μBIO

MicroBio Engineering

Bioenergy Technologies Office (BETO) 2019 Project Peer Review

Integrated Low Cost and High Yield Microalgal Biofuel Intermediates Production

DE-EE0007691

03/06/2019

Advanced Algal Systems

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MicroBio Engineering Inc.

The goal of this project is to meet DOE-BETO's 2020 MYPP performance goal of developing technologies that enable mature modeled annual average algae yields of 3,700 gallons per acre-year of biofuel intermediates by:

- Developing a non-GMO approach to produce strains with higher productivity for total biomass, or individually for proteins, or carbohydrates or lipids.
  - Demonstrating the long-term strain productivity and robustness in outdoor ponds on wastewater.
  - Using genomics to identify the genetic basis of improvement.
- Leveraging mixotrophy in algae cultivation to improve productivity.
- Converting algal biomass to biofuel intermediates through fermentations of carbohydrates to ethanol and proteins to fusel alcohols and anaerobic digestion of the biomass residuals to biogas.
- Verifying economic and environmental performance through TEA/LCA

Quad Chart Overview

Timeline

- Project start date: October 1, 2016
- Project end date: September 30, 2020
- Percent complete: 58% (as of 1/31/19)

	Total Costs Pre FY17 **	FY 17 Costs	FY 18 Costs	Total Planned Funding (FY 19-Project End Date)
DOE Funded	-	418,323	1,295,482	3,295,859
Total Cost Share	-	207,896	400,957	674,877

Partners: Percent of total funding FY 17-18, MBE 26%, Sandia 26%, Cal Poly 21%, Heliae 24%, Polle 3%. **NOTE: Sandia DOE funded costs are estimated

Targets and Barriers addressed

- Targets addressed: 3,700 gal BFI/acre year by 2020
- Barriers addressed: non-GMO productivity improvements, long–term outdoor cultivation on recycled nutrients, and practices to measure and improve robustness. Conversion of whole biomass to biofuel. (AFT A, B, C, E, G, I and J)

Objective

To use non-GMO approaches to improve algae biomass productivity and/or composition. Demonstrate baseline productivity and composition outdoors and compare to long-term improved productivity and composition.

End of Project Goal

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 GGE

1 - Project Overview

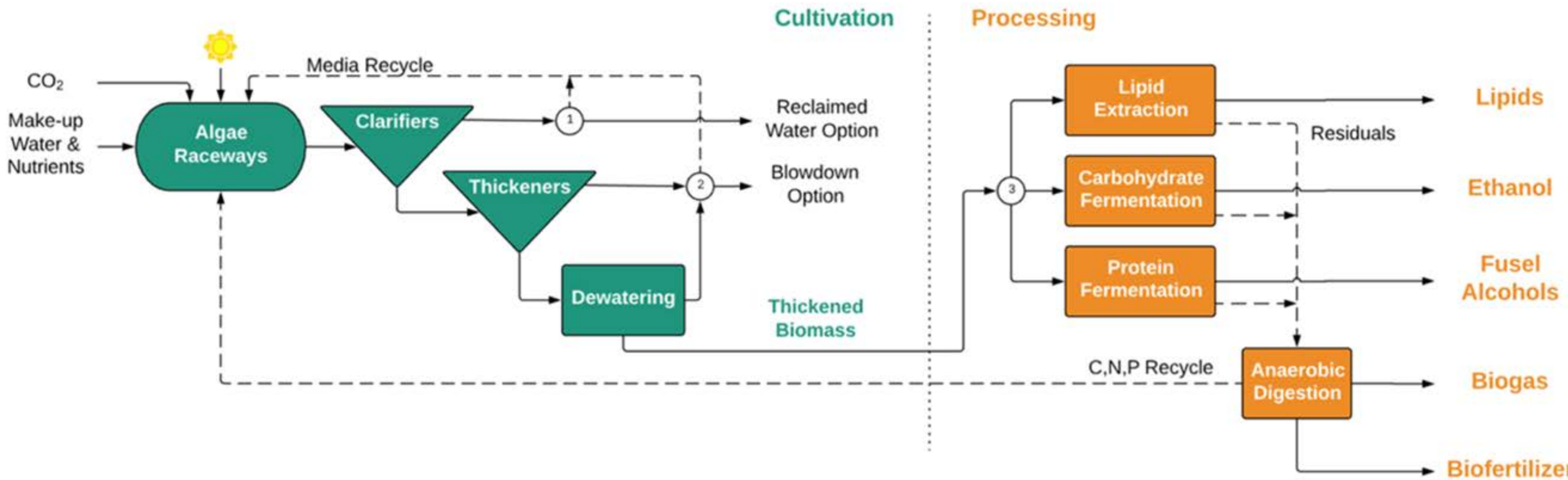
Improvements in algal productivity, robustness and conversion necessary to meet DOE-BETO's aggressive 2020 targets. To that end, this project seeks to:

1. Develop more productive algal strains through selective enrichment cultivation
2. Identify the genetic basis of improvements
3. Test improved strains in outdoor wastewater ponds compare to baseline productivity and robustness.
4. Use mixotrophic cultivation to further increase algal productivity

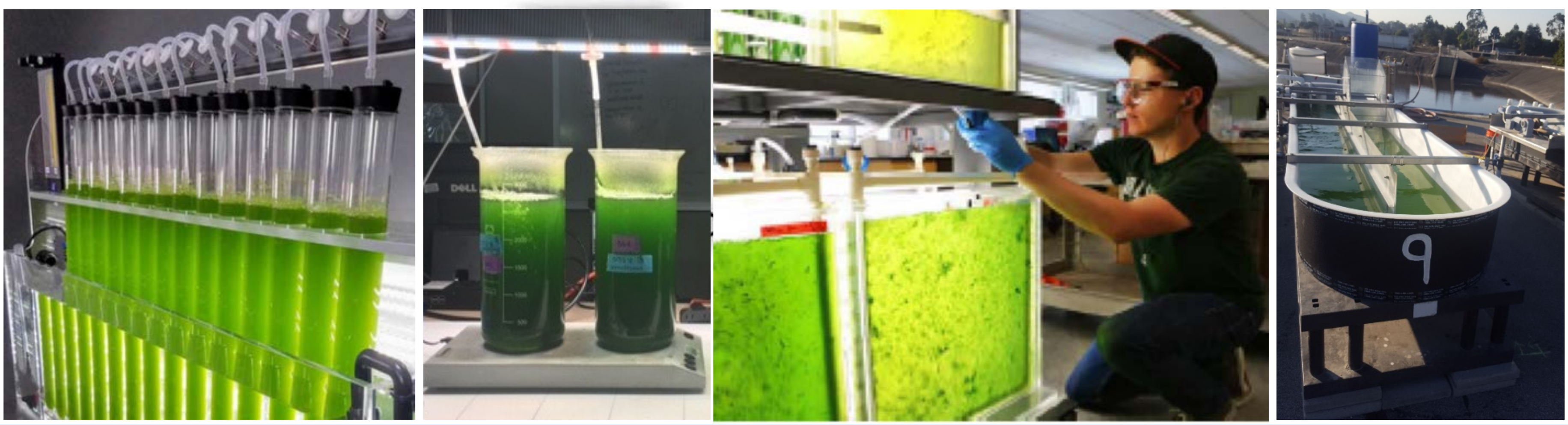
3. Convert algal carbon to biofuel intermediates through extraction, fermentations and digestion of residues

1 - Project Overview

Full-scale process flow diagram



Algal strain laboratory and outdoor culturing facilities:
flasks, PBRs, pond-simulating reactors panels, ponds



2 – Approach (Management)

Task 2: Project management task contains milestones for all Tasks 2-9. Dr. John Benemann, PI

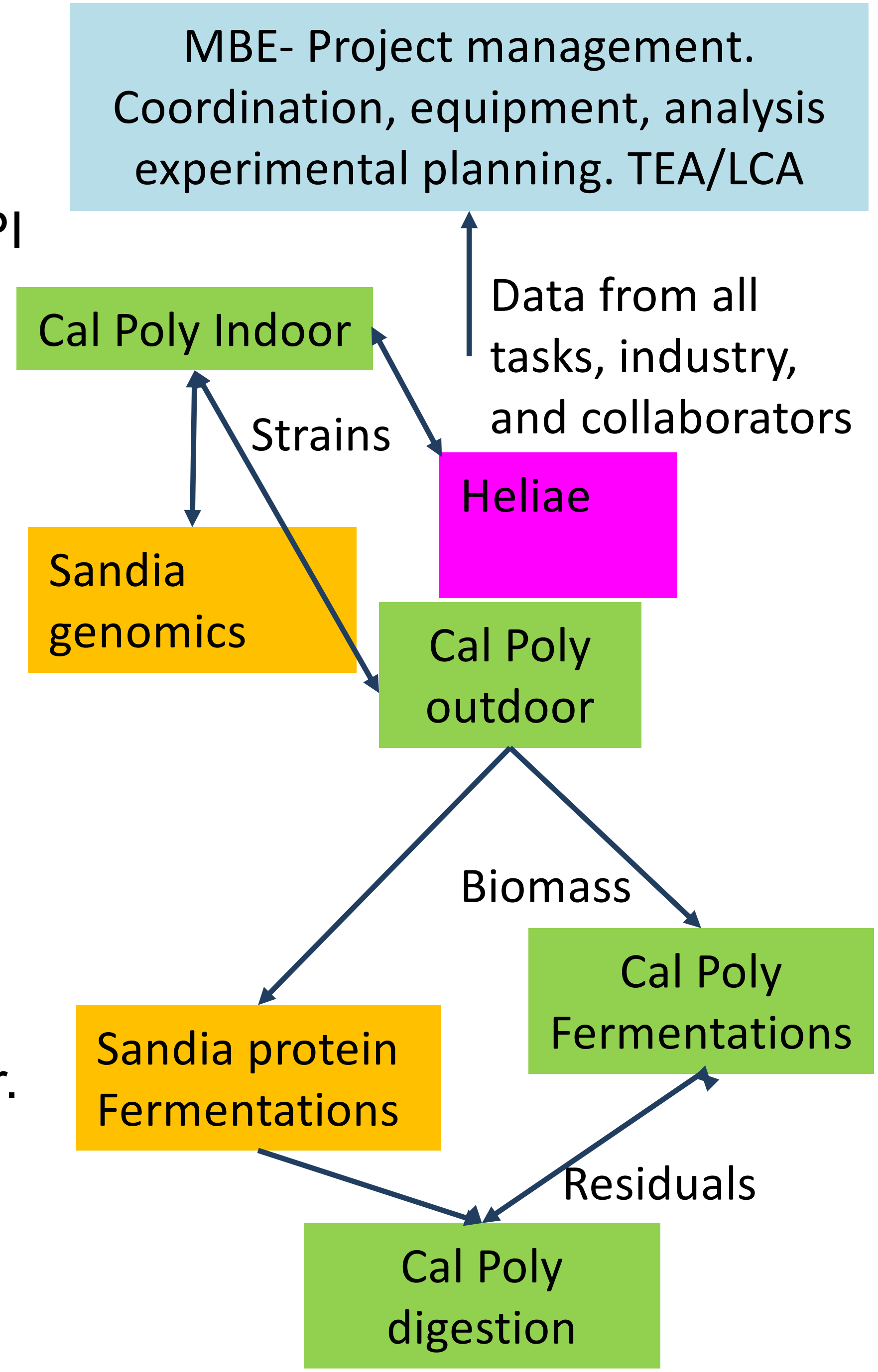
MicroBio Engineering, Inc. – John Benemann, Project management and guidance (Task 2), also TEA / LCA (Task 4), open pond cultivation (Task 3), PBR design & assembly (Task 5).

Cal Poly - Dr. Tryg Lundquist, supervision and operations; Dr. Aubrey Davis enrichment cultures, strain isolation (Task 5). Ms. Ruth Spierling carbohydrate fermentations, pond operations, anaerobic digestion (Task 3, Task 6, Task 8).

Heliae – Mike LaMont and Steven Pflucker, mixotrophic cultivation (Task 9).

Sandia – Dr. Ryan Davis, protein fermentations; Dr. Todd Lane, Dr. Krissy Mahan genomics (Task 7).

Consultant – Dr. Juergen Polle, algal strains, cultures, genomics.



2 – Approach (Technical)

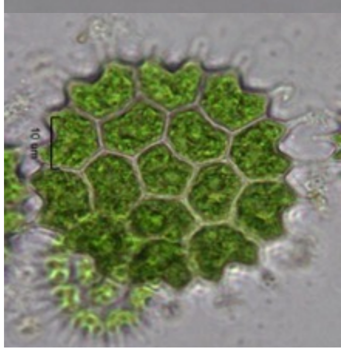
Subtask 5: Strain screening and development

1. Identify two strains suitable for selective enrichment. Apply a multi-level screen approach to choose two strains that are productive, colloidal, able to grow in outdoor wastewater ponds and have some genome information available
2. Develop photobioreactors for improved strain selection and screening
3. Develop selection regime for improving productivity and biofuel intermediate content

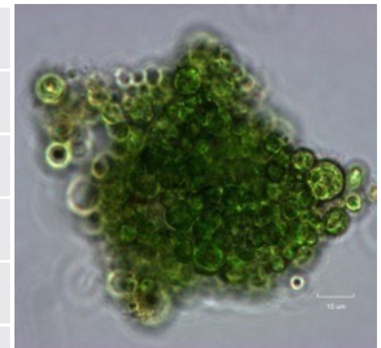
RELEVANCE: This task addresses the DOE, BETO MYPP technology portfolio in the high-impact area of Algae Feedstock R&D by unlocking algal potential through non-GMO improvements. Meeting the 2019 MYPP performance goals will require significant advances in algal productivity.

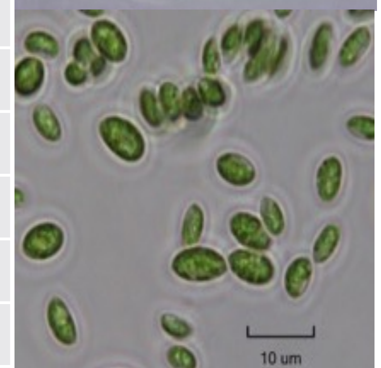
Task 3 &5: Technical Accomplishments

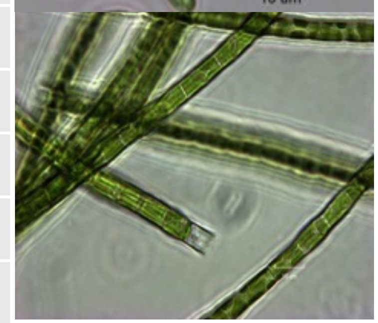
Screened 15 strains indoors and 8 strains outdoors



Name	Number	**Location**
Scenedesmus obliquus	DOE 0152_Z	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 sp.	RWW4	Lab/Out
Pseudopediastrum boryanum	RWW8	Lab/Out







Task 5: Technical Accomplishments

Developed Pond-simulating photobioreactors

- Used for testing strains before cultivation in ponds

Multi-level screen approach to choose promising strains:

- Biomass productivity in PBRs
- Compositional analysis during linear growth
- Mixotrophic capabilities
- Characteristics during cultivation in PBRs
- Productivity and robustness outdoors
- Availability of genomic information

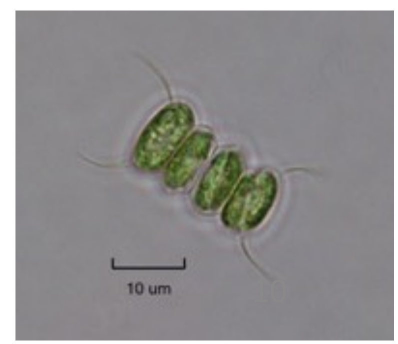
Strains Chosen for Selective Enrichment:

Strain 1: *S. obliquus* (DOE0152z)

- Stable, productive and colloidal in PBRs and wastewater ponds
- Genome available

Strain 2: *D. armatus* (UTEX B 2533)

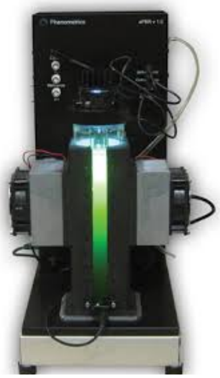
- Stable, productive and colloidal in PBRs and wastewater ponds
- Morphologically distinct from strain 1



Task 5: Technical Accomplishments

Develop Selection Methodology: Growth improvements

ePBRs operated as turbidostats maintained at low density-

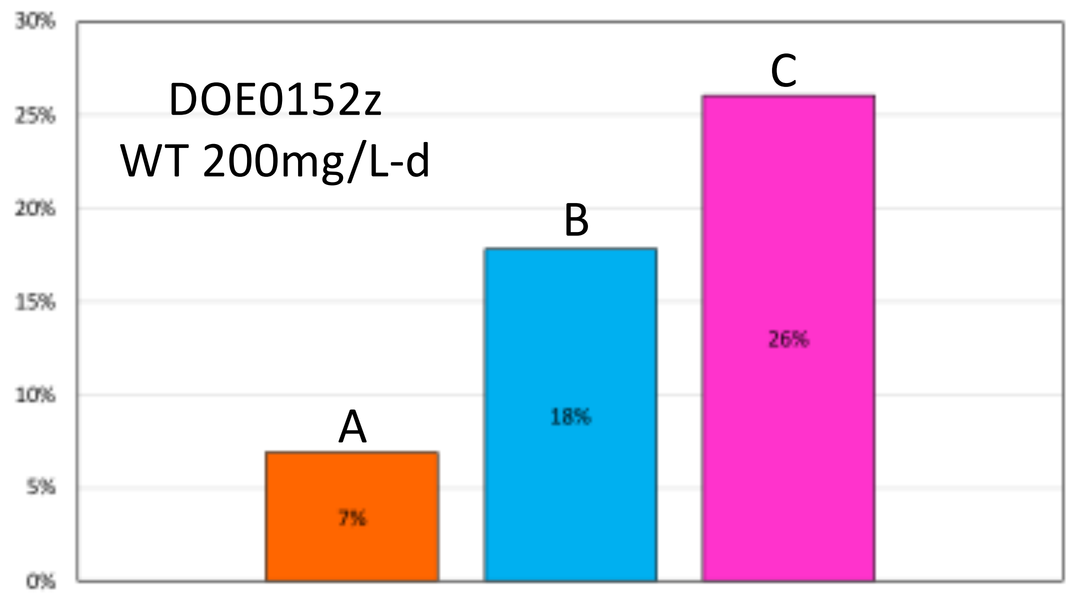


Modest productivity improvements at low culture densities after selection. Encountered technical challenges with contamination and biomass adhesion.

Selection through high dilution rate in bubble columns -

Growth rate improvements tested under pond-simulating conditions

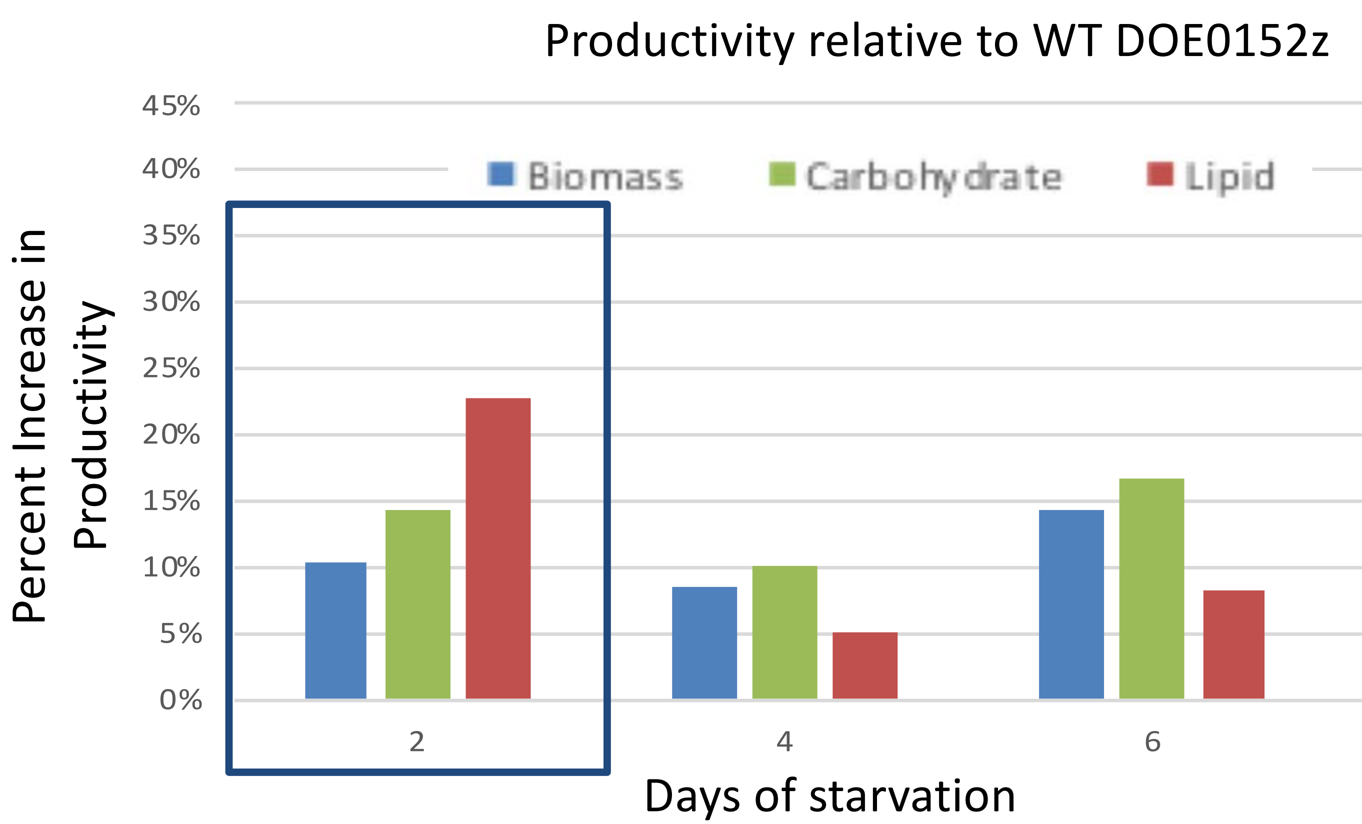
% Increase Productivity (mg AFDW/L-day)



Task 5: Technical Accomplishments

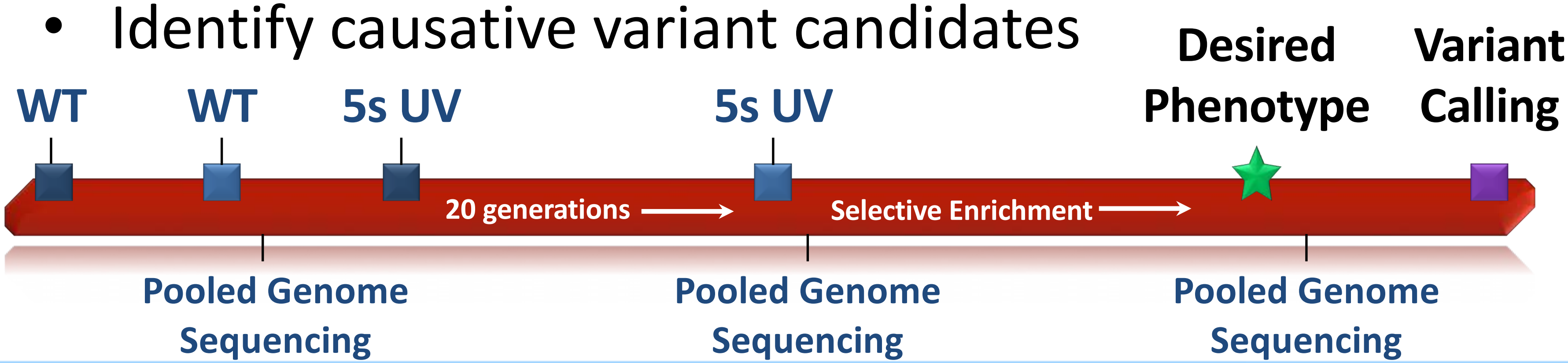
Develop Selection Methodology: FACS sorting for improvements in lipid productivity

- Designed to enrich for rapid lipidogenesis (short duration limitation)
- Promising approach resulted in cultivars with higher lipid productivity on day 2 of N-limitation relative to WT.
- Modest increases in biomolecule content, but improvements largely driven by growth improvements.



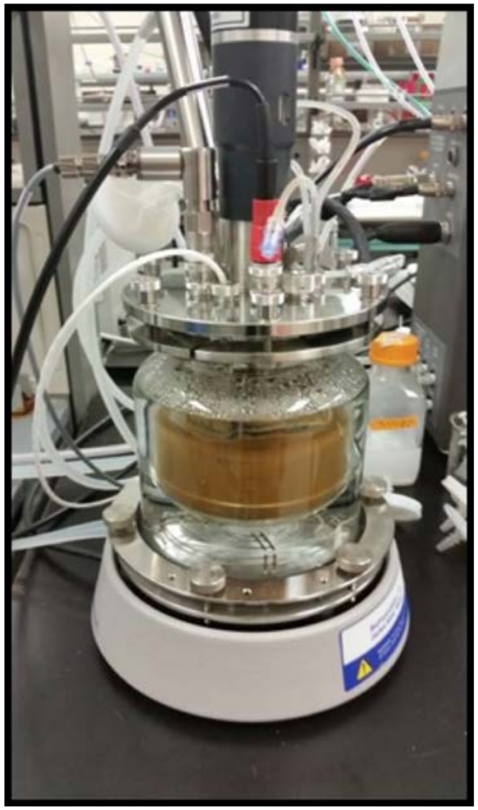
Task 7 Sandia (genomic analysis): Approach

- Strain improvement by UV mutagenesis and selective enrichments
- Use genomics to identify the genetic basis of improved strains
- Identify sequencing strategy for non-axenic cultures
- Develop bioinformatics pipeline for genetic characterization and variant analysis
- Identify causative variant candidates

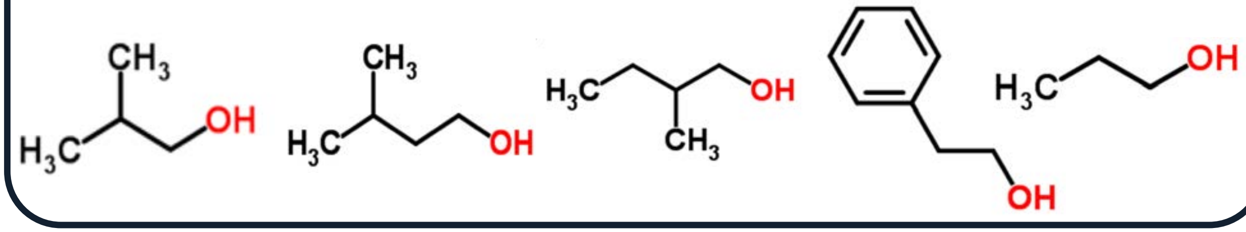


Task 7 Sandia (fermentation): Approach

Development of *E. coli* strains for protein + carbohydrate conversion to fusel alcohols in co-culture system



Fusel Alcohol Product Mix



Process Byproducts

- Residual Lipids
- Acetate

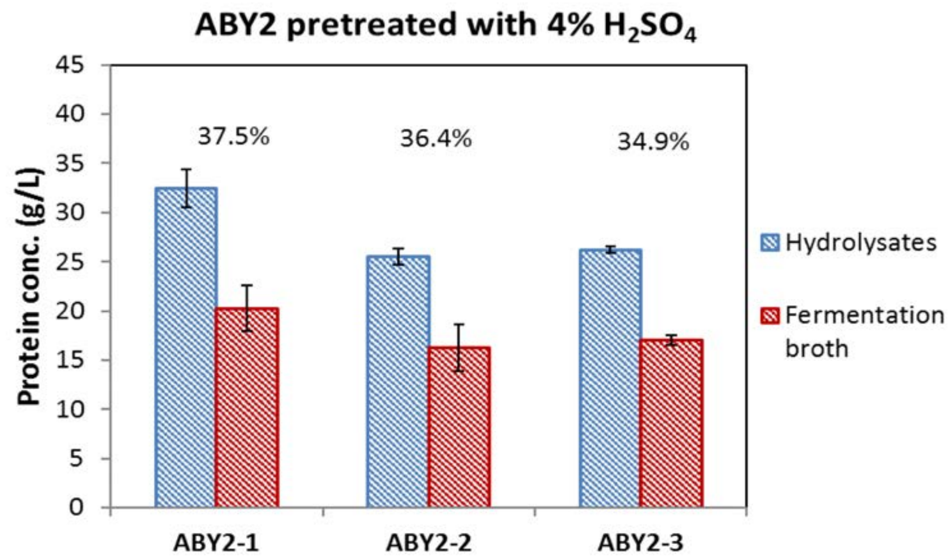
Wu and Davis *Algal Res* 2016 | Huo et al Nature Biot. 2011 | Wu et al Algal Res 2016

Task 7: Technical Accomplishments

Genomics:

- Performed genome resequencing on axenic and non-axenic culture material Illumina HiSeq X Ten
- Developed variant analysis pipeline

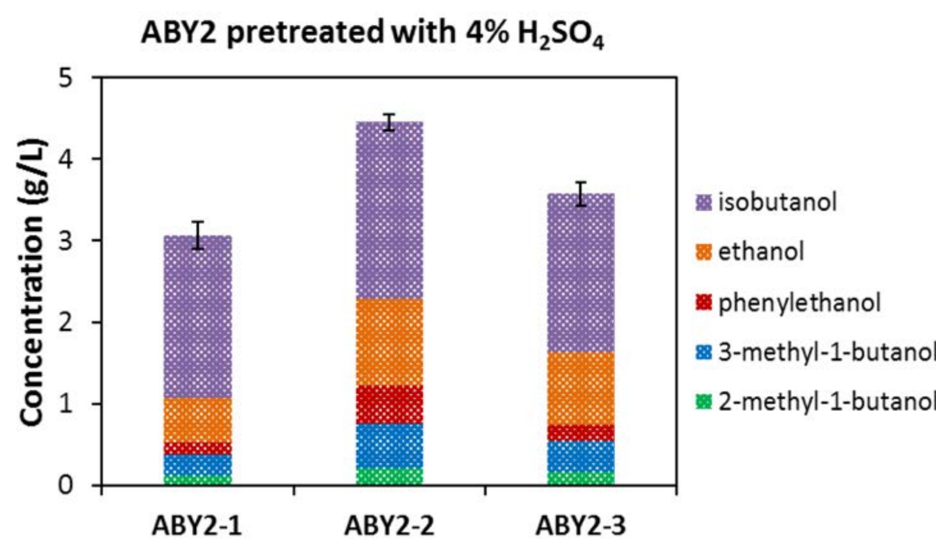
RELEVANCE: Identify genetic basis of strain improvements



Fermentation:

- Hydrolysis yield very good (38 g/L)
- Detection of high FFA, fermentation inhibitor
- 0.211- 0.264 g alcohols/g protein

RELEVANCE: Conversion efficiency

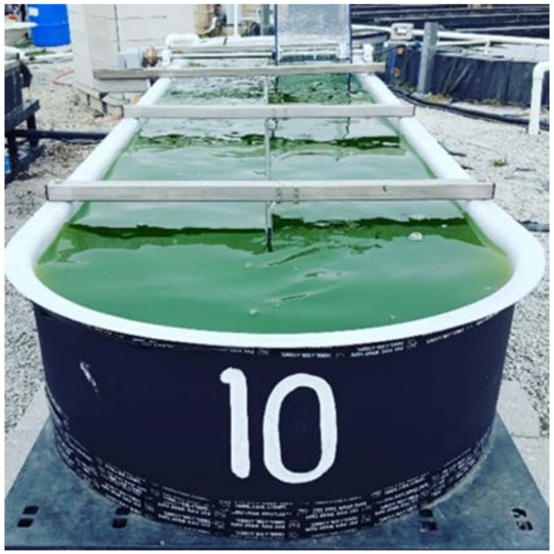


Task 3: Outdoor Work Approach

- Establish annual average numbers for baseline productivity for total biomass and carbohydrates, lipids, and proteins (g AFDW/m^2-day)
- Measure biochemical oxygen demand (BOD) for estimating autotrophic versus heterotrophic growth
- Determine outdoor hardiness/robustness of pure cultures (resistance to predation and invasion)
  - Robustness –days between culture restart due to any cause
  - Ponds were cleaned when contamination >20% of biovolume
- Compare improved strain productivity and robustness to wild type.
- Produce and process biomass for subsequent fermentation tasks

Task 3: Outdoor Work Approach

Ten outdoor ponds operated in duplicate

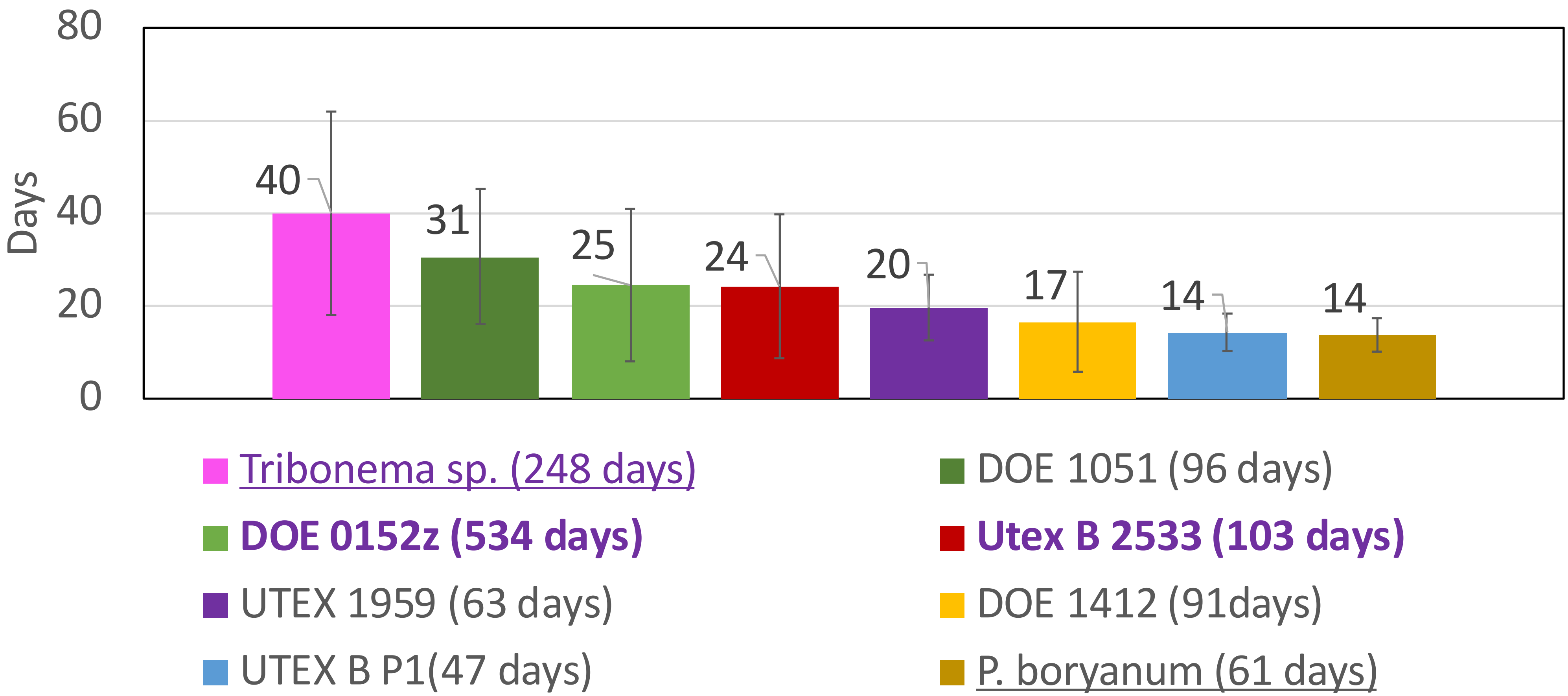


- Water sources: Reclaimed wastewater, and primary clarified wastewater
- All productivity determined from pond effluent.
- Gross and net productivity determined.
- For reclaimed wastewater gross=net
- Diluted to a 2-4 day hydraulic residence time
- Where HRT=V(L)/Q (L/day)
- 30cm depth
- AFDW sampled 3x per week
- Productivity compared as a percent of the polyculture over the growth period and robustness (days) used to down-select strains

Task 3: Outdoor Technical Accomplishments

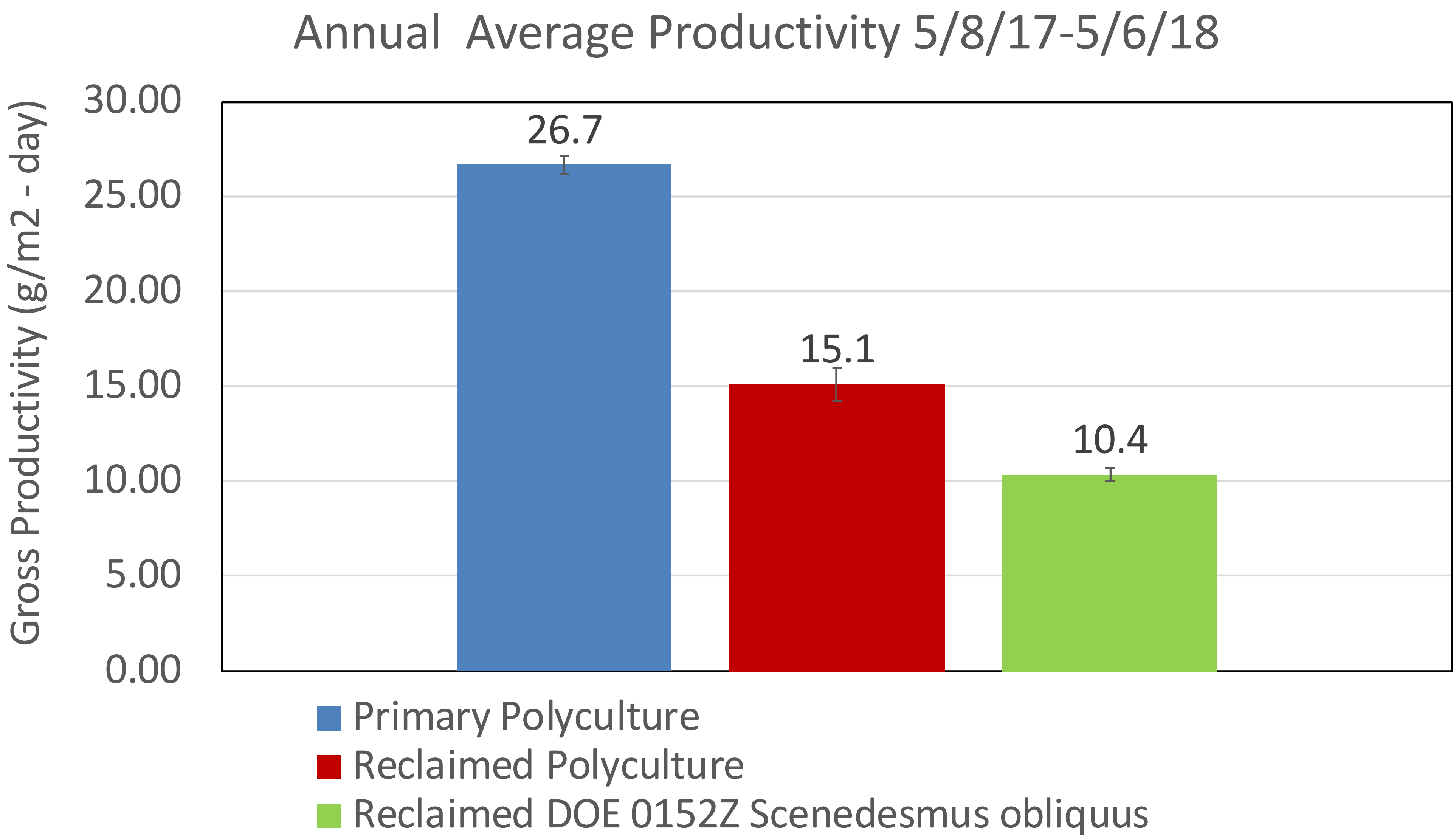
No restarts , or pond crashes due to any cause for polycultures, most robust pure culture strain (*Tribonema* sp.) was isolated from the polyculture. (Bold: two strains chosen for further work).

Pure Culture Robustness (days between restart) 5/4/17-10/29/18



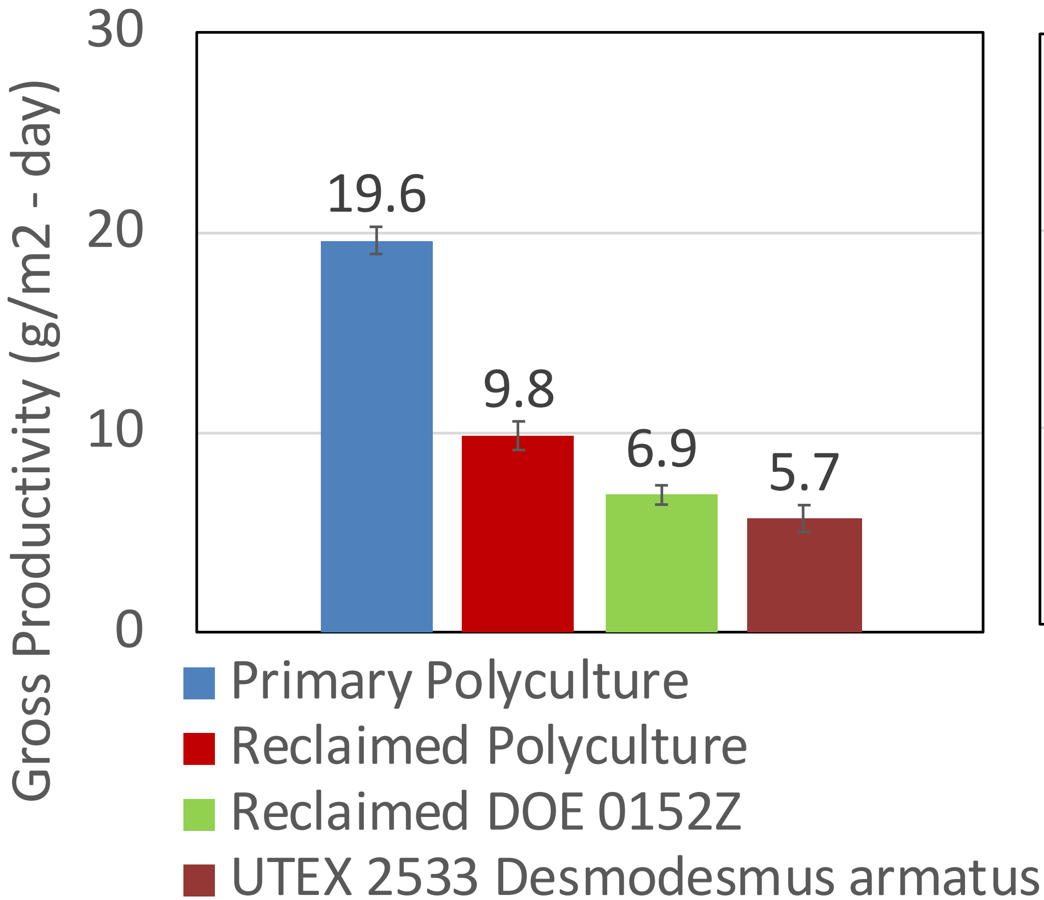
Task 3: Outdoor Technical Accomplishments

Productivity DOE 0152z *Scenedesmus obliquus* 5/8/17-5/6/18, average 25 days cultivation (primary)

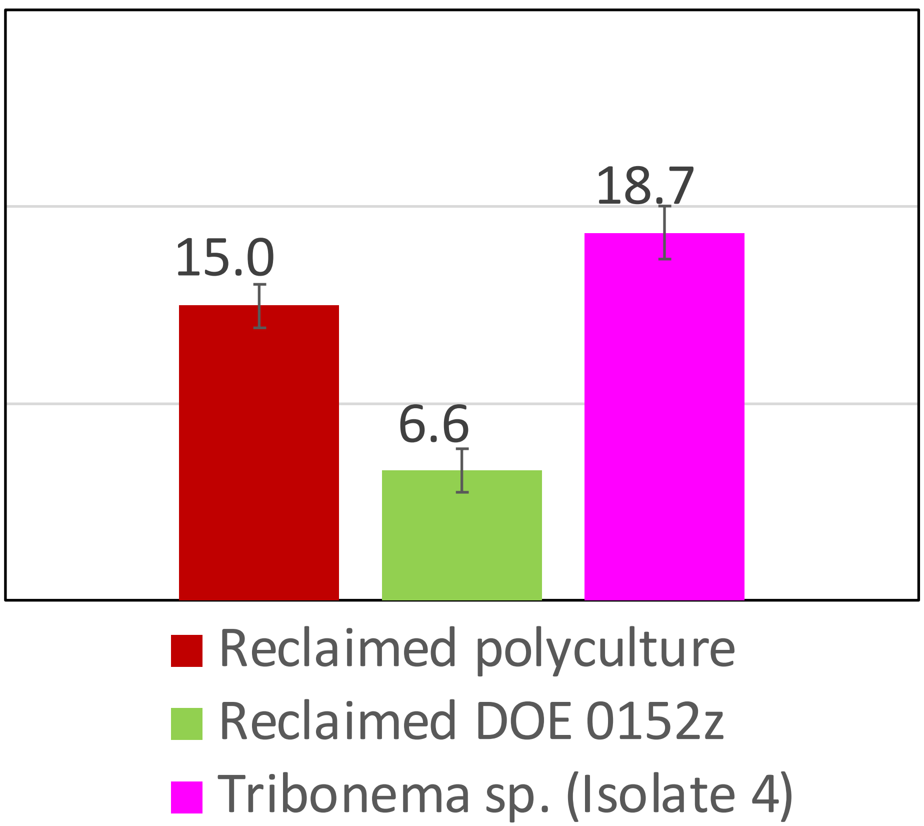


Task 3: Outdoor Technical Accomplishments

Productivity UTEX B 2533 *Desmodesmus armatus* 2/23/18-3/29/18, average 24 days cultivation (secondary)

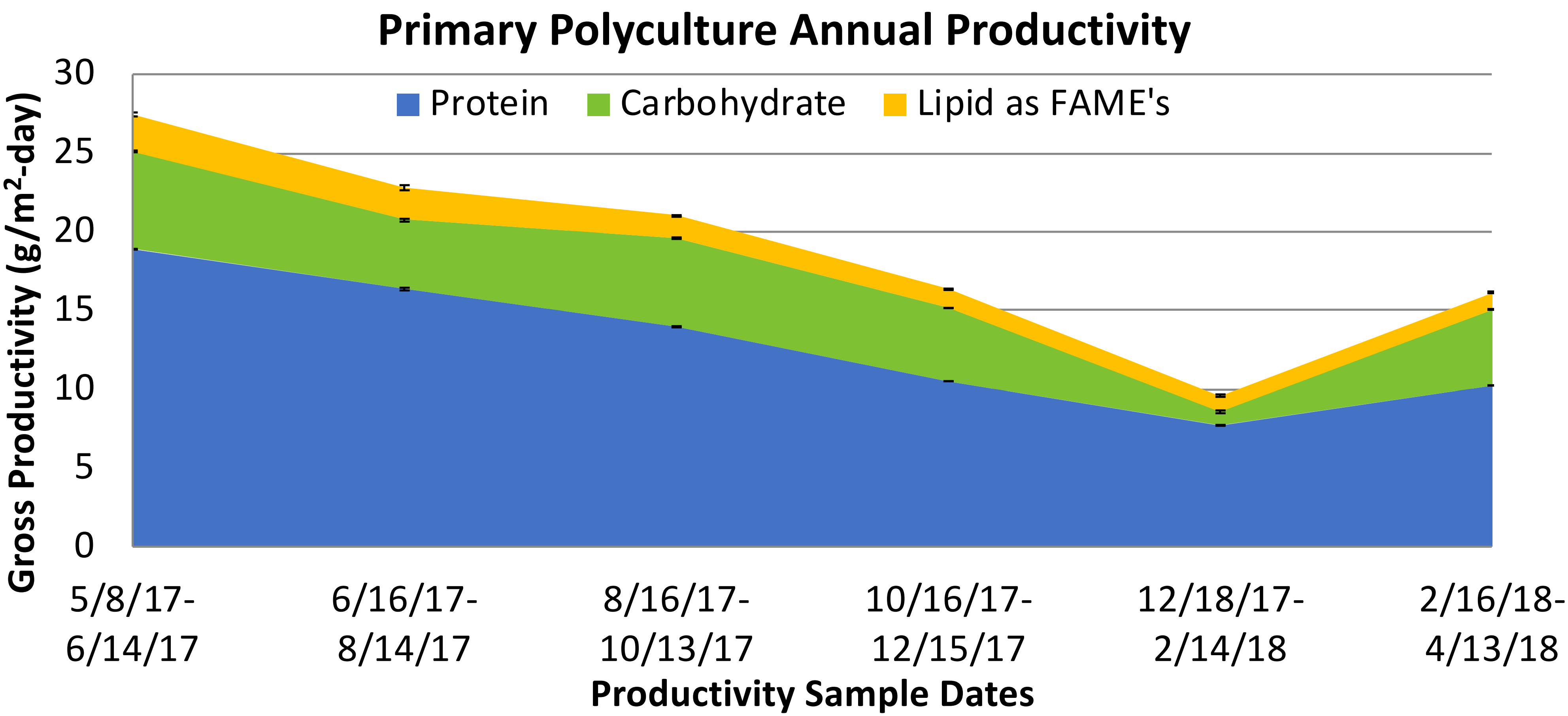


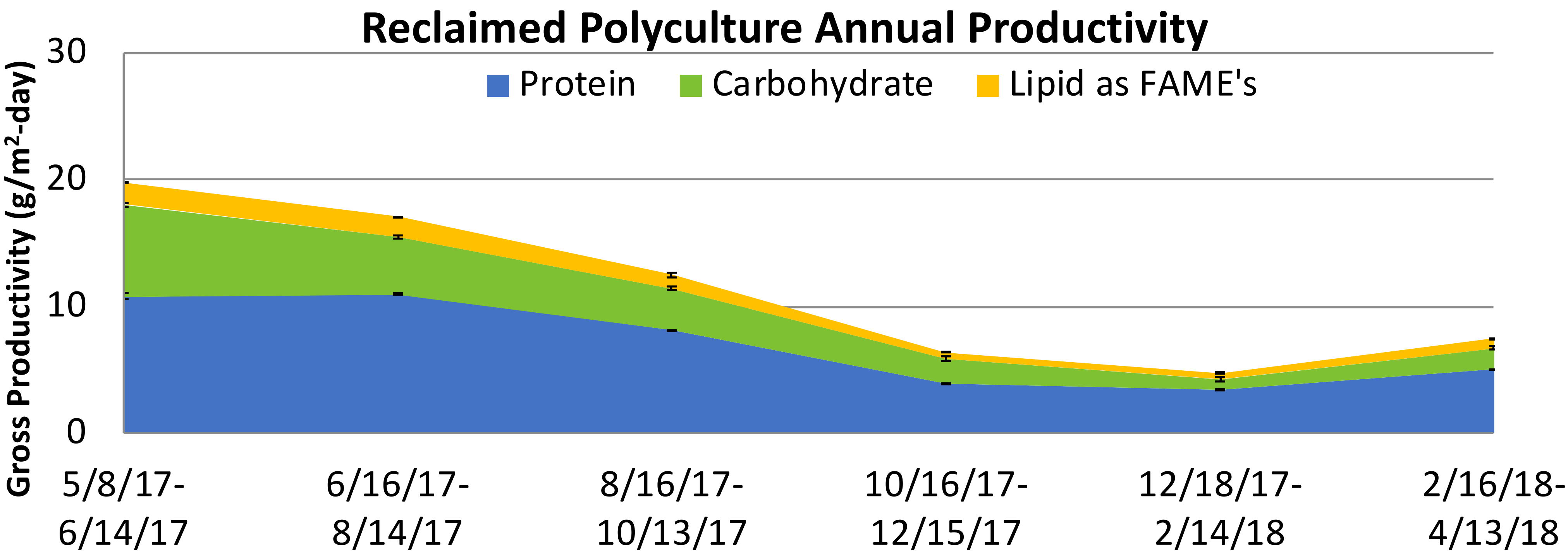
Productivity *Tribonema* sp. 7/5/18-01/07/19, average 40 days cultivation (tertiary)

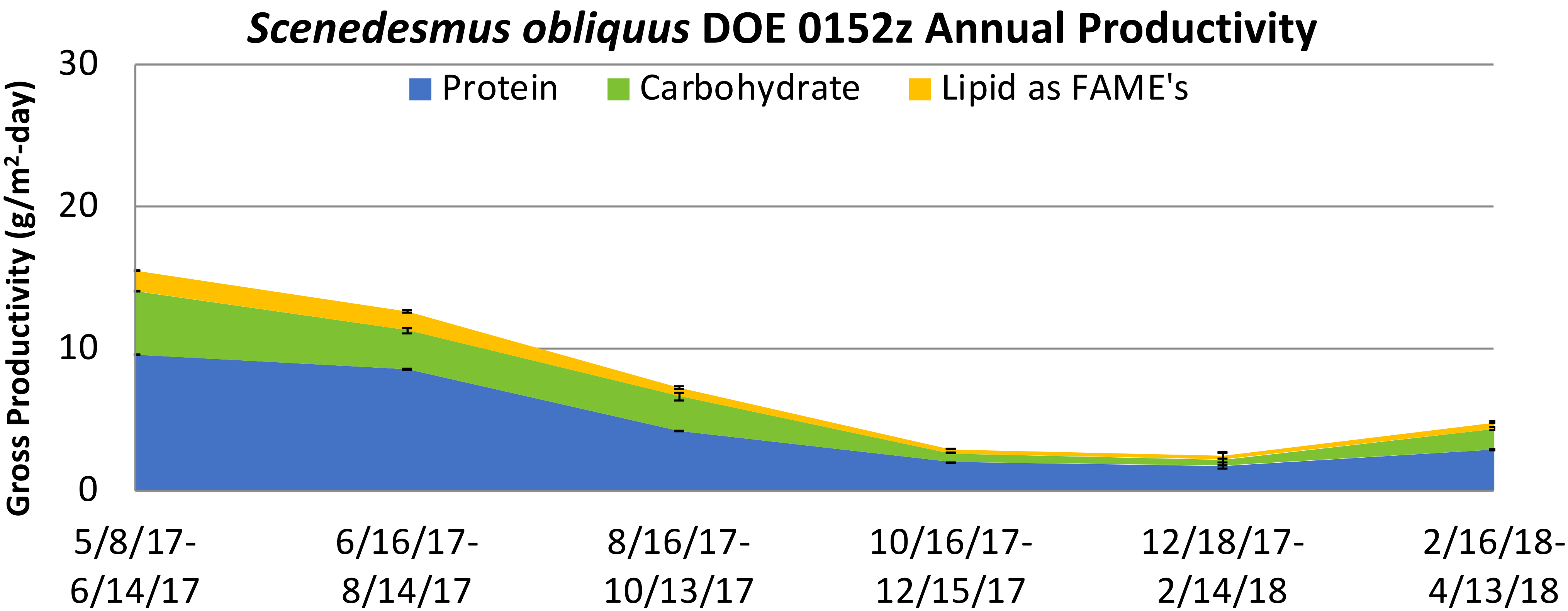


Task 6: Approach and technical accomplishments
Monitored long-term outdoor biochemical productivity using NREL biochemical methods

Relevance: Long-term outdoor cultivation of algal cultures for productivity and robustness, improvements in cultivation practices, long term study of biochemical composition using standard methods, nutrient and water recycling



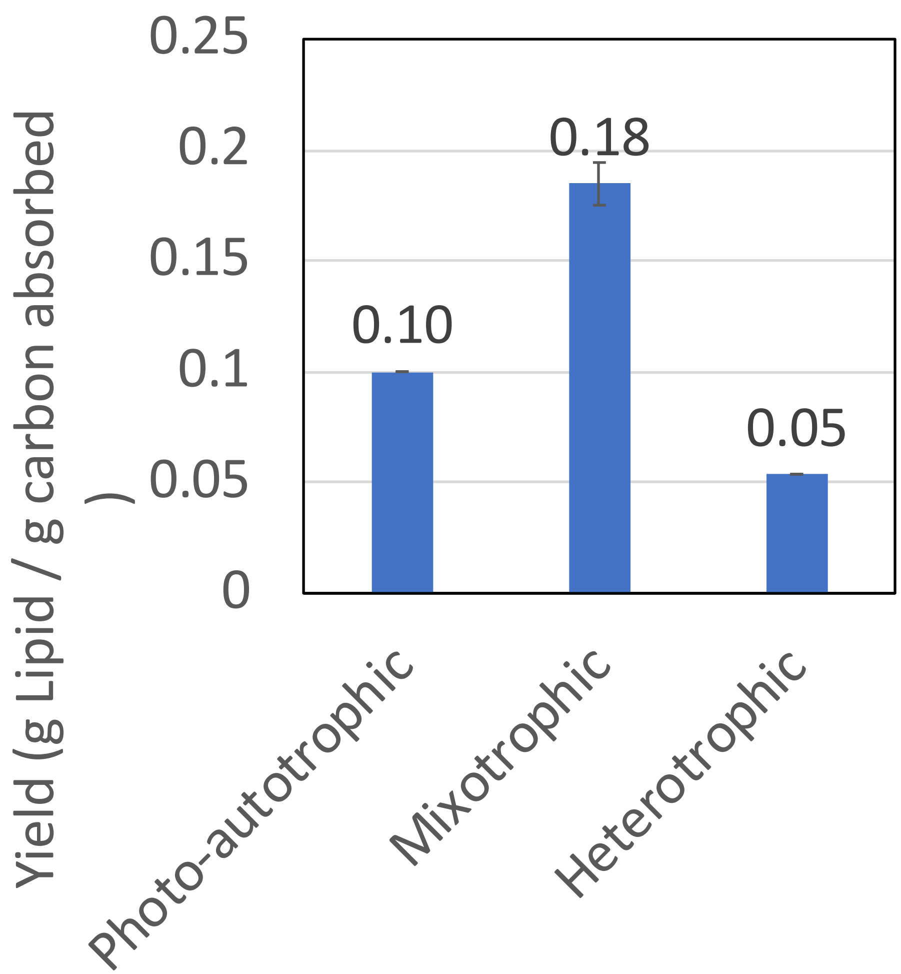

Reclaimed Polyculture Annual Productivity
Protein
Carbohydrate
Lipid as FAME's
Gross Productivity (g/m²-day)
30
20
10
0
5/8/17-6/14/17
6/16/17-8/14/17
8/16/17-10/13/17
10/16/17-12/15/17
12/18/17-2/14/18
2/16/18-4/13/18

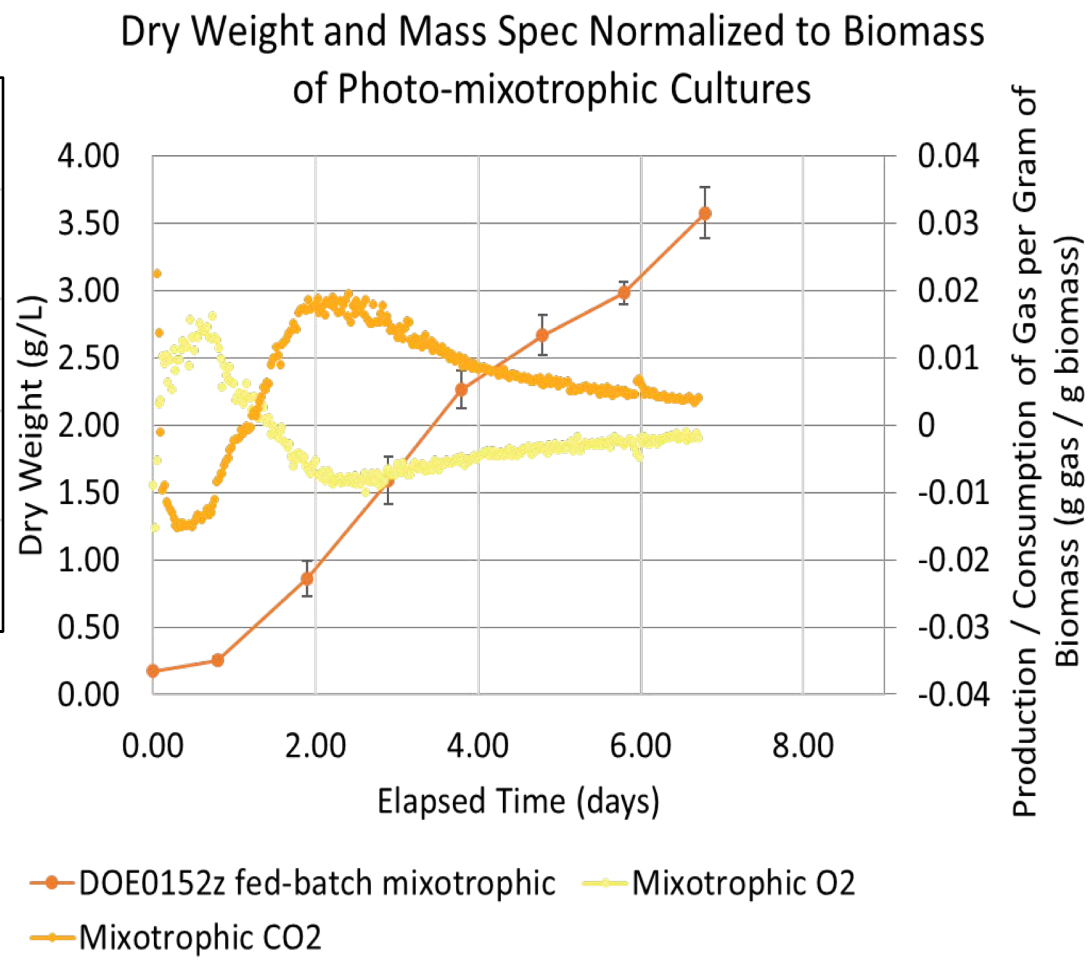

Scenedesmus obliquus DOE 0152z Annual Productivity
Protein
Carbohydrate
Lipid as FAME's
Gross Productivity (g/m²-day)
30
20
10
0
5/8/17-6/14/17
6/16/17-8/14/17
8/16/17-10/13/17
10/16/17-12/15/17
12/18/17-2/14/18
2/16/18-4/13/18

Task 9: Heterotrophic Growth Approach (Heliae)

- AMP = Advanced Mixotrophic Platform
- Glass bubble columns with 8 reactors per unit
- Variable temperature, light intensity, 3 inlet gas rotometers, and pH auxotstat dosing
- Acetate used to simulate organic carbon from anaerobic digestion
- Mass spec used to monitor O_2 and CO_2
- DCMU = (3-(3,4-dichlorophenyl)-1,1-dimethylurea),PSII blocker and DBMIB = dibromothymoquinone, a PS1 blocker used

Task 9: Heterotrophic Growth Technical Accomplishments: *Scenedesmus obliquus* (DOE 0152z) Yields of 0.18 g Lipid/g Carbon for mixotrophic growth





Task 9: Heterotrophic Growth Technical Accomplishments: Inhibition of one or both photosystems in photo-mixotrophic cultures yields higher growth rates than heterotrophic cultures

RDT2900			RDT2976		
	AVG growth rate (g/L/day)	% diff to mixo		AVG growth rate (g/L/day)	% diff to mixotrophic controls
Photo	0.205	83.629	Mixo control	0.655	0.000
Mixo	0.501	0.000	Mixo + DBMIB	0.573	13.467
Mixo w/ DCMU	0.416	18.474	Mixo + DBMIB and DCMU	0.355	59.352
Hetero	0.174	96.619	Hetero	0.138	130.364

Relevance: Understanding the basis of mixotrophy. Productivity improvements with mixotrophy.

Task 8: Approach HTL of residuals

- High ash
  - Residuals often greater than 50% ash
    - HTL limit was 30% ash to prevent filter clogging
- Recycling of nutrients inhibited growth of the selected strains
  - Recycling of HTLaq was tested on 6 strains
  - *Pseudopediastrum boryanum* (RWW08) 17% reduction in volumetric productivity at 400x dilution
  - *Scenedesmus obliquus* (DOE 0152z) 46%, and 38% reduction at 400X
- Options included
  - Pretreating HTLaq with aerobic or anaerobic treatment. CHG.
  - Longer term adaptations
  - Investigating less sensitive strains
    - *Chlorella sorokiniana* (DOE 1412)
  - Using a different process

Task 8: revised approach anaerobic digestion

Advantages

- Process less sensitive to ash
- Nutrients better conserved
- Improves TEA/LCA over HTL (see Task 4)
- Fuel flexibility (LRNG, pipeline etc.)
- Improved productivity on digester effluent (ASAP project)

Relevance

Complete conversion of algal carbon to biofuel after the primary fuel extraction process (lipid extraction, carbohydrate and protein fermentations). Solubilization of nutrients for recycling.

Task 4: TEA/LCA approach: Input experimental results into the engineering model

- MicroBio Engineering's Environmental Sustainability and Process Economics (ESPE) model
- Location (climate data): San Joaquin Valley
- Facility Size: 400-ha pond surface
- CO_2 Source: MEA concentrated from anthropogenic source
- CO_2 Transfer Efficiency: 80%
- Blowdown: 10%
- Culture Media: Medium Strength Wastewater
- Harvest Efficiency: 95% stage, 86% overall
- Economic model assumptions harmonized to NREL standards for general methodology and additional indirect cost factors (engineering and construction fees, contingency, field expenses, etc.)

Task 4 TEA/LCA: Technical Accomplishments:

Intermediate Goals Exceeded , Improved Strains and Mixotrophy will Further Improve Results

Task	Intermediate Goals	Protein Scenario	Carbohydrate Scenario	Lipid Scenario
Task 4: TEA (RSP $/GGE)	$9.40	$6.11	$6.98	$5.58
Task 4: LCA (gCO2e/MJ)	79% Reduction*	9.31	6.79	-15.6
Application Economics: BFI Yield	2704	4,293	2,009	3,182

Relevance**: Outdoor annual average protein and productivity data** used to develop protein scenario values. Shorter term outdoor data used for carbohydrate scenario.

Future Work: Lab strain improvement (Task 5) and genomics (Task 7)

- Continue selection for growth rate and productivity improvements
- In parallel, continue selection for improved lipid or carb content
- Stack traits through sequential selection for growth improvements followed by lipid or carbohydrate productivity improvements
- Variant analysis of chloroplast and mitochondrial genomes
- Genomic characterization of *S. obliquus* DOE-0152z strains with desired phenotypes
- Genomic characterization of secondary strain: *Desmodesmus armatus* UTEX B 2533
- Develop additional variant calling tools

Future Work: Outdoor work (Task 3), fermentations (Task 6 &7), and Digestion (Task 8)

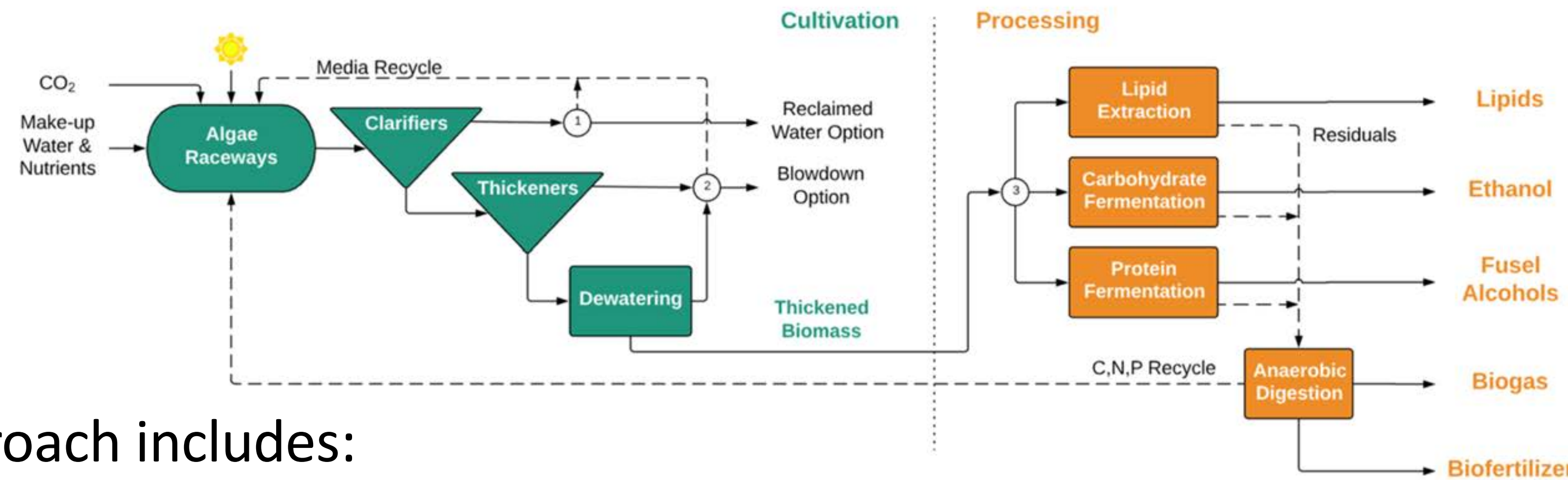
- Continue testing improved strain culture against wild type and polycultures
- For strains that demonstrate improvement develop long-term productivity and robustness data
- Continue testing cultivation conditions and strains that improve productivity and robustness
- Continue long-term outdoor tracking of biochemical composition of strains over time to identify compositional stability
- Evaluate conditions that reduce fermentation inhibitors in protein and carbohydrate fermentations
- Digest fermentation residuals and measure gas yields and regrowth on digestate

Future Work: Heterotrophic growth (Task 9) and TEA/LCA (Task 4)

- Heterotrophic work
  - Outdoor mixotrophy
  - Investigate multiple potential sources of waste organic carbon
  - Investigate C, N & P recycling
- TEA/LCA
  - Continue to update the models with experimental data as it becomes available
  - Contribution and sensitivity analysis to help guide experimental work
  - Update of protein fermentation economic model based on SNL provided information

Summary

Meeting the BETO MYPP goal of developing technologies that enable mature modeled annual average algae yields of 3,700 gallons/acre year requires significant advances in productivity, co-products, and conversion of the whole biomass to fuel



Approach includes:

- A multilevel screen approach to identify promising strains for improvement
- Improvement through rapid dilution and selective enrichment
- Mixotrophic productivity improvements
- Conversion of the whole biomass to biofuel
- Long-term, outdoor demonstration of productivity, robustness, and biochemical composition for both wild-type and improved strains

Summary

Accomplishments

- Screened 15 pure culture strains indoors and 8 pure culture strains outdoors. Selected three strains for improvement.
- 27% improvement in growth rate over wildtype for *Scenedesmus obliquus* (DOE 0152z).
- Produced 3 annual average productivities outdoors of 26, 15, and 10 g/m^2-day for the primary polyculture, reclaimed polyculture, and reclaimed *Scenedesmus obliquus* (DOE 0152z)
- Met or exceeded intermediate goals for mixotrophy, protein fermentations, biochemical composition and the TEA/LCA.

Relevance

- Intermediate results for protein carbohydrates and lipids 4,293, 2,009 and 3,183 GGE/acre-year and 6.11, 6.98, 5.58 $/GGE, respectively.
- Project targets critical barriers including productivity improvements, long-term cultivation outdoors, improvements in cultivation practices, nutrient recycling, improvements in conversion and more.

Future work

- Continue strain improvement work in conjunction with outdoor work and biochemical composition determinations

Acknowledgements

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Todd Lane
Krissy Mahan
Ryan Davis

heliae

Steven Pflucker
Mike LaMont

Pacific Northwest National Laboratory

Dan Anderson

Additional Slides

Responses to Previous Reviewers' Comments

- Reviewer: I think the TEA of this project would benefit greatly from including a reputable member of industry practiced in upgrading oils to make them suitable as a refinery feedstock. The industry partner should advise on the cost of upgrading (including the removal of metals) the different oils generated by different HTL operating conditions to make them acceptable to a refiner and at what discount the refiner would want in order to take the oils.
- Response: HTL was removed from the process because algal residuals were too high in ash for the HTL process and because recycling nutrients from HTL reduced the algal productivity. The remaining fuels in this process include, lipids as fatty acid methyl esters, ethanol, fusel alcohols, and biogas. Of these fuels, fusel alcohols are the least understood in terms of market value; however, they are also a drop in fuel similar to ethanol. Significant work in BP3 will revolve around better understanding the market and value of fusel alcohols.
- Reviewer: It was not clear from the presentation what sort of innovation will be implemented that is expected to enable productivity targets to be met. Trying to simultaneously generate strains with high lipid, carbohydrate, or protein would suggest the lack of a clear path to improved economic outcomes, and strains that are enriched for one component may exhibit lower productivity overall.
- Response: All of the selection approaches applied in this project incorporate selective pressures for growth improvement relative to wild type or, minimally, continued growth in conjunction with enhanced biomolecule content. These approaches are geared toward unlocking biomass productivity potential in combination with improvements in biofuel precursor content, or the rate at which cells transition into biomolecule synthesis (ie. the rapid induction of lipidogenesis). Moreover, there is an association between rapid growth and protein content, therefore, the incorporation of selective pressure for enhanced overall productivity is synergistic with improvements in the content of this particular biofuel intermediate. Incorporation of mixotrophy using waste carbon and or wastewater will also yield productivity improvements.

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: Wastewater treatment 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 the wastewater contains 30-60 mg NO_3-N/L and the pond is operated at a 2 day hydraulic residence time meaning that the wastewater can easily support productivities over 45 g/m2-day assuming biomass is 10%N. Similarly phosphorus concentrations are typically around 10mg PO_4-P/L.

- Reviewer: It is unclear how much of the yield target is accomplished from heterotrophic growth versus photosynthetic growth.
- Response: For the outdoor work performed on wastewater, heterotrophic growth is determined using Biochemical Oxygen Demand (BOD). This method is derived from standard equations used in wastewater treatment biological processes and is unable to separate bacterial heterotrophy from algal heterotopy and/or mixotrophy. For the axenic mixotrophic work in the lab heterotrophic growth is measured in the closed reactor by sampling the headspace gases using a GCMS.

Presentations and Posters

- 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.
- 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.