33 g/m^2-d**
- Cultivation in Synthetic Primary Clarified Effluent (SPCE) also tested
- Developed bacterial control measures
- A patent application was filed for inventions developed with this project

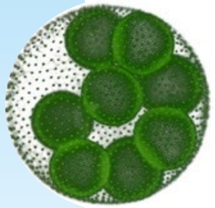


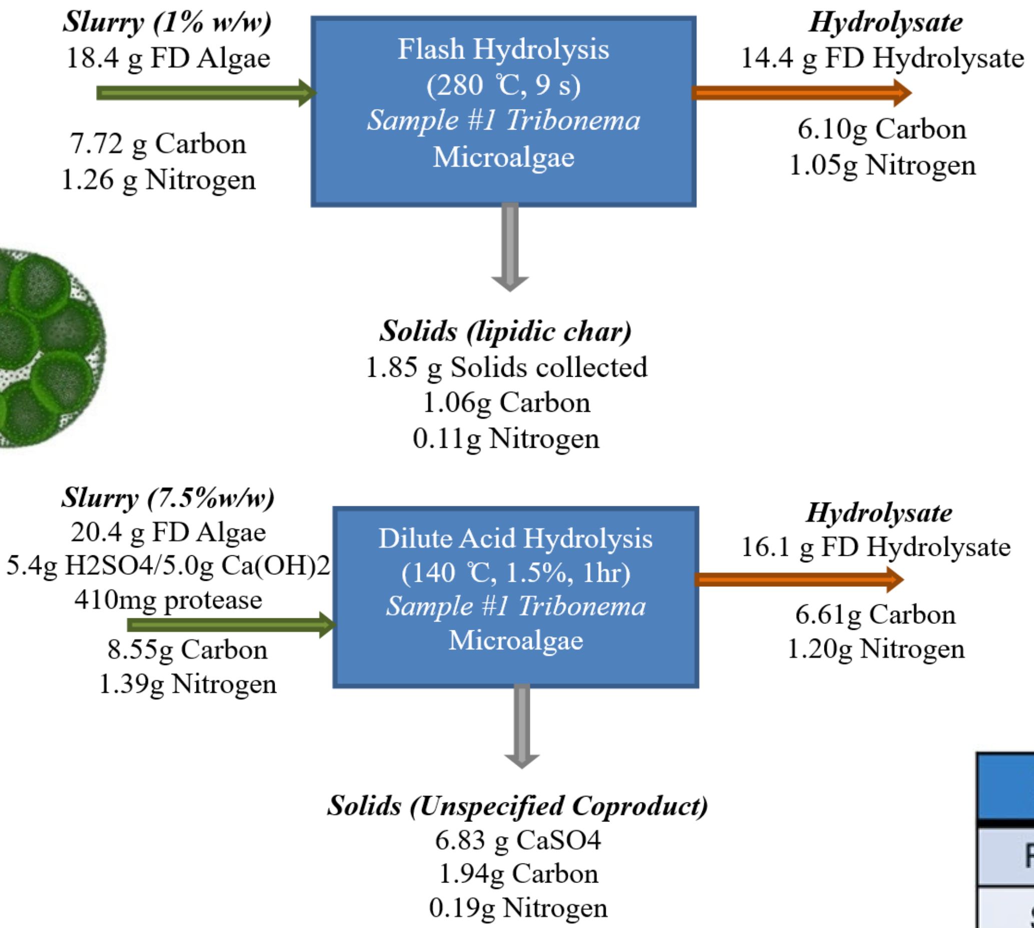


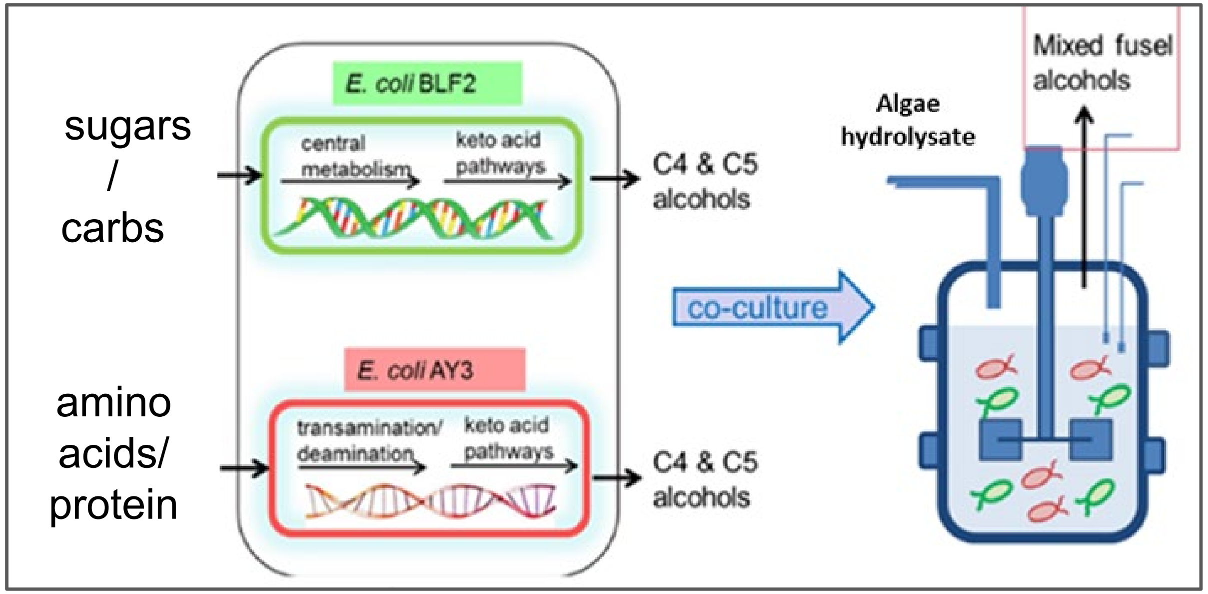


Task 9 heliae

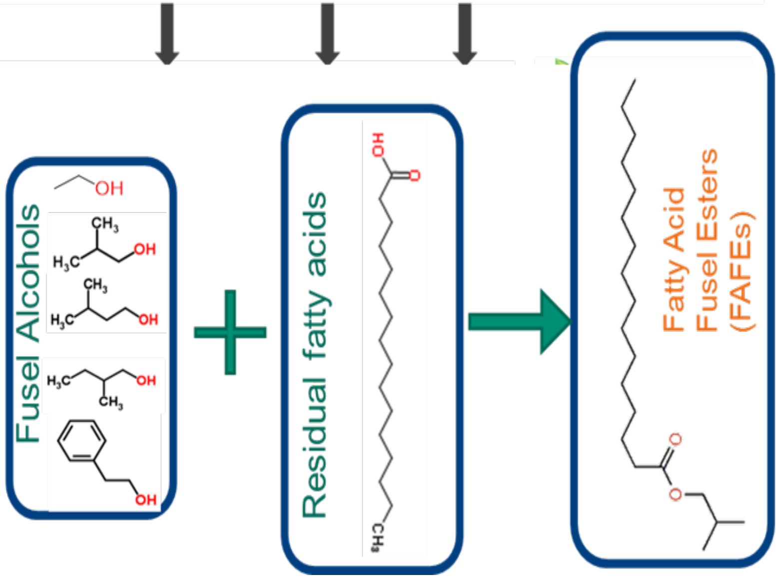
Process optimization for bioconversion of ABYII strains







Property	Fusel Alcohols
RON/MON	110/98
Sensitivity	12
bRON at 20%	115
HOV (kJ/kg)	637
LHV (MJ/L)	26.8



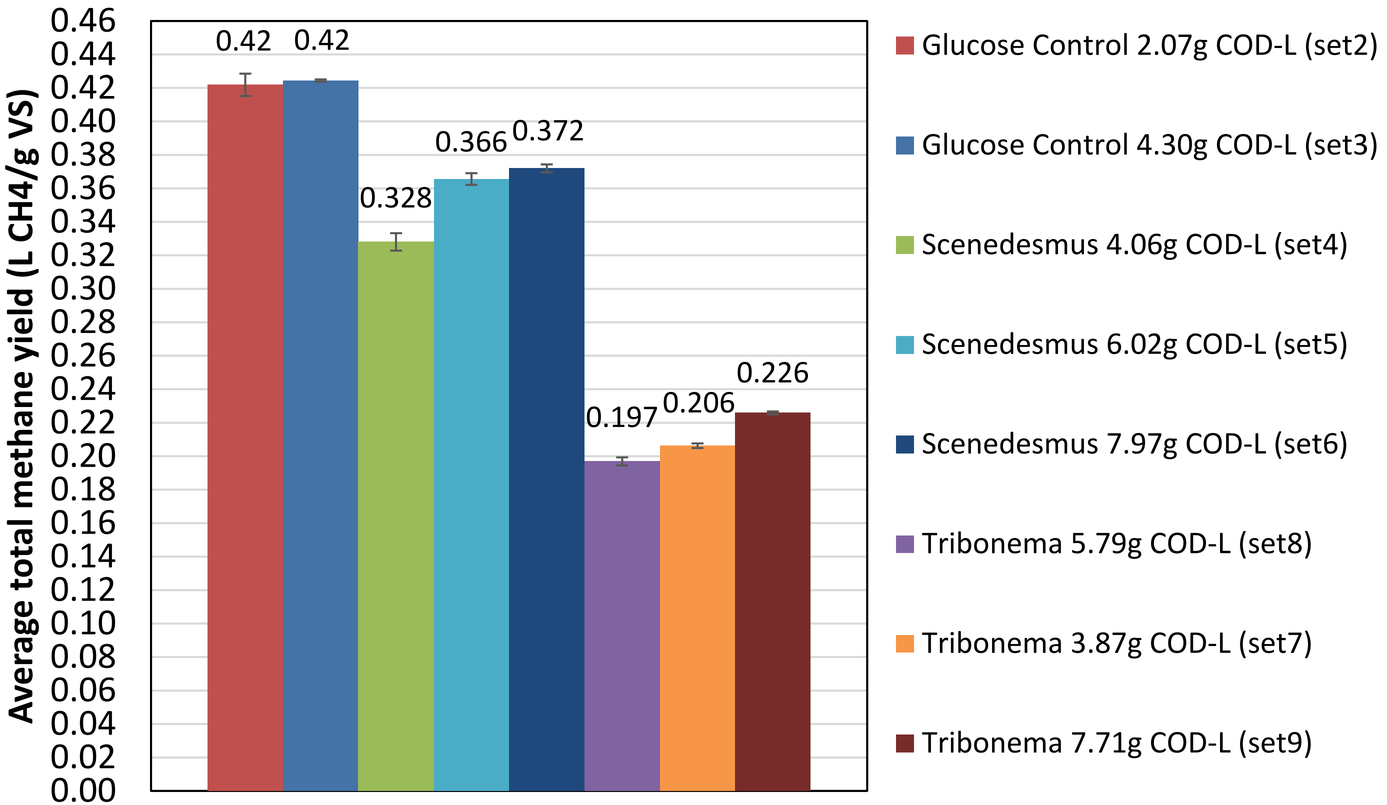
ABYII-developed process provides means for upgrading of dominant fractions of algae biomass to fungible gasoline and diesel fuels

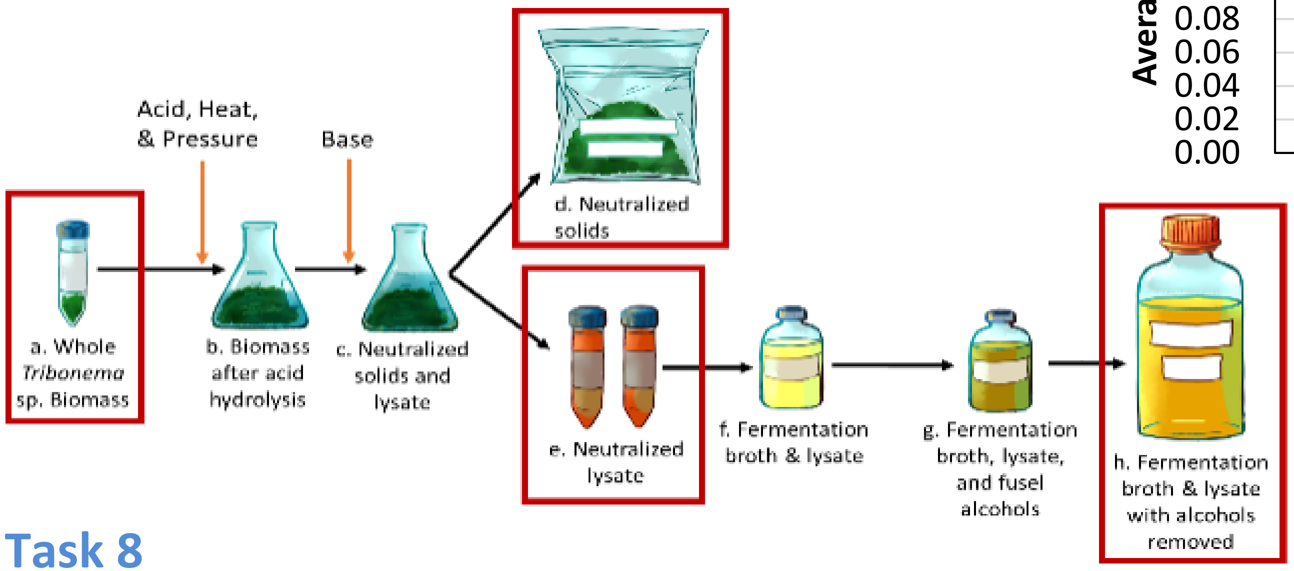
Monroe et al. 2020 (https://www.sciencedirect.com/science/article/abs/pii/S0016236120304038)
https://www.greencarcongress.com/2019/10/20191011-cooptima.html

Anaerobic Digestion: The desired yield of 0.34 L CH_4/g VS was exceeded with *Scenedesmus* intermediate residual material.

- *Scenedesmus obliquus* and *Tribonema minus* whole biomasses were hydrolyzed, neutralized and separated into solids for anaerobic digestion (d. in figure below) while the liquid was saved for fusel alcohol fermentation.

- The highest yield of the *Tribonema* digestion was 0.226 L CH_4/g VS while the highest yield for the *Scenedesmus* was 0.372 L CH_4/g VS.





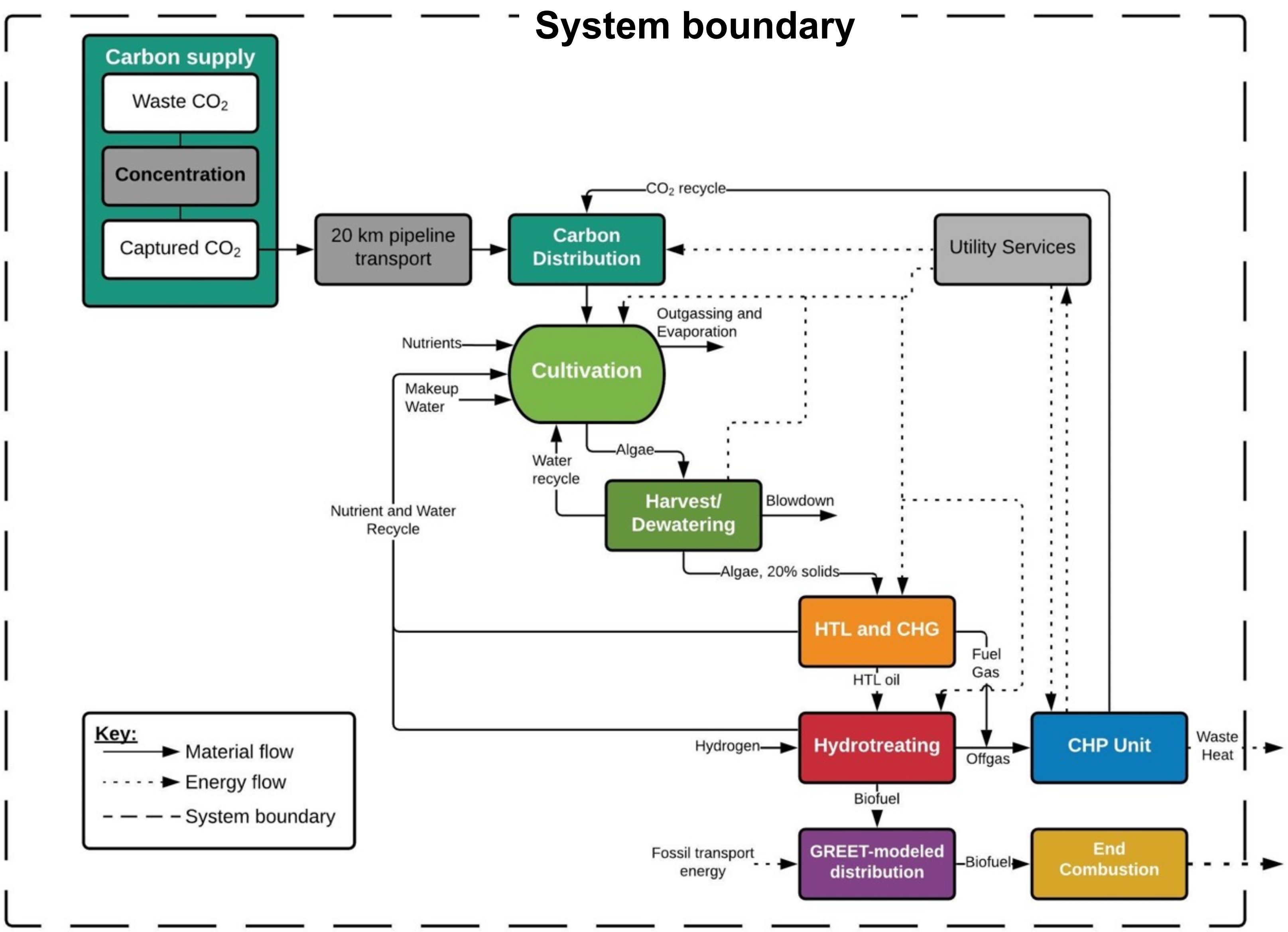
- Final residual materials (h. in figure) were received with the plan of performing anaerobic digestion, but it could not be completed due to COVID restrictions.

Task 8

CAL POLY

Economic Analysis and Life Cycle Assessment (TEA and LCA) Autotrophic Pathway Key Assumptions and Process Flow

Parameter	Autotrophic Pathway
Facility size	400 ha of pond surface area
Location	San Joaquin Valley, CA
Annual average productivity	15 g/m^2-day
Growth media	Reclaimed wastewater
Biofuel product	Renewable diesel
Coproducts	Naphtha

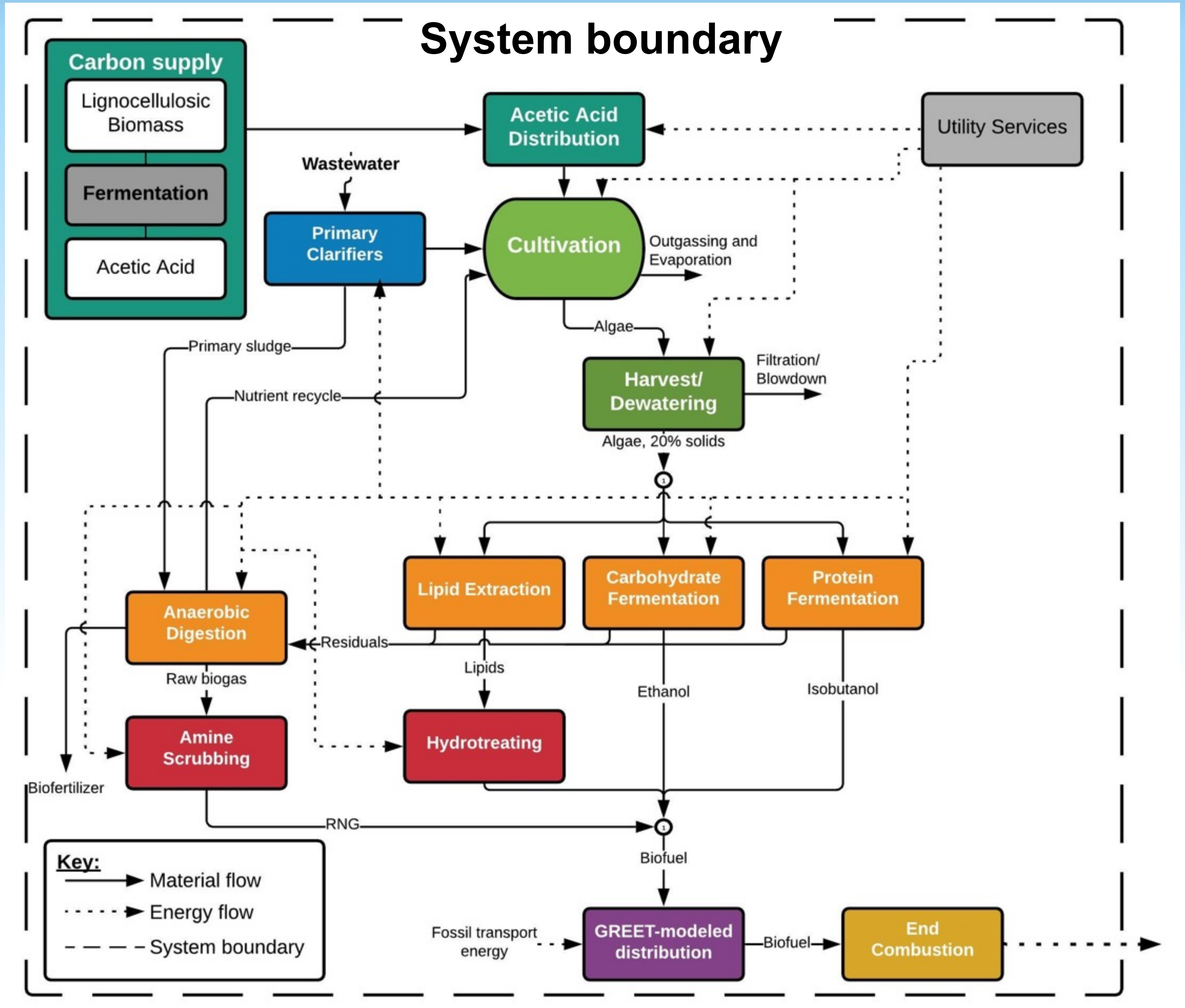


Task 4

MicroBio Engineering

Economic Analysis and Life Cycle Assessment (TEA and LCA)
Mixotrophic Pathway Key Assumptions and Process Flow

Parameter	Mixotrophic Pathway
Facility size	400 ha of pond surface area
Location	San Joaquin Valley, CA
Annual average productivity	31 g/m^2-day (Combined photoautotrophic and mixotrophic net output)
Growth media	Wastewater and acetic acid
Biofuel product	1.) Ethanol and RNG 2.) Fusel alcohols and RNG 3.) Renewable diesel and RNG
Acetic acid cost[1]	$293/tonne of acetic acid
Acetic acid GHG emissions[2]	180 kg CO_2e/tonne of acetic acid
Coproducts	1.) WWT, biofertilizer 2.) WWT, biofertilizer 3.) WWT, biofertilizer, naphtha



Task 4

[1]Budsberg, E., Morales-Vera, R., Crawford, J. T., Bura, R., & Gustafson, R. (2020). Production routes to bio-acetic acid: life cycle assessment. Biotechnology for Biofuels, 13(1).
[2]Morales-Vera, R., Crawford, J., Dou, C., Bura, R., & Gustafson, R. (2020). Techno-Economic Analysis of Producing Glacial Acetic Acid from Poplar Biomass via Bioconversion. Molecules, 25(18), 4328.

Economic Analysis and Life Cycle Assessment (TEA and LCA) Results and Relevance

Parameter	Autotrophic Pathway	Mixotrophic Pathway	End of Project Goals
TEA results ($/GGE)	$8.20	**Carb pathway: $4.05 Protein pathway: $4.90 Lipid pathway: $2.74**	$4.96
LCA results (% reduction)	41%	**Carb pathway: 65% Protein pathway: 66% Lipid pathway: 78%**	60% reduction*

*when compared to typical greenhouse gas emissions (GHG) of California gasoline.

Relevance:

- Exceeded MYPP goals of 3,700 gallons/acre-year in all three mixotrophic pathways.
- Demonstrated the importance of coupling biofuels production with wastewater treatment from an economic and life cycle perspective.
- Exceeded minimum 50% GHG emissions reduction set forth by the Renewable Fuel Standard (RFS).
- Met final project TEA and LCA targets for the mixotrophic pathways modeled.

MicroBio Engineering

Summary

- Screened a variety of microalgal strains obtained from collections and bioprospecting efforts. Chose three strains for continued investigation.
- *Tribonema minus* demonstrated to be easy to harvest, stable and more productive than native polycultures in long-term outdoor studies.
- Sequenced the genomes of three promising strains, re-sequenced *S. obliquus* UTEX 3031.
- Demonstrated more productive *S. obliquus* strains through an ALE approach.
- Validated productivity improvements relative to the wild type in long-term outdoor cultivation.
- Developed a variant analysis pipeline to characterize genetic variants in improved isolates.
- Used mixotrophic cultivation to greatly increase UTEX 3031 productivity in outdoor raceways.
- Applied single pot strategy to ferment carbohydrates and proteins into biofuel intermediates.
- Developed methods for the conversion of residual algal carbon to biofuel through digestion with the simultaneous solubilization of nutrients for recycling.
- Modeled the complete process through TEA and LCA incorporating data from each task.
- Met or exceeded intermediate goals for mixotrophy, protein fermentations, biochemical composition and the TEA/LCA.
- Exceeded MYPP goals of 3,700 gallons/acre-year in all three mixotrophic pathways.
- Met final project TEA and LCA targets for the mixotrophic pathways modeled.

Quad Chart Overview

Timeline
- Project start date: October 1, 2016
- Project end date with no cost extension: March 30, 2021

	FY20 Costed	Total Award
DOE Funding	$967,009	$3,790,212
Project Cost Share	$375,276	$1,283,730

Project Partners
- MBE 26%
- Sandia 26% (estimated)
- Cal Poly 21%
- Heliae 24%
- Dr. Juergen Polle 3% (consultant)

Project Goal

To develop the capability for 3,700 gal/ac-yr of biofuel intermediates through microalgal strain improvement, mixotrophic approaches and novel conversion technology and to validate advances in field-relevant settings to facilitate process modeling through TEA and LCA.

End of Project Milestone
- 33 g/m^2-day outdoor annual average productivity with recycled nutrients and media
- High productivity with high biofuel precursor content: 40% lipid, or 60% carb, or 50% protein
- $4.96/ GGE and 60% reduction in GGE

Funding Mechanism
- FOA: DE-FOA-0001471
- Advancements in Algal Biomass Yield, Phase 2 (ABY2)
- FY: 2016

Thank you

Additional Slides

Responses to Previous Reviewers' Comments

- Reviewer: The project seems to want to tackle too many individual tasks which is resulting in a disjointed project. The overall goal states using non-GMO tool but there is a task dedicated to genomics to identify the genetic basis of strain improvement. Technical accomplishment utilizing genomics has been stated without sharing any of the data. Have any improvements been determined?
- Response: The FOA required addressing all the goals that the tasks addressed. Following the Go/No-Go project review, the workplan was streamlined to focus primarily on biomass productivity improvements and increases in protein content for fusel alcohol production. The aim of the project is to apply non-GMO methods (directed evolution) to obtain strain improvements. Future work beyond the present project could incorporate genetic engineering approaches informed by the association of genetic changes elucidated during this research in the improved phenotypes. Initial genomic effort focused on developing a variant analysis pipeline. The pipeline was then used to identify mutational 'hot spots' within the genome resulting from UV exposure that would otherwise produce false positives during analysis. Strains demonstrating productivity improvements have been developed in the lab and the genomic analysis associates the phenotype with the underlying genetic modifications.

Responses to Previous Reviewers' Comments

- **Reviewer:** The use of wastewater could potentially reduce the overall operational costs by the reduction of nutrient inputs, though care may need to be taken as high biomass yield can sometimes not be achieved without supplemental nutrients.
- **Response:** Treated wastewater is a co-product of algae biomass production that significantly reduces the cost of the biofuel produced. Nutrients are generally replete under normal operating conditions using wastewater. In general, wastewater contains 30-60 mg NO_3-N/L and the ponds are operated at a 2 day hydraulic residence time. Thus the wastewater easily supports productivities over 45 g/m2-day assuming biomass is 10% N. Similarly, phosphorus concentrations are typically near 10mg PO_4-P/L.
- **Reviewer**: High ash content appears to have led the team to abandon HTL as a downstream conversion technology and focus on AD instead. It is unclear if the team has considered how this change might influence other objectives.
- **Response**: The change in focus from HTL to AD as a downstream conversion technology was carefully considered. Beyond concerns about high ash content, algal regrowth studies on HTL residuals indicated significant challenges. Based on previous results, fewer challenges are anticipated in growing algae on AD centrate.

Publications, Products, Patents, Presentations and Posters

Publications

- Liu F, Lane P, Hewson J, Stavila V, Tran-Gyamfi MB, Hamel M, Lane TW, Davis RW "Development of a closed-loop process for fusel alcohol production and nutrient recycling from microalgae biomass" *Bioresource Technology* 2019, 283, 350-357.
- Fang Liu, Eric Monroe and Ryan W. Davis (Nov 2018) "Engineering Microbial Consortia for Bioconversion of Multisubstrate Biomass Streams to Biofuels", Biofuels - Challenges and Opportunities, Mansour Al Qubeissi, IntechOpen, DOI: 10.5772/intechopen.80534
- Davis, A.K., Anderson, R., Spierling, R., Leader, S., Mahan, K., Lundquist, T., Benemann, J., Lane, T., Polle, J. Submitted. Characterization of a novel strain of *Tribonema minus* demonstrating high biomass productivity in outdoor raceway ponds. *Bioresource Technology*.
- Mahan, K.M., Polle, J., McKie-Krisberg, Z., Lipzen, A., Kuo, A., Grigoriev, I., Lane, T., Davis, A. Submitted. Annotated Genome of the yellow-green alga *Tribonema minus*. *Genome Announcements.*

Products

- JGI PhycoCosm Portal creation underway for *Tribonema minus*.
- JGI PhycoCosm Portal creation underway for *Desmodesmus armatus*.

Presentations & Posters

- Mahan, K., Davis, A., Polle, J. Lundquist, T., Benemann, J. 2020. Identification of Genetic Variants Associated with Phenotype Improvements in Two Green Algal Strains. Algae Biomass Summit. Online.
- Plfucker, S. 2020. High Productivity Mixotrophic Culturing of *Scenedesmus obliquus* on Wastewater/Waste Carbon for Biofuel Production. Algae Biomass Summit. Online.
- Davis, A. (Presenter). 2020. Improved Strains for Combined Phycoremediation and Biofuels Production. Presentation. Algae Biomass Summit. Online.
- Shinde S, Davis RW 2020. "Algal biomass conversion to fusel alcohols and further upgrading to high-performance diesel fuels" ACS National Meeting, Philadelphia, PA.
- Davis, A., Spierling, R., Camerena, P., Ali, N., Lundquist, T., Benemann J. 2019. Domestication of Microalgae: Selection for Improved Growth. Presentation. Algal Biomass, Biofuels & Bioproducts. Boulder, CO.
- Lesne, C., Scott, M., Anderson, R., Leader, S., Spierling, R., Davis, A., Lundquist, T., Benemann J. 2019. Annual Lipid, Carbohydrate and Protein Composition of *Tribonema* sp. in Outdoor Raceway Ponds. Poster. Algae Biomass Summit. Orlando, FL.
- Mahan, K., Davis, A., Lundquist, T., Benemann J., Lane, T. 2019. Identification of Genetic Variants Associated with Phenotype Improvements in Two Green Algal Strains. Presentation. Algae Biomass Summit. Orlando, FL.
- Spierling, R., Anderson, R., Scott, M., Lesne, C., Leader, S., Davis, A., Lundquist, T., Benemann J. 2019. Productivity and Robustness of Algal Monocultures and Polycultures in Open Pond Cultivation. Presentation. Algae Biomass Summit. Orlando, FL.
- Anderson, R., Leader, S., Lesne, C., Spierling, R., Lundquist, T., Benemann J., Davis, A. 2019. Performance of Native *Tribonema* sp. in Outdoor Raceway Ponds. Poster. Algae Biomass Summit. Orlando, FL.
- Monroe E, Davis RW 2019. "The algae slaughterhouse - integrated conversion of algae biomass to fuels and chemicals using biocatalyst consortia" Algae Biomass Summit, Orlando, FL.

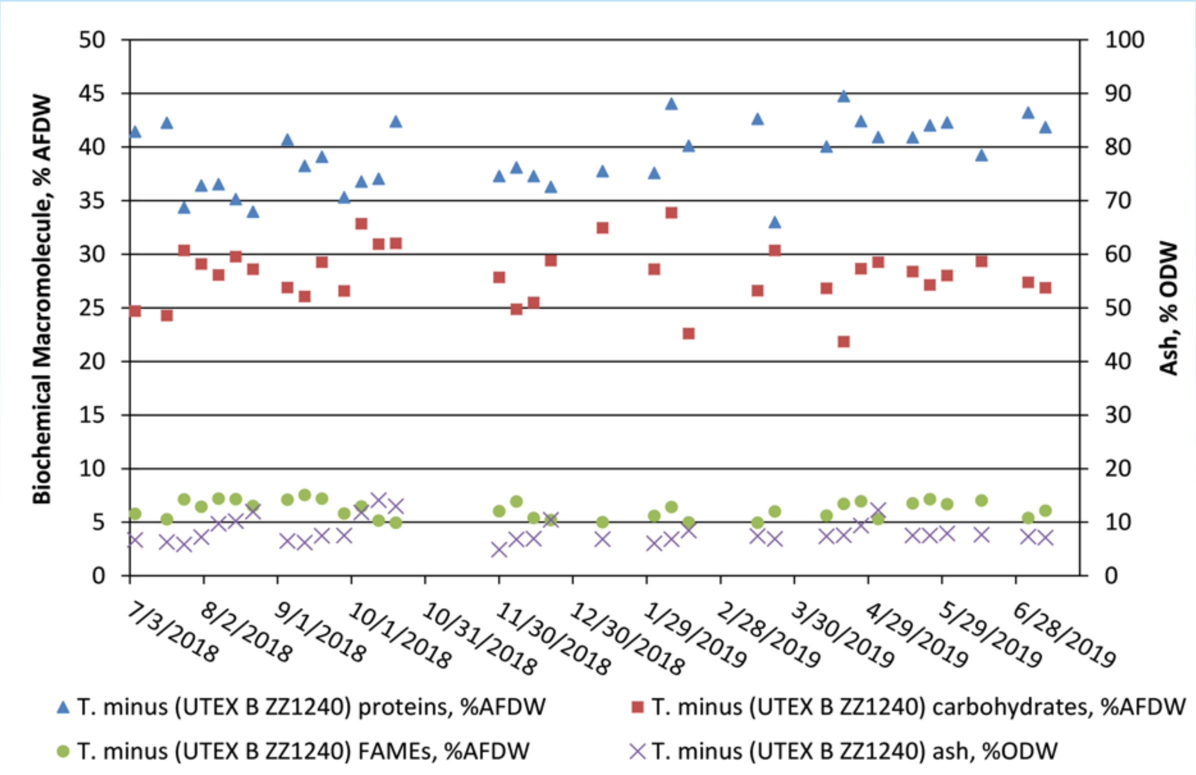
Publications, Products, Patents, Presentations and Posters

Presentations & Posters (continued)

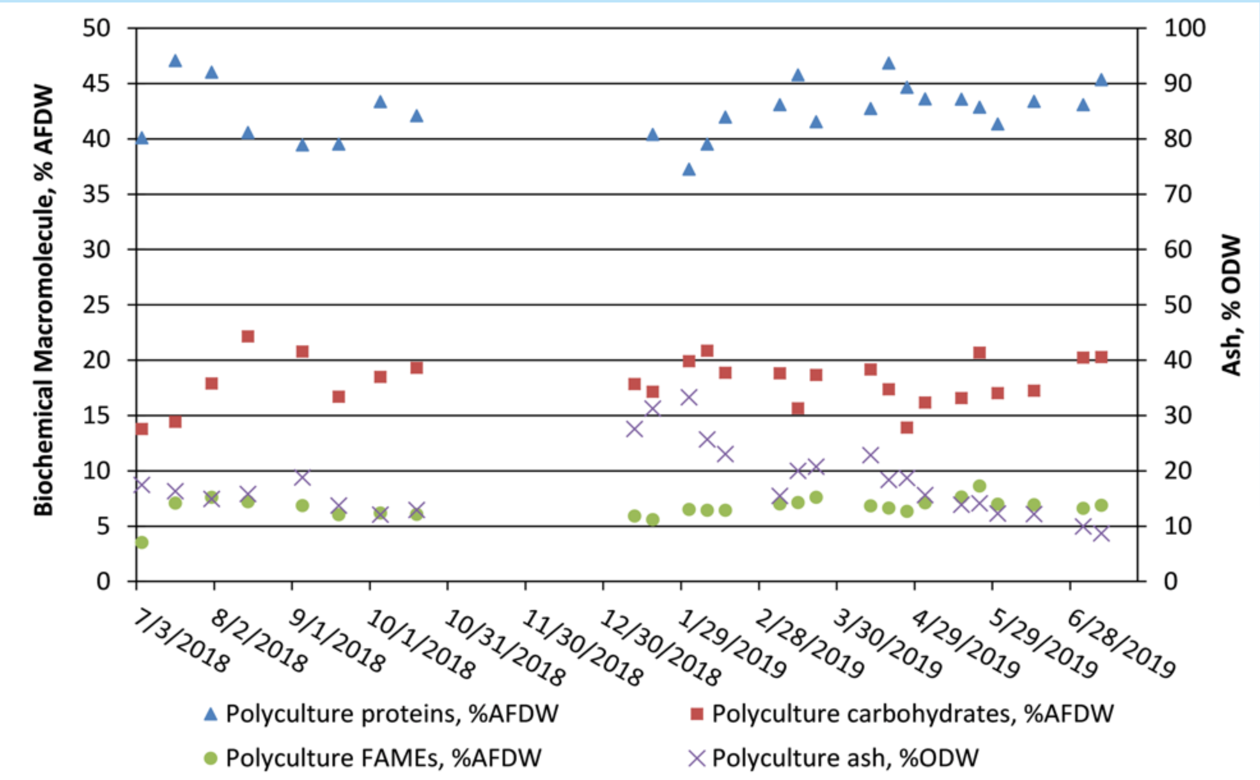
- Davis RW, Benemann J. 2019. "Integrated conversion of algae biomass to fuels and chemicals using biocatalyst consortia" Biofuels and Bioenergy (BBC2019), San Francisco, CA.
- Davis, A., Spierling, R., Lundquist, T., Benemann J. 2018. Domestication of Microalgae: Selection of Strains for Large-Scale Cultivation. Presentation. Algae Biomass Summit. Houston, TX.
- Spierling R., Davis, A., Scott, M., Leader, S., Lundquist, T., Benemann, J. 2018. A Comparison of Pure Culture and Polyculture Productivity in Raceway Ponds. Presentation. Algae Biomass Summit. Houston, TX.
- Leader, S., Scott, M., Lesne, C., Spierling, R., Davis, A., Lundquist, T., Benemann, J. 2018. Annual Average Lipid, Carbohydrate, and Protein Productivities of Outdoor Algal Monocultures and Polyculture Ponds. Poster. Algae Biomass Summit. Houston, TX.
- Hennig, E., Leader, S., Spierling, R., Davis, A., Lundquist, T., Benemann, J. 2018. Outdoor Productivity of Filamentous *Tribonema* sp. Isolated from a Reclaimed Wastewater Polyculture. Poster. Algae Biomass Summit. Houston, TX.
- Davis RW, Wu BC, Benemann J. 2018. "Comparative assessment of suspended and attached microalgae cultivation for coupling water treatment and production of biobased commodities" 7th International Conference on Algae Biomass, Biofuel, and Bioproducts (ABBB), Seattle, WA.
- Monroe E, Davis RW. 2018. "Changing the value proposition of algal biofuels: production and testing of a tunable suite of high-performance fuels from low-cost algae biomass" 7th International Conference on Algae Biomass, Biofuel, and Bioproducts (ABBB), Seattle, WA .
- Davis RW. 2018. "The fusel alcohol platform for high performance biofuels from whole algae biomass" ACS National Meeting, New Orleans, LA.
- Monroe E, Davis RW. 2017. "Production of Fusel Alcohols as High Performance Fuels from Whole Algae Bioconversion" Algae Biomass Summit, Salt Lake City, UT.
- Hennig, E., Davis, A., Spierling, R., Lundquist, T., Benemann, J. 2017. Productivity of Culture Collection Strains and Wastewater isolates Indoors and Outdoors. Poster. Algae Biomass Summit. Salt Lake City, UT.

Tribonema minus biochemical profile over one year of outdoor cultivation and comparison with native polyculture

T. minus (UTEX B ZZ1240)



Native polyculture



S. obliquus (UTEX 3031) biochemical profile over one year of outdoor cultivation and comparison with native polyculture