Multi-Scale Characterization of Improved Algae Strains

U.S. Department of Energy (DOE)
Bioenergy Technologies Office (BETO)
2017 Project Peer Review

Advanced Algal Systems

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March 7, 2017

Los Alamos NATIONAL LABORATORY EST.1943

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NNSA National Nuclear Security Administration

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Goal Statement

Goal

The goal of this project is to aid the realization of cost-effective algae biofuels, by developing approaches for increasing algae strain productivity and by generating a better understanding of the relationship between laboratory and outdoor productivity data

Outcomes & Relevance

- *Accelerate the advancement of the algae biofuels industry through*
  - *New strain improvement methods*
  - *Informing the scale-up process*
- *Enable BETO in reaching their biofuel and cost targets*

Quad Chart Overview

Timeline

- Start Date: 10/1/12 (10/1/15 since last Merit Review)
- End Date: 9/30/18
- Percent complete = ~ 75%

Barriers

- *AftC. Biomass Genetics & Development.* "The productivity and robustness of algae strains… could be improved by selection, screening…"
- Validated methods for non-GM strain improvement; Understanding 'flask to farm'
- 3,700 gal biofuel intermediate by 2020
- <$3/GGE by 2030

Budget

	Total Costs FY 12 –FY 14	FY 15 Costs	FY 16 Costs	Total Planned Funding (FY 17-Project End Date)
DOE Funded	475k	350k	500k	1.3M
Project Cost Share (Comp.)*	None	None	None	None

Partners

- *Intrafunded Partners*
  - ATP^3 testbed facility
  - New Mexico Consortium
- *Interfunded Partners*
  - PNNL (M. Huesemann, separate AOP funds)
  - Greenhouse AOP and other LANL AOPs

1- Project Overview

Challenge

Outdoor productivity needs to be ~doubled by new strain identification or improvement, but algae strains developed in the lab often do not perform as well or as predictably when moved outdoors.

Question

How do we go from flask to farm? How do we improve and downselect strains indoors in a way that increases our chances of developing strains that demonstrate high performance outside?

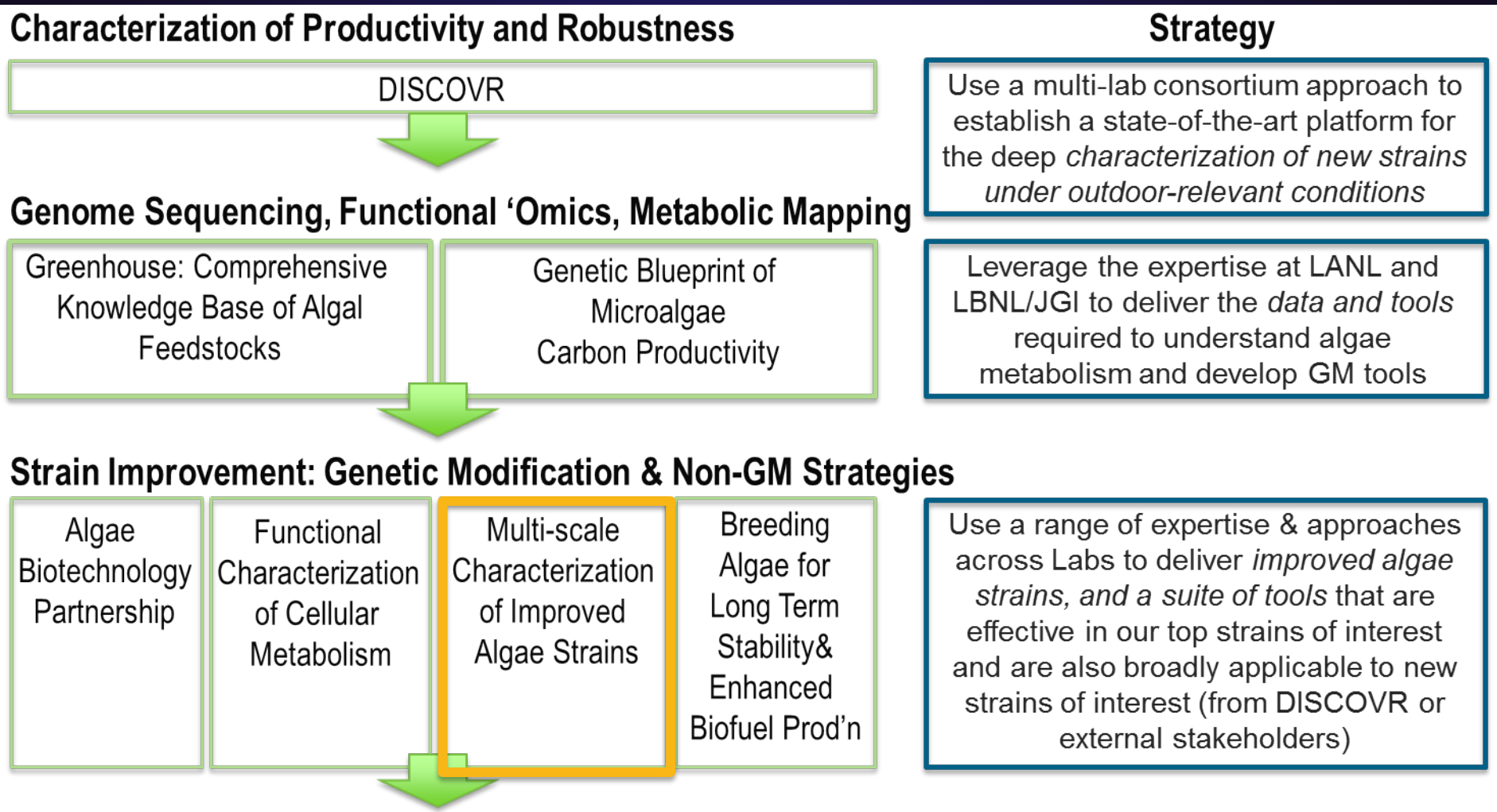
Opportunity

Establish methods for generating improved algae strains and evaluating their performance at multiple scales, from the bench to outdoors, by integrating national lab capabilities in algal biology, flow cytometry, and environmental simulation experiments, and by leveraging BETO's investment in algae testbed facilities.

Technical Objectives

1. Strain Improvement
2. Strain Transition
3. Molecular Mechanisms

1- Project Overview



GOAL: *Deliver deeply characterized and improved strains, with accompanying data and tools, to stakeholders including industry, academics, and other BETO projects (e.g. BioFoundry)*

2 – Approach (Management)

- Meetings with the project (LANL) team and individual researchers regarding current experiments, approach, and progress are held weekly
- Team members contribute to presentations, quarterly/milestone reports, and Go/No-Gos
- Discussions with PNNL and ATP^3 regarding collaborative work are held on an as-needed basis
- PI is responsible for ensuring progress and execution of project plan, making final scientific decisions, meeting milestones, and complying with reporting requirements

2 – Approach (Technical)

3 Objectives

1. **Strain Improvement**: Use non-genetic modification strategies to improve strain productivity and robustness, including flow cytometry and adaptive evolution.
   - *Advantages*: Uses natural genetic/phenotypic variation when possible; non-GM strategies permit us to move outside easily.
2. **Strain Transition**: Develop and utilize strategies for transitioning our improved strains from laboratory flask experiments to outdoor ponds.
   - *Advantages*: Allows us to delineate the relationship between our lab-scale systems and outdoor data; enables our efforts to “bring the outdoors in”.
3. **Molecular Mechanisms**. Identify and validate the underlying biochemical phenomena that results in improved strain phenotypes.
   - *Advantages*: Takes our results to the next level by identifying new gene targets.

Potential Challenges

- Improved strains do not grow well outdoors (are not fit)
- Increases in productivity do not translate to outdoor systems.

Critical Success Factors

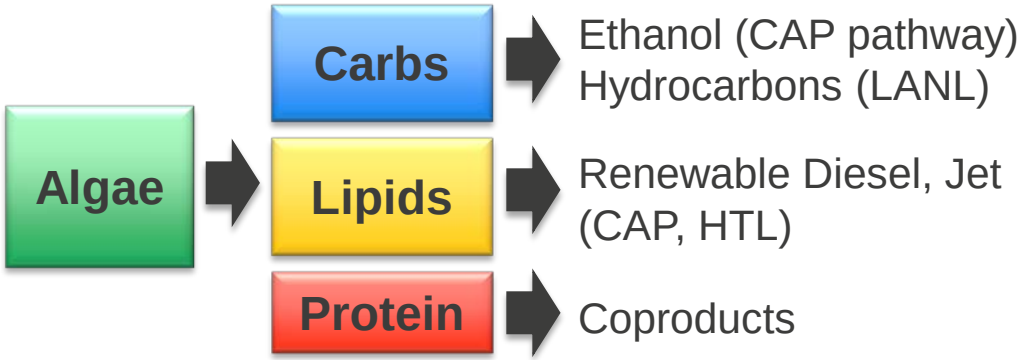
- Relative indoor performance translates to outdoor systems
- *Outdoor testbed facilities remain available*

2 – Approach (Technical)

Our platform strains are on par with the current state of technology

[Marine Strains] **Season** (Months)	**2016 SOT g/m²-d**	**2020 Target g/m²-d**
Spring (MAM)	11	22
Summer (JJA)	13	26
Fall (SON)	7	14
Winter (DJF)	5	10
Annual Average	9	18

Biochemical composition is important for reaching BETO's biofuel intermediate targets



Platform Strain	**May g/m²-d (1000L)**	**Biochem Data**	**Water**
Picochlorum soloecismus	15.6	40% Carb 30% Lipid	Marine
Chlorella sorokiniana 1228	13	34% Lipid	Fresh to Brackish

Approach: Use 2 strains that perform at the ~SOT level as platform strains to develop our methods around improving lipid and biomass productivity.

3 – Accomplishments (Technical)

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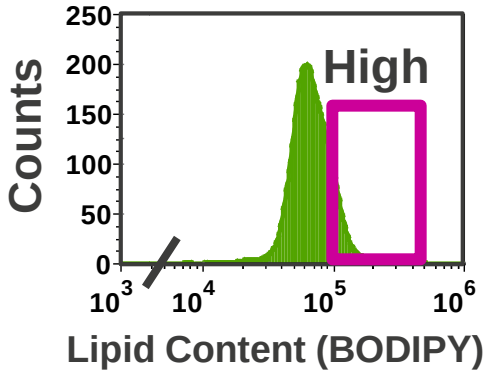
3 – Accomplishments, Strain Improvement

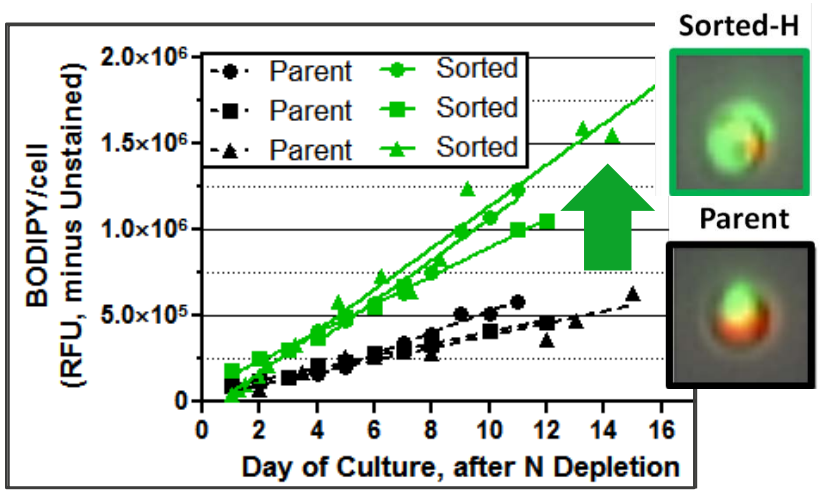
Fluorescence-activated cell sorting is used to improve productivity

Sort Top Lipid Accumulating Cells



Rounds of Sorting

Regrowth



Strain Name	Year of Sort	Improvement in Lipids Over Parent	Stability
Picochlorum soloecismus	2011 NAABB	70%	Re-sorted 2015
***N. salina* 1776**	2014 w/ Twary	30%	Still stable
***C. sorokiniana* 1228**	2014	70%	Still stable
***C. sorokiniana* 1602**	2014	80%	Stable at last check (late 2014)

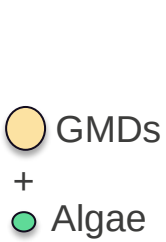
- 1000s of single cells interrogated per second in a flowing sample stream
- BODIPY staining permits sorting on neutral lipids → FAMEs → Fuel
- Generated new phenotypes for 4 strains
- Strains are stable for 2+ years

- Demonstrated success of alternate sorting strategies (Twary AOP; Cellana ABY1)
- Developed alternate staining protocol for recalcitrant strains

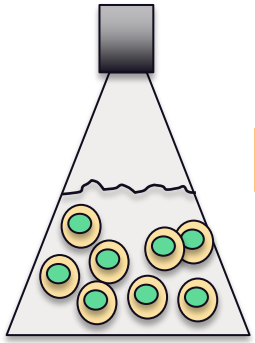
3 – Accomplishments, Strain Improvement

Fluorescence-activated cell sorting is used for improved productivity

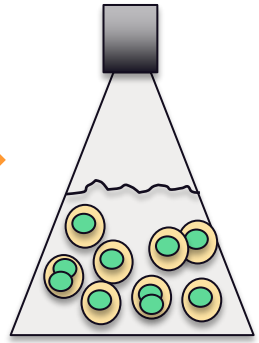
A Cell Sorting Method for Increasing Biomass Productivity



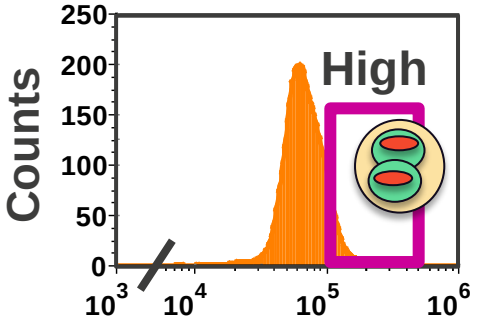
Form aGMDs



Grow culture



The most rapidly dividing cells end up with higher levels of chlorophyll per GMD

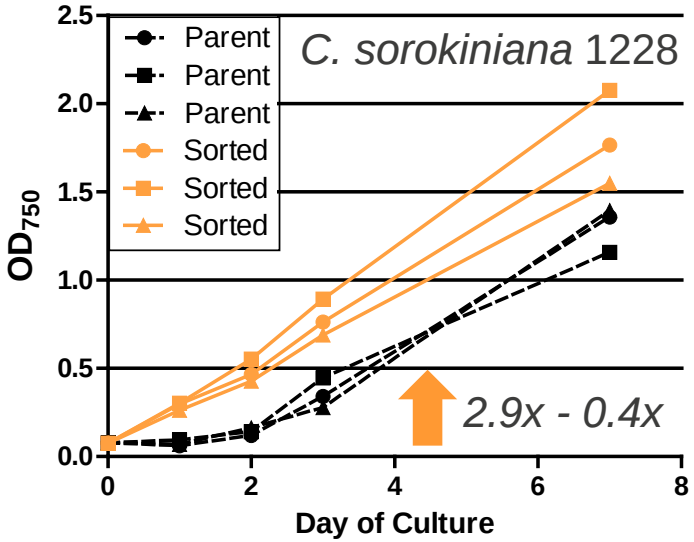


Autofluorescence of Gel MicroDroplet-encapsulated Algae

Break GMDs, free Algae

Regrowth NO GMDs after final sort

Test Growth



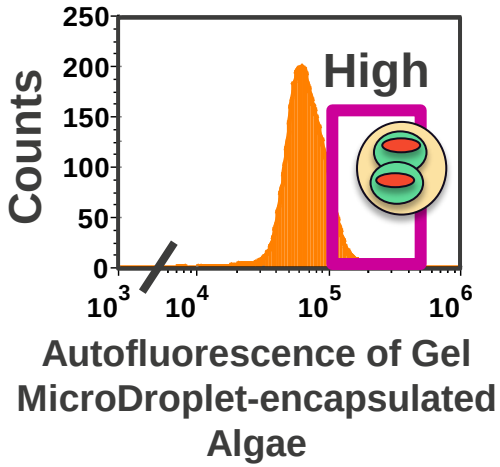
3 – Accomplishments, Strain Improvement

Fluorescence-activated cell sorting is used for improved productivity

A Cell Sorting Method for Increasing Biomass Productivity

18 month Go/No-Go: Validation of biomass growth assay coupled with mutagenized population by generating at least one strain with improved biomass accumulation of 30%.

Status: On track. Demonstrated 40+% increase in biomass accumulation using natural variation in genetic/phenotype diversity



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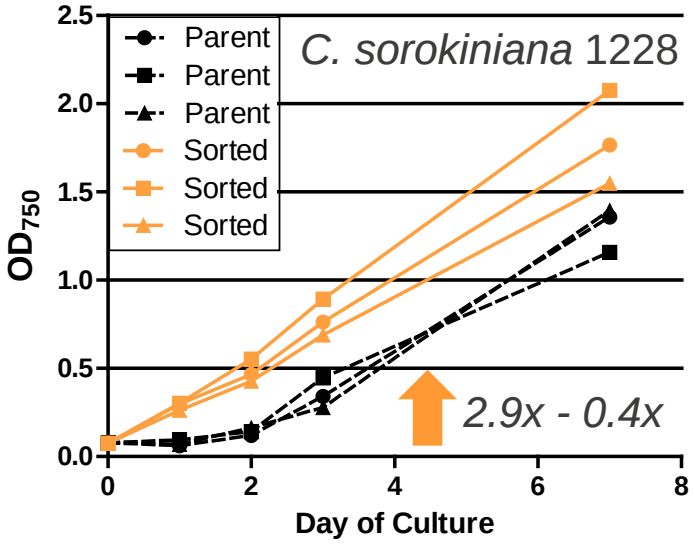

3 – Accomplishments (Technical)

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3 – Accomplishments, Strain Transition

A pipeline was established for downselecting & transitioning strains outdoors

Lab-Scale Strain Improvement & Characterization

LANL

Improve strains by cell sorting

Characterize at the flask level

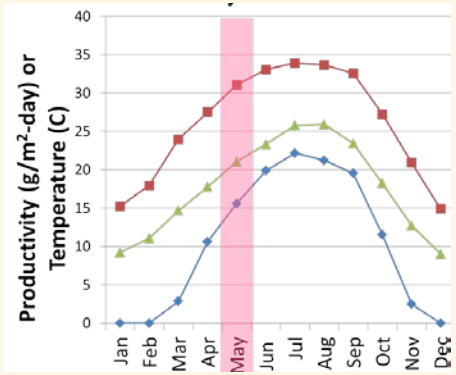
PNNL

Characterize further for biomass growth model input

Productivities Predicted by the ssBAT

PNNL

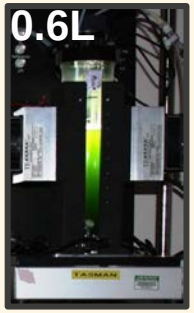
Predict areal productivities (g/m^2-d) and make scripts



Outdoor Conditions Tested Indoors at Two Scales

LANL

Use scripts in Phenometrics ePBRs (n=37, CO_2/light/temp)



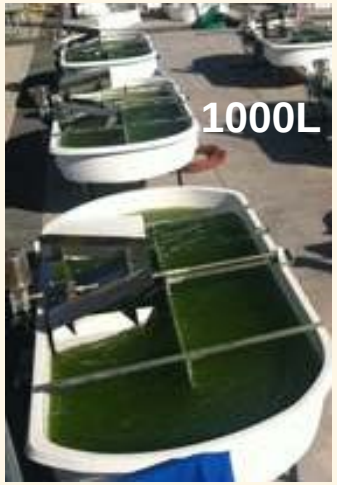
PNNL

Use scripts in indoor, environmentally controlled ponds (n=3, CO_2/light/temp)

Strains Tested Outdoors

ATP³

Use outdoor testbeds to grow LANL strains



Pacific Northwest NATIONAL LABORATORY

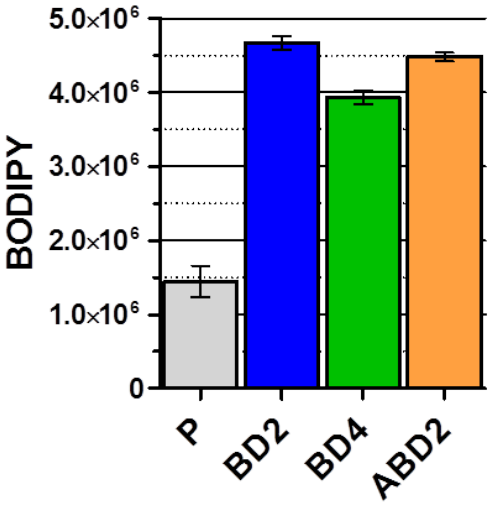
ATP³ Algae Testbed Public-Private Partnership

Samples were analyzed at LANL using the same methods developed for the flask system

3 – Accomplishments, Strain Transition

Case study in moving a parent and cell sorted Chlorella *strain outside*

Cell sorted C. *sorokiniana* 1228 showed increased lipids



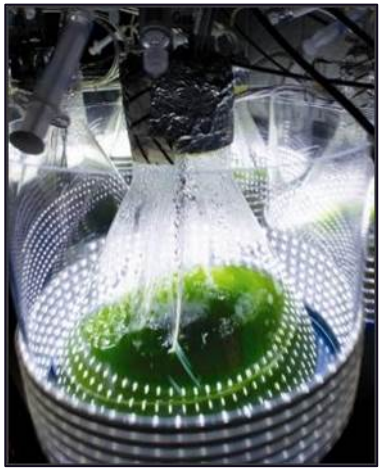
'Spinner' Flasks:

25°C,

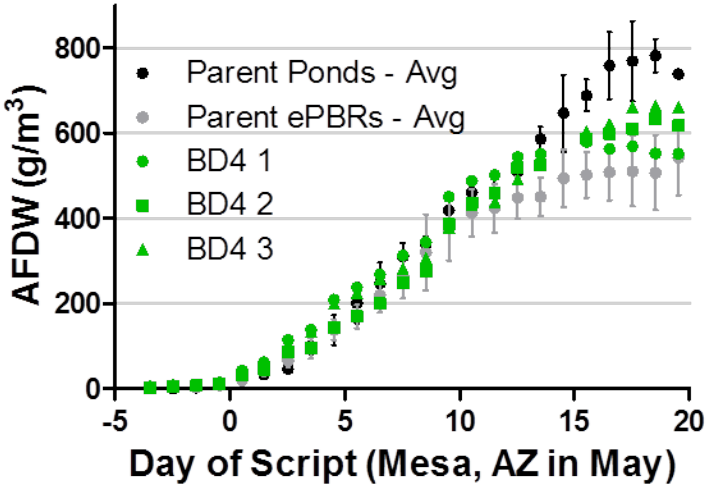
800 µE/m^2-s^2

16:8 light:dark on/off

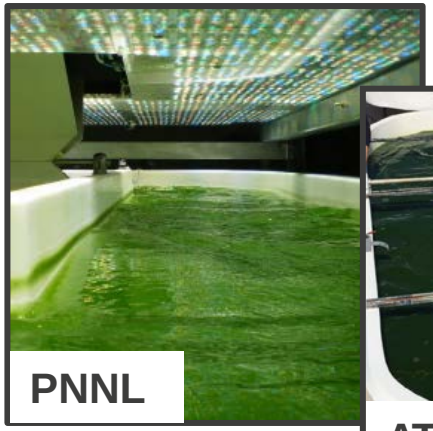
Batch cultures



Simulated May at ATP^3 site to downselect



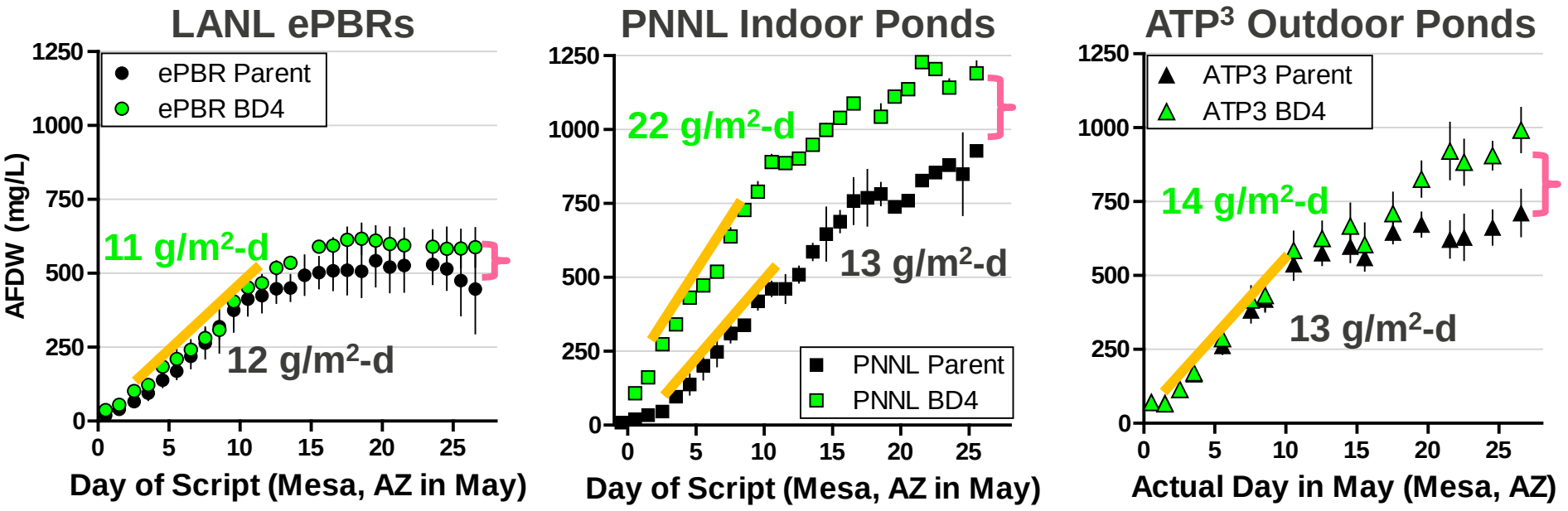
Simulated in indoor & tested in outdoor ponds



May 2015

3 – Accomplishments, Strain Transition

Two Indoor Cultivation Systems Approximate Outdoor Performance



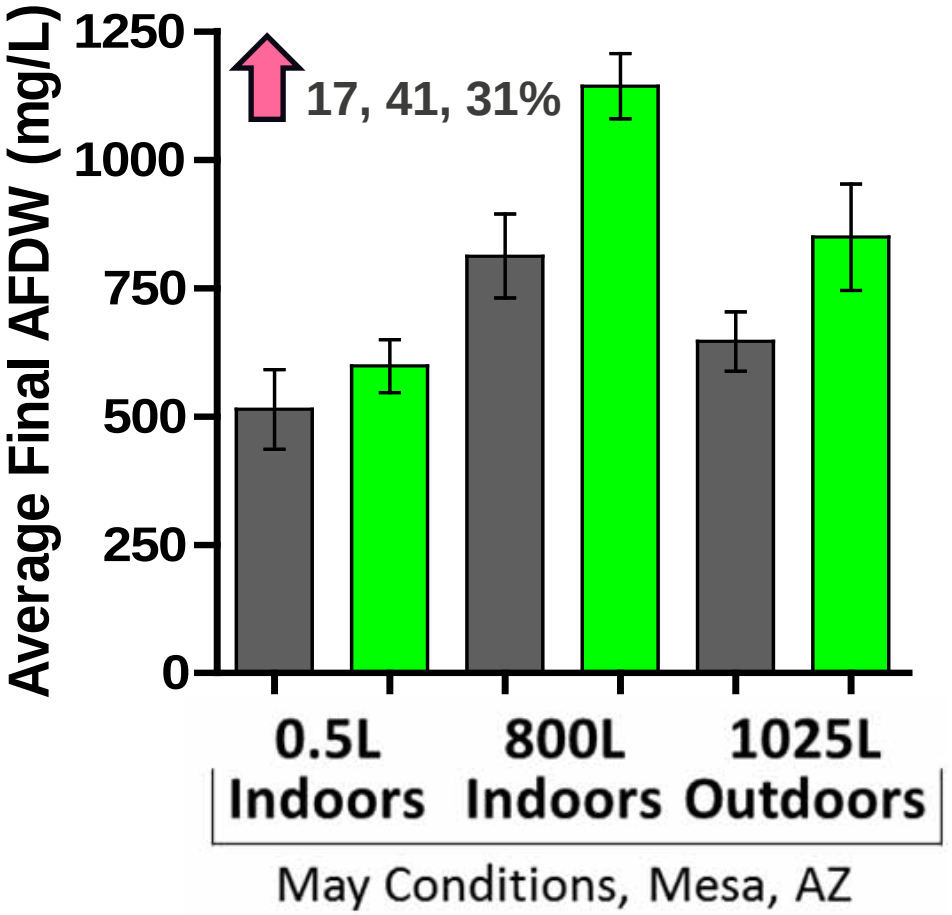
- Indoor data brackets outdoor data → consistent with previous work
- Outdoor simulation experiments reasonably mimic actual outdoor performance:
  - **Productivities** (g/m^2-d) are similar across systems, except BD4 grew faster in PNNL Ponds.
  - An **Improvement** in total biomass was observed for BD4
- Repeat of outdoor data 1 year later showed similar productivity →

May/June 2016	
P	**14 g/m^2-d**
BD4	**16 g/m^2-d**

3 – Accomplishments, Strain Transition

Cell sorted phenotypes can translate to larger scales and to outdoor ponds

- BD4 shows an increase in total biomass accumulation at all scales – maintains biomass phenotype at larger scales, and outdoors
- Carbon storage was insufficient to assess BD4 lipid accumulation over Parent in these experiments – need to understand C partitioning of this strain better under outdoor conditions

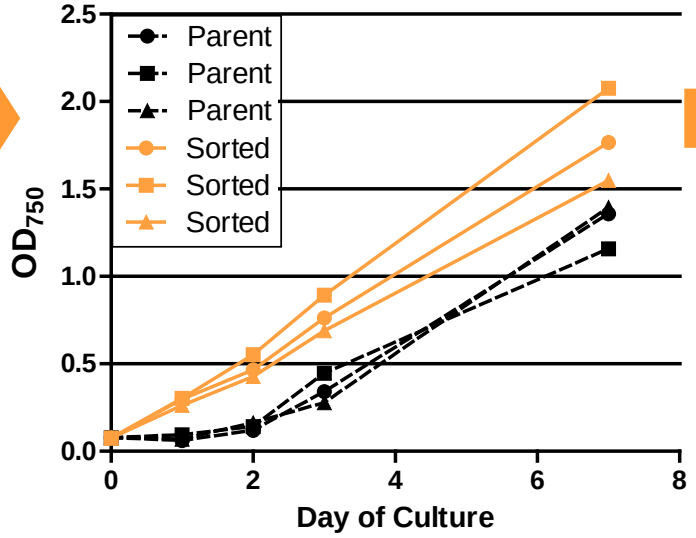


3 – Accomplishments, Strain Transition

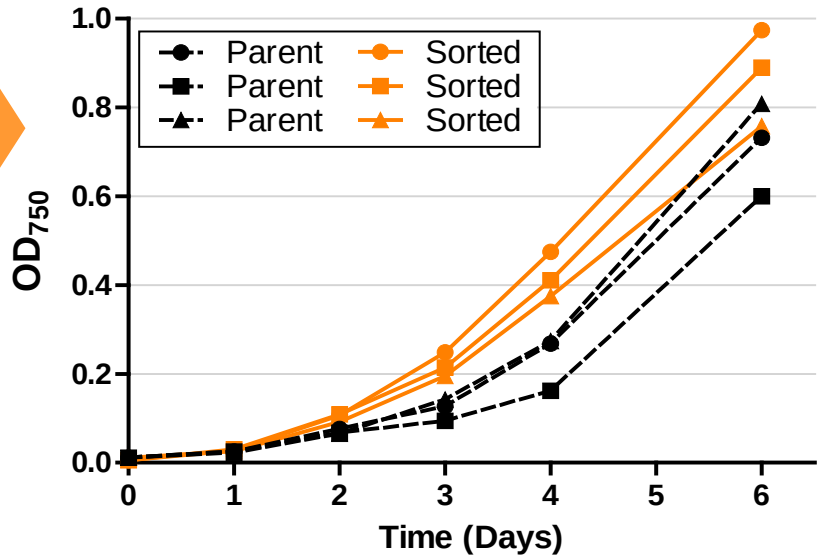
Next: Transitioning the sorted population with increased growth phenotype

Regrowth NO GMDs after final sort

24h Light Shaker Flasks



ePBRs, ATP^3 May Script



- Improved biomass accumulation phenotype holds in outdoor simulations in ePBRs
- Sorted and Parent strains are going to be tested at ATP^3 in the spring

3 – Accomplishments (Technical)

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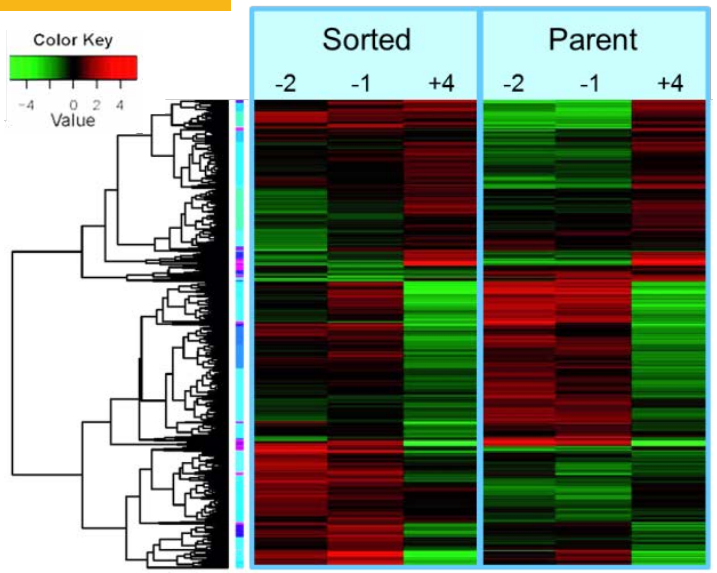
3 – Accomplishments, Molecular Mechanisms

Understanding cell sorted phenotypes using 'omics and gene modification

Transcriptome Data of Parent and Sorted *Picochlorum*

- Transcriptome data (limited time course) was collected under the NAABB project
- Genes of interest were identified from this data as well as data from other strains



Transformants Show Increased Lipid Accumulation

- Colony picking and characterization of PCR-positive clones showed up to 20% increases in lipid accumulation
- Higher replicate experiments that followed showed that phenotypes are transient

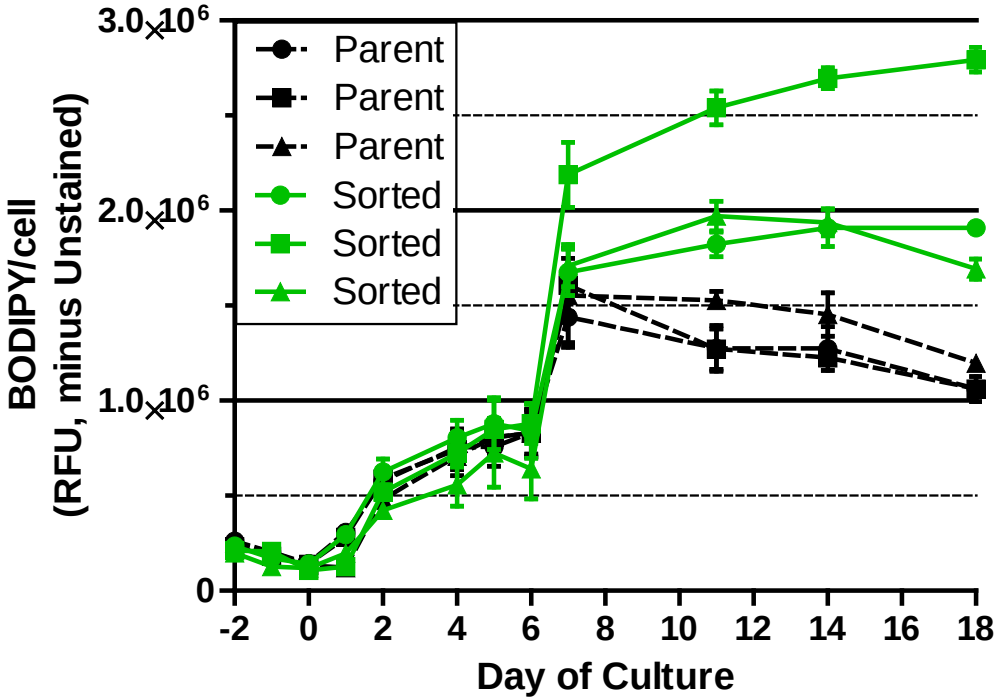
Targeted Transformation Efforts

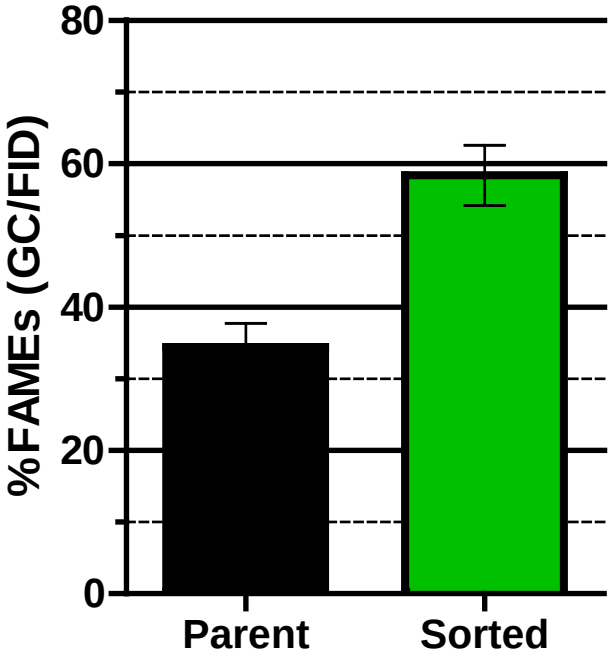
- Targeted insertion of overexpressing genes, knock-outs, and knock downs

3 – Accomplishments, Molecular Mechanisms

Collecting transcriptome data for Chlorella sorokiniana 1228

Triplicate Spinner Flasks, Parent and BD4





- 16:8 light:dark, pH controlled CO_2, ~25°C, nitrogen starvation
- Transcriptome data to be collected from 8 time points and all 6 cultures
- RNA extraction and sample preparation is happening now

4 – Relevance to R&D and Industry

A work in progress: Bringing the outdoors in

- **Adapting laboratory experiments** to better reflect outdoor performance

Variable	Before Crude Simulation Conditions	Now Environmental Simulation + Outdoors	Next Adapt Lab Protocol to Match Outdoor Experiments
System	Flasks (<1L)	**ePBRs, in/out 1000L, out 60,000L**	**ePBRs, in/outside 1000L, out 60,000L**
Temp	Constant (25C)	**Script/outdoors**	**Script/outdoors**
Lights	800 µE, on/off	**Script/sunlight**	**Script/sunlight**
Media	Rich	Rich	**Cost Effective**
C Partitioning	Batch, long deplete, high [biomass]	Batch, long deplete (for comparison)	**Outdoor relevant conditions, bigger scale**
Productivity	OD_{750} & cell count	**Slope g/m^2-day**	**Harvested g/m^2-day**

- Established and are **expanding upon our understanding of how to downselect these strains inside at a cost-effective scale, in an outdoor-relevant way**.
- Use of non-GM methods permits us to **move outdoors easily** to gather multi-scale performance data.
- Non-GM strain **improvement methods have garnered interest** from industrial partners and are **being leveraged in other projects**.

4 – Relevance to BETO and the MYPP

Strategies for strain improvement and 'flask to farm' data are needed

- Strategies for **strain improvement and relating laboratory to outdoor data are needed** to reach cost targets

- **Using production-relevant strains** to increase lipid/biomass productivity
  - There is a **need for 22-26 g/m^2-d** algae productivity (Spring/Summer) to reach <$3/GGE advanced biofuel by 2030 (BETO target)
  - Have **observed up to 22 g/m^2-day** in 1000L indoor simulations of May (16 g/^{2}-d outside); aim to get that consistently above 20 g/m^2-day outdoors

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5 – Future Work

1. Strain Improvement

- Continue to develop and **validate biomass accumulation improvement** assay
- Continue to develop the **incorporation of mutagenesis** into improvement strategies
- Push **salt tolerance** and work on temperature tolerance

Milestone: Go/NoGo for validation of gel-microdroplet technology (Q2)

2. Strain Transition

- Continue to partner with ATP^3 to conduct **outdoor runs**
- Continue to strengthen our understanding of the **relationship between ePBR & outdoor data**

Milestone: Two improved strains sent to ATP^3 for outdoor trials (Q3)

3. Molecular Mechanisms

- Stand up **directed genetic modification** strategies in *Picochlorum*
- **Collect and analyze transcriptome data** for 1228 and use to identify new gene targets
- **Re-sequence genome** of BD4 and interesting mutants from mutagenesis work

Milestones: Three gene targets identified from *C. sorokiniana* 1228 transcriptome (Q4)

Summary

1. Overview

We are **establishing methods for generating improved algae strains and evaluating their performance at multiple scales**, from the bench to outdoors, using national lab core expertise

2. Approach

We are using **two platform strains that meet the BETO state of technology** and value proposition to achieve **3 objectives**: Strain Improvement, Strain Transition, and Molecular Mechanisms

3. Progress

- We are establishing **methods for increasing productivity & robustness** using cell sorting and other non-GM approaches
- **Sorted populations are at least as fit as parents** up to 1000L, outdoors
- **Relative phenotypes between parent and sorted hold** across scales
- We are progressing toward **understanding biology behind new phenotypes**

4. Relevance

We are **developing approaches for strain improvement and strain downselection that can be leveraged in other projects** (DISCOVR, competitive projects) and handed off to **external stakeholders**

5. Future Work

We will continue to **develop strain improvement methods**, **evaluate strains** across scale, and **gather an understanding of the underlying biological phenomena** behind the improved phenotypes

Acknowledgements

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Pacific Northwest NATIONAL LABORATORY

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- M. Wigmosta
- S. Edmundson
- P. Chen

ATP3 Algae Testbed Public-Private Partnership

- J. McGowen
- T. Dempster

New Mexico CONSORTIUM

- S. Negi

U.S. DEPARTMENT OF ENERGY | Energy Efficiency & Renewable Energy

BIOENERGY TECHNOLOGIES OFFICE

Table of Outcomes and Impacts

Type of Outcome	Outcome Description	Impact	Relationship to BETO Priorities (see MYPP Table 2-6)
Physical product	Generation of algae strains with improved phenotypes	Improves algae productivity for biofuel production	Develop productive and robust algal feedstocks
Strategies	A suite of non-GM based strategies for generating custom strains	Establishes a capability expected to benefit future partners	Laboratory tools and technologies to expedite algal strain development & industry
Data	Productivity data for improved algae strains, at 1000L scale	Provides data on the relationship between lab-scale & outdoor data	Engineer productive and robust strains for increased production scales
Strategies	Approach for down-selecting improved strains for transition to outdoors	Informs the scale-up process	Laboratory tools & technologies to expedite development of algal strains for large-scale cultivation
Fundamental knowledge	Identification of molecular mechanisms underlying improved strain phenotypes	Increases the ability to make rational decisions regarding strain modifications	Explore and identify underlying biological phenomena

Presentations/Publications Since Last Peer Review

Publications

- Huesemann M, **Dale T**, Chavis A, Crowe B, Twary SN, Barry A, Valentine D, Yoshida R, Wigmosta M (2017). Simulation of outdoor pond cultures using indoor LED-lighted and temperature controlled raceway ponds and Phenometrics™ photobioreactors. *Algal Research*, 21, 178-190.
- Olivares J, Unkefer CJ, Sayre RT, Magnuson JK, Anderson DB, Baxter I, Blaby IK, Brown JK, Carleton M, Cattolico R, **Dale T**, et al. (2016). Review of the algal biology program within the national alliance for advanced biofuels and bioproducts. *Algal Research*. http://dx.doi.org/10.1016/j.algal.2016.06.002
- Bagwell CE, Abernathy A, Barnwell R, Milliken CE, Noble PA, **Dale T**, Beauchesne KR, Moeller PD (2016). Discovery of bioactive metabolites in biofuel microalgae that offer protection against predatory bacteria. *Frontiers in Microbiology*, 7, article 516, 1-12.

Presentations

2016 Sharpening Our Tools: Algal Biology Toolbox Workshop. **Dale T**. *Getting from flask to farm – progress and challenges in transitioning strains to larger scales.* Invited oral presentation.

2015 Algae Biomass Summit. **Dale T**, Twary SN, Barry AN, Huesemann M, Chavis A, Chen P, McGowen J. *Multi-scale Characterization of Improved Algae Strains.* Oral presentation.

2015 5th International Conference on Algal Biomass, Biofuels, and Bioproducts. **Dale T**, Twary SN, Barry AN, Huesemann M, Chavis A, Chen P, McGowen J. *Multi-scale Characterization of Improved Algae Strains.* Oral presentation.

2015 Arizona State University, Arizona Center for Algae Technology and Innovation weekly meeting. **Dale T**. *Biotechnology and Bioengineering for the Advancement of Algal Biofuels.* Invited speaker.

2015 LANL Complex Natural and Engineered Systems Review. **Dale T**. *Biotechnology and Bioengineering for the Advancement of Algal Biofuels.* Oral presentation.

2015 Indiana University Biotechnology Seminar Series. **Dale T**. *Multi-scale Characterization of Improved Algae Strains.* Invited speaker.

(Also 2 other 2016 talks on cell sorted strains grown outdoors – see Twary AOP & Cellana ABY1)

Previous Peer Review Comments/Responses

Comment:
Solid demonstration of progress and value of methodology. External system selected for modeling had modest productivity. Model may need rebalancing for higher productivity systems. Demonstrated for 3 systems. Needs to be extended further especially for salt water systems.

Response:
The BAT has been utilized for two marine strains, *Nannochloropsis salina* and *Picochlorum* sp., and four freshwater strains, *C. sorokiniana* 1412 and the three RAFT strains. We agree that marine strains have a real advantage when considering water resources. However, to date, most freshwater strains show a higher productivity than marine strains (a possible exception being the strains discussed in Cellana's talk, which are as-yet unpublished). Also, the use of ocean water greatly limits the geospatial options for algae cultivation. Therefore, it has seemed prudent to investigate both marine and freshwater species. To this end, we have focused on *Picochlorum* sp., a **marine** strain, and *Chlorella sorokiniana*, a freshwater strain. It is interesting to note that both of these strains may have some saline tolerance that would allow them to be cultivated in a variety of salinities, thereby increasing their geographic applicability. This will be a focus of future study for this project.

Comment:
This project addressed objectives and several technical barriers of the algae program. It is good that this project will be leveraging BETO- funded test-beds. The project's focus of improving strains and ultimately predicting their outdoor performance is a highly-relevant objective, but DOE should assure it is complimentary with its other efforts.

Response:
BETO's strain improvement portfolio covers/has covered strain prospecting from natural sources (Cellana/Cornell; NAABB; RAFT), genetic modification strategies (NAABB, LANL Algal Biotechnology AOP, REAP), culture consortium studies (INL), and cell sorting (LANL this project, CABComm). The cell sorting approach is complementary and has the strength that it can be used in conjunction with some of the other approaches in the portfolio.

Previous Peer Review Comments/Responses

Comment:
This work was not funded adequately to do the proposed work. It needs to redirect its efforts to seriously measuring productivity in units of relevance to outdoor production. I cannot imagine a process that starts cultures from scratch every three weeks with a minimal inoculum, and waits for growth to occur. Rather all the commercial models I know of (e.g. Cornell project) predict that cultures are inoculated at high density, and harvested semi-continuously, keeping the culture near its sweet spot where it absorbs almost all the light at its dilutest, and all of it at its densest. This requires a doubling time near to once per day... and several people to do the work.

Response:
While it is true that in a commercial setting a culture will not typically be started at a very low density and grow out to saturation, the growth experiments shown in the presentation were designed with two goals in mind. First, we aimed to compare the parent and cell sorted populations as completely as possible, and this included measuring maximal specific growth rates from the exponential growth part of the growth curve as well as the final maximal biomass accumulation at saturation. It was important to measure the full growth curves of these strains and compare that data to similar experiments conducted in flasks, as a goal of this project is to examine the performance of these strains at multiple scales. The second goal was to measure an areal productivity (g/m^2-day) that would be meaningful in an outdoor setting. PNNL has previously demonstrated that areal productivities measured in outdoor ponds, during the linear growth phase of a culture, can be mimicked in the PNNL indoor ponds and accurately predicted by the Biomass Assessment Tool. In this presentation we also showed that the ePBRs can obtain similar areal productivity values during linear growth, thereby demonstrating that this measurement of areal productivity is one that can be made across multiple scales, in the lab and outdoors. Therefore, it is a useful measure for ranking strain performance and downselecting strains for outdoor growth. It is also important to note that the Biomass Assessment Tool can simulate various semi-continuous harvesting strategies, in order to predict which dilution rate is optimal for a strain of interest. This part of the BAT is already in use in other BETO-funded projects, and as our strains are incorporated into the BAT, we can predict and measure productivities during semi-continuous harvesting in the future. The most promising strategies can then be tested outside at the ATP^3 testbed.

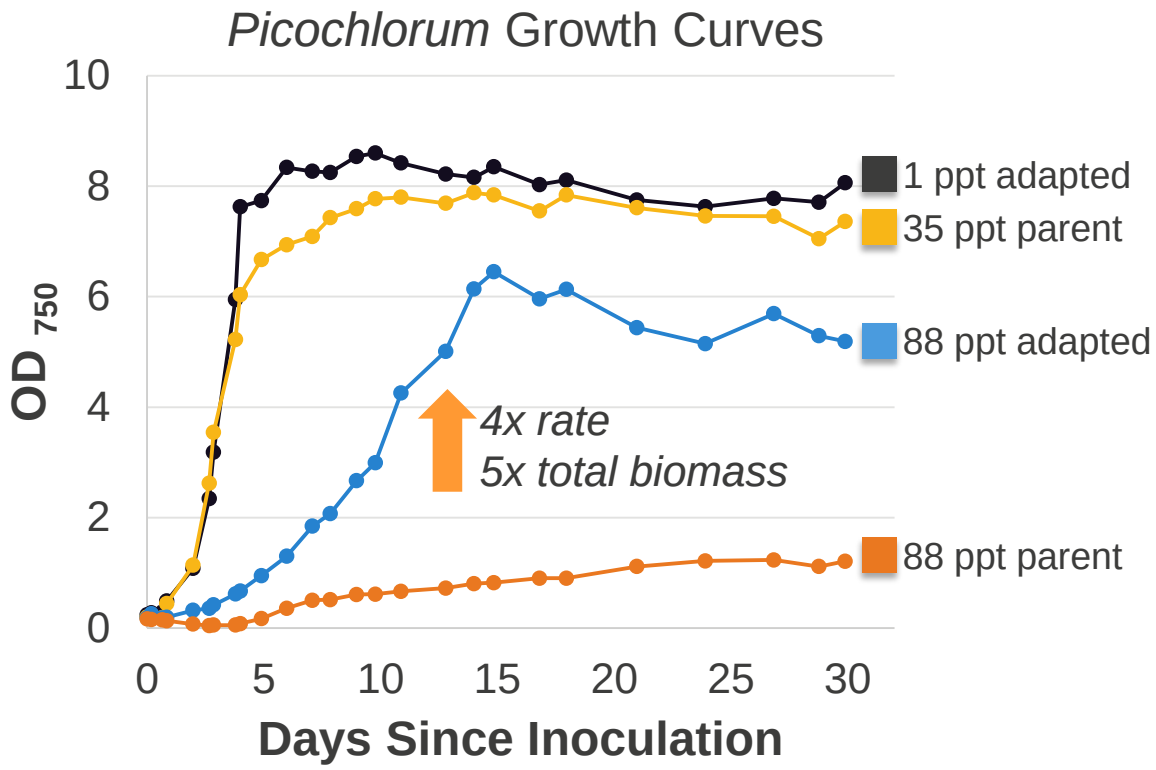
3 – Accomplishments, Strain Improvement

Adaptive evolution and mutagenesis are additional strategies being applied

Salt Tolerance

- *Picochlorum soloecismus*:
  Baseline: 2-70 ppt salinity
  Adapted to: 1-88 ppt
- *C. sorokiniana* 1228:
  Baseline: 0-9 ppt
  Adapting to: 17+ ppt

NOTE:
Average seawater: 35 ppt
Drinking water: 0.1 ppt
Limit on agriculture irrigation: 2 ppt



EMS Mutagenesis

- Developed a protocol for random mutagenesis by EMS in Cs1228
- Permits the broadening of genetic diversity and increases the chance of meeting our productivity targets and/or finding new phenotypes.