



# Algal Biology and Cultivation Technical Accomplishments, Progress and Results

Presented by Richard Sayre, NAABB Scientific Director

# Algal Biology Task Framework

## Increase Productivity

- Trait identification from research & production strains
  - Genomics and transcriptomics
  - Systems biology
  - Genetics enabled trait screening
  - Lipid & isoprenoid genes and pathways
  - Growth and energy genes and pathways
- Phenotypic characterization in PBRs
- Molecular biology tool development for production strains
  - Genome enabled expression constructs
  - Transformation method development
- Crop protection from competitors and predators
- Assessing traits for improved production

## Research Strains

*Chlamydomonas reinhardtii*  
*Botryococcus braunii*

## Production Development Strains

*Nannochloropsis salina* (SW)  
*Auxenochlorella protothecoides* (FW)  
New or modified strains (*C. sorokiniana*)

Strains to Cultivation Task

## New Strain Isolation and Development

- Isolation of new strains
- Screening strains for biomass and lipid productivity
- Optimizing culture conditions for candidate strains
- Adaptive evolution for selection of new strains
  - Flow cytometry for high lipid productivity
  - Chemostat selection for decreased nutrient requirements

# Algal Biology Milestones and Deliverables



| Milestones (M), Decision Points (GN) and (DL) Deliverables   | Time (mo)<br>Status |
|--|---------------------|
| A.4.ML.1: 500 algal isolates screened, ≥10 promising high lipid strains tested in culture. (report)  | 18<br>Complete      |
| A.1.DL.1: Genes for increased yield, productivity, nutrient utilization, or crop protection cataloged. Transgenic tools demonstrated for <i>C. reinhardtii</i> , <i>B. braunii</i> , <i>Chlorella</i> . (report) | 18<br>Complete      |
| A.3.DL.1: 1 <sup>st</sup> generation of <i>Nanochloropsis</i> & <i>Chlorella</i> strains obtained by adaptive evolution with demonstrated improvement in growth or lipid yield over parent strain. (report)      | 18<br>Complete      |
| A.4.ML.2: 1500 algal isolates screened, ≥30 best strains verified and deposited to UTEX (report)   | 36<br>Complete      |
| A.ML.2: Transgenic strains incorporating best trait(s) demonstrated in culture. (report)   | 36<br>Complete      |



# Algal Biology Highlights



## Benchmark: Algal biology for biofuels prior to 2008

- Bio-prospecting efforts lead to the identification of high yielding strains but many (ASP) were not maintained or publically available.
- Only 8 algal genomes were publically available
- Very few gene targets identified for improved yield and few transgenic algae generated. None had substantial improvements in yield.

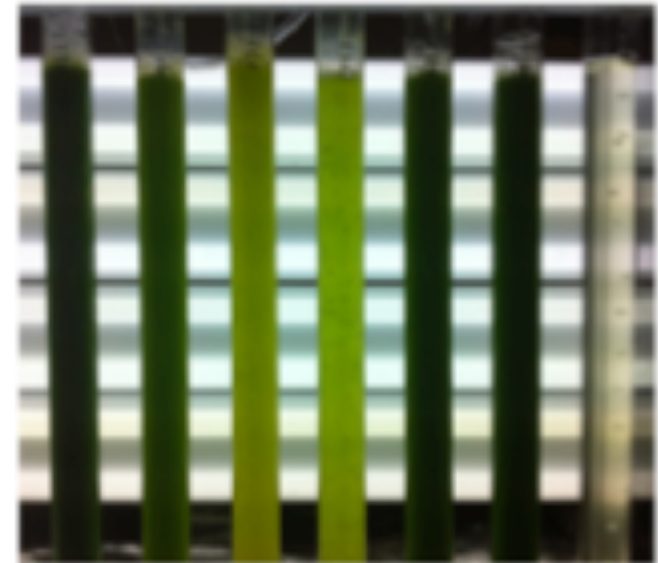
## NAABB highlights and improvements:

- Screened over 2,000 isolates and 30 strains with improved yield relative to benchmark strains were deposited at UTEX.
- Sequenced 8 new genomes and over 250 transcriptomes analyzed using new bioinformatic tools.
- Identified over 50 gene targets for improved yield and oil production.
- Demonstrated up to a 5X increase in biomass yield and a 5X increase in oil yield per unit biomass in transgenic algae.

# New Strain Isolation and Development

## Milestones 1 and 2: Selecting for improved algal strains:

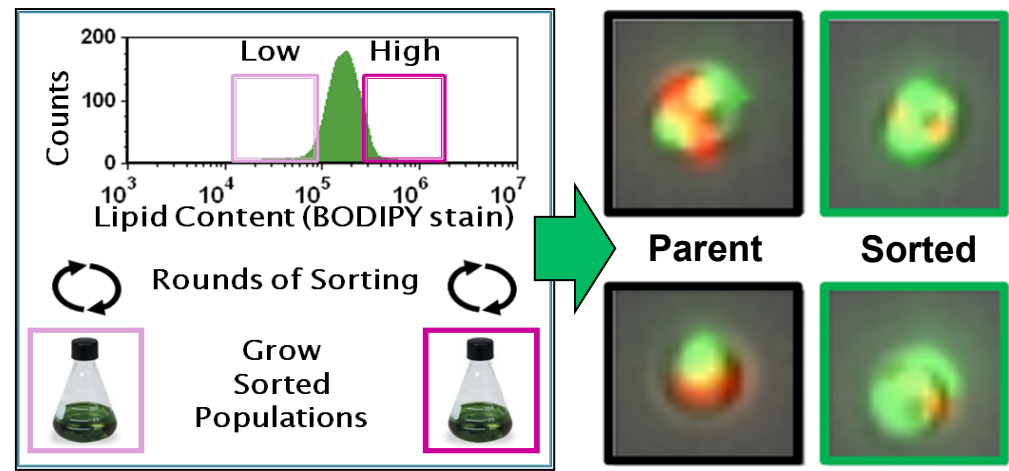
- Over 2,000 independent algal isolates were collected across the US. More than 60 strains were identified that outperformed bench mark production algal strains.
- New micro-GC/MS techniques were developed for measuring lipid quantity and fatty acid profiles
- A genetic bar-coding system was developed for tracking algal strains



# Adaptive Evolution

## Deliverable 2: Adaptive evolution for improved strains:

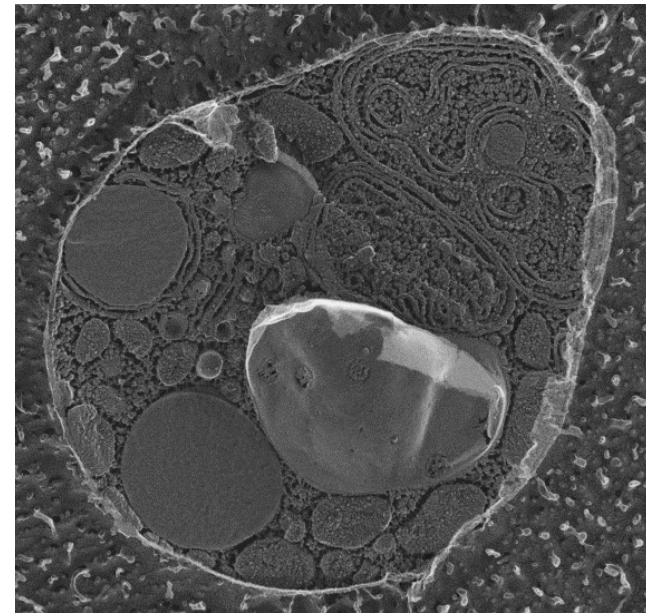
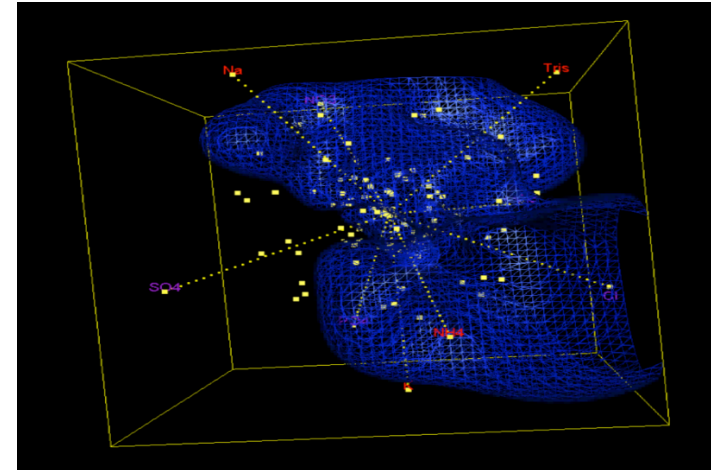
- Directed evolution (FACS for increased oil) approaches were used to isolate *Pichoclorum* strains with greater oil yield (150% improvement).
- Transcriptomics studies indicated that fatty acid synthesis genes were up-regulated in selected strains with increased oil content relative to wild type
- *Auxenchlorella protothecoides* strains were screened and identified for improved growth (26%) under low phosphate conditions





# Systems Biology Tools

- A multidimensional macronutrient growth space model was developed using multiple *Chlamydomonas* strains and mating types demonstrating that transmission genetic approaches can be used to map QTLs and breed for improved algae strains
- Rapid freezing Deep Etch Electron Microscopy (DEEM) was used to image algal sub-cellular organelles and oil storage compartments. DEEM studies demonstrated that algae use both new synthesis and lipid remodeling strategies for accumulating storage lipids





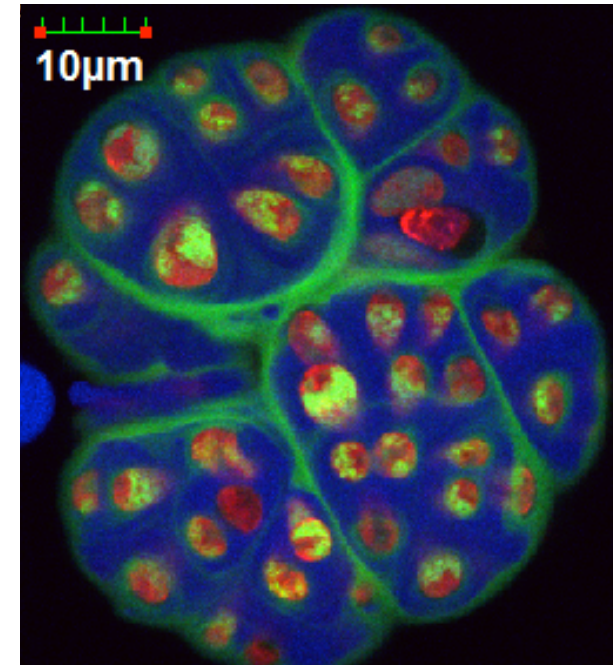
# Metabolic engineering strategies to enhance biomass and oil production in GM algae



| Trait  | Push/<br>Transport  | Partition  | Pull   | Buffer/<br>Storage   | Novel  |
|--|---|--|--|--|--|
| Light capture and photosynthetic electron transfer | <ul style="list-style-type: none"> <li>• Self-modulating antennae size</li> </ul>                                 |  |  | Modulate electron acceptor/donor pool size   |  |
| CO <sub>2</sub> fixation                           | <ul style="list-style-type: none"> <li>• Carbonic anhydrase-2 (HCA-2)</li> <li>• Rubisco-HCA-2 fusions</li> </ul> | <ul style="list-style-type: none"> <li>• Fructose Bisphosphatase</li> </ul>  | <ul style="list-style-type: none"> <li>• Glutamine synthase</li> <li>• Gln phospho – transferase</li> </ul>          |  |  |
| Carbon metabolism                                  |   | <ul style="list-style-type: none"> <li>• Isocitrate lyase</li> <li>• Malate synthase</li> <li>• Malate DH (mito)</li> <li>• 2-keto glutarate-DH</li> </ul> |  |  |  |
| Oil synthesis                                      | <ul style="list-style-type: none"> <li>• Acyl CoA Carboxylase</li> </ul>  |  | <ul style="list-style-type: none"> <li>• Diacyl glycerol acyl transferase</li> <li>• Acyl carrier protein</li> </ul> | <ul style="list-style-type: none"> <li>• Oleosin</li> <li>• Lipid droplet protein</li> </ul> |  |
| Bioflocculation                                    |   |  |  |  | <ul style="list-style-type: none"> <li>• Hyalluronic acid</li> <li>• Chitin binding</li> </ul> |
| Anti-contaminant                                   |   |  |  |  | <ul style="list-style-type: none"> <li>• AMPs</li> </ul>                                       |

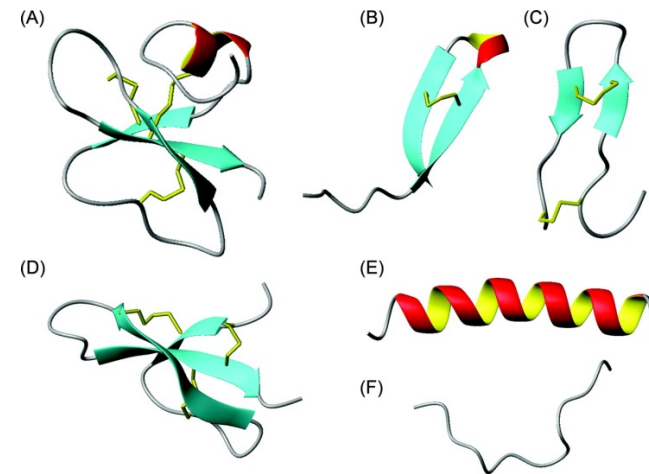
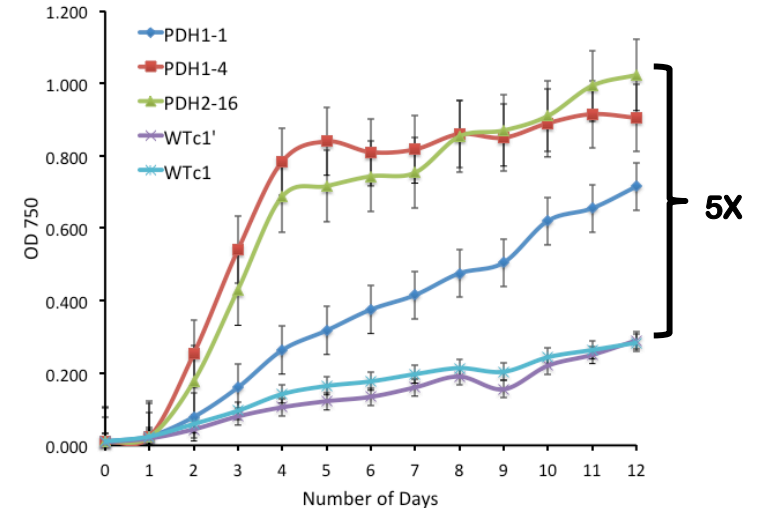
# Algal Biology Tools

- A plant floral organ ABC-transporter that export lipids was functionally expressed in yeast and is being tested in algae
- Multiple genes involved in squalene synthesis were identified from *Botryococcus braunii* and a mechanism for squalene secretion was described
- Cyanobacteria with increased oil production have been achieved by altering flux through glycolysis



# Algal Biology Tools

- **Molecular transformation tool boxes** were developed for the genetic transformation of *Auxenochlorella protothecoides*, *Chlorella sorokiniana*, and *Nannochloropsis salina*
- **A transgenic pipeline** production platform was developed and over 50 independent transgene constructs were expressed in *Chlamydomonas* and other species.
- **A 5-fold increase in biomass accumulation** was achieved by altering electron transfer flux in chloroplast thylakoids in transgenic algae.
- **A 2-fold increase in growth rate** was achieved by expression of self-adjusting light harvesting antennae.
- **A 2-fold increase in oil accumulation** was achieved (+/- N) by miss-targeting expression of glyoxylate cycle enzymes to non-target organelles
- **A 5-fold increase** in oil accumulation was achieved by overexpressing an oil storage protein.
- **Anti-microbial peptides** (AMP) were identified that kill bacteria and rotifers without harming algae



# Summary



## Milestones 1 and 2, Isolation, selection and deposition of new strains with improved yield characteristics:

- **Over 2,000 independent algal isolates** were screened. At least 16 potential new production strains were screened in culture and shown to outperform the benchmark strains, *Nannochloropsis salina* (SW) and *Auxenchlorella protothecoides* (FW).
- **One of those isolates, *Chlorella sorokiniana*-1412**, performs well in outdoor trials and has a very high growth temperature optimum of 37 °C. The genomes of two independent *C. sorokiniana* strains were sequenced and assembled. Molecular transformation of the nuclear genome has been demonstrated.
- **Over 30 novel algal isolates** with improved yields have been deposited at the UTEX Culture Collection in Austin, TX

## Deliverable 1, Genes for increased yield and crop protection identified:

- **Successfully sequenced and assembled 8 alga genomes *de novo*** plus re-sequencing of 2 adapted mutants. Sequenced 215 transcriptome samples from 10 investigators across 8 institutions. Generated genomic annotations for 3 genomes.
- **Created two complementary web-based platforms** for display, analysis, and distribution of “omics” data.

# Summary

## Deliverable 1, Genes for increased yield and crop protection identified:

- Many gene targets were identified from RNAseq analyses of altered gene expression during oil induction including glyoxylate cycle genes, ACCase gene family members, lipases, and genes involved in nitrogen metabolism
- Over 50 transgene targets were screened for increased yield and oil production in transgenic *Chlamydomonas*, *Auxenochlorella protothecoides* and *Nannochloropsis salina*.
- Genes synthesized for expression of AMPs that kill rotifers and bacteria but not algae were transformed into *Chlamydomonas*. Currently being screened for efficacy in culture.
- Genes for botryococcene synthesis identified.
- Improved yields in transgenic *Chlamydomonas* were used for LCA predictions

## Milestone 3, Transgenic strains with best traits demonstrated in many cases in ePBRs:

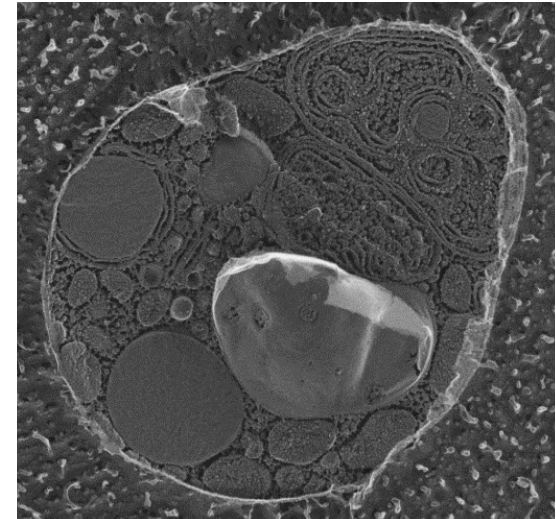
- Overexpression of glyoxylate cycle enzymes **increased oil yield 2X** but with a 30% yield penalty.
- Overexpression of miss-targeted glyoxylate enzymes **increased lipid yield 2X** with or without nitrogen deprivation and with no yield penalty.
- Enhancing photosynthetic electron transfer properties resulted in a **5X increase in growth**.
- Expressed of human carbonic anhydrase-2 in algal chloroplasts **increased growth rates 30%**
- Expressed the **volatile hydrocarbons** limonene and pinene in *Chlamydomonas*

**Conclusions:** The pathway to application for GM algae has been demonstrated but will require demonstrating scalable, enhanced productivity in outdoor cultivation systems.

# Algal Biology Synopsis

## Impact

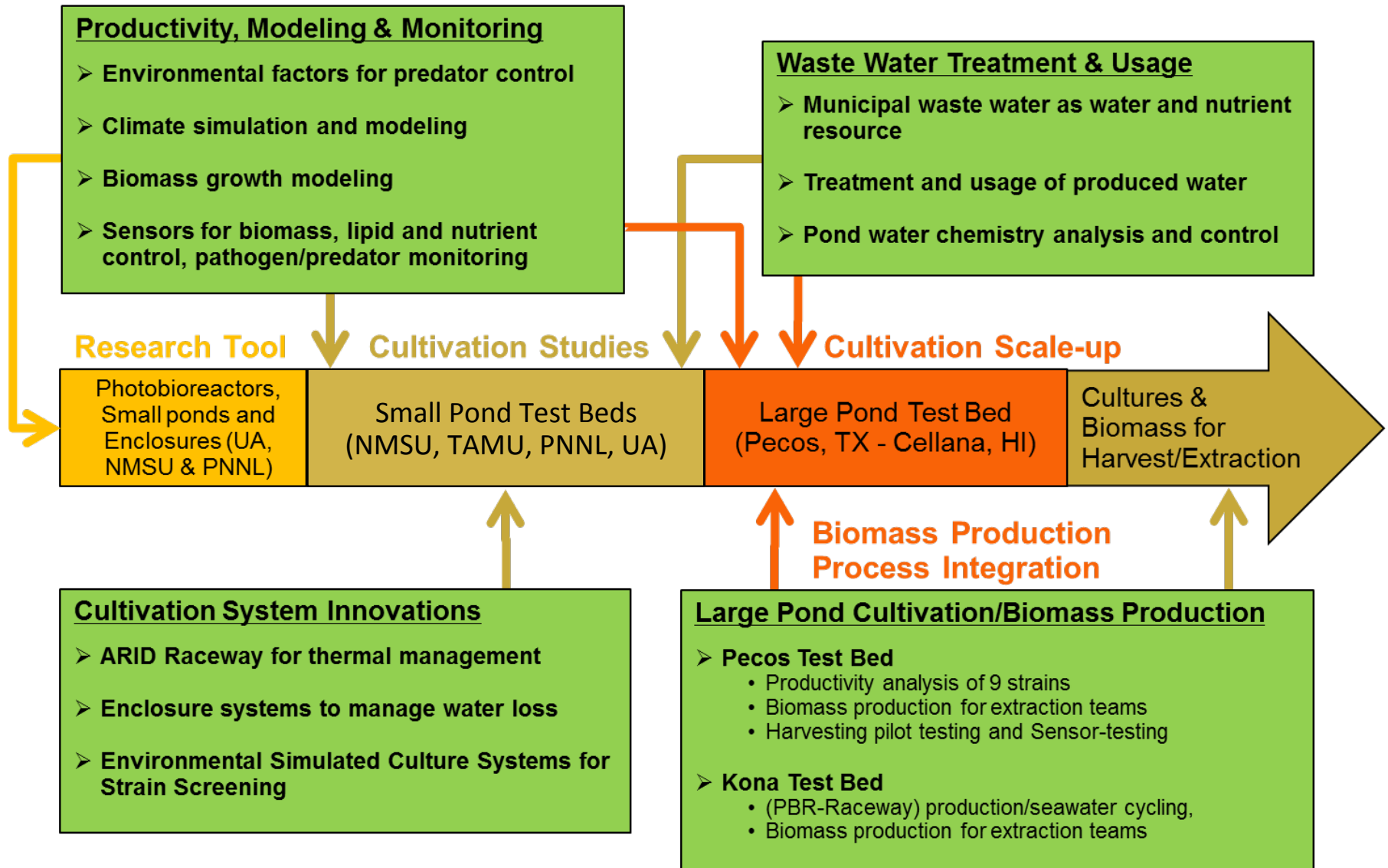
- **New improved strains isolated** (productivity, lipid, and temperature tolerance) e.g. *Chlorella sorokiniana* 1412 strain. Daily productivity in lab (PBR) >30g/m<sup>2</sup>/day, grows well at 40 C and is saline tolerant.
- **Tool boxes** for transformation and expression of transgenes were developed for several species.
- **Identified promising gene targets and generated transgenic strains expressing those genes** with 5X improvement in biomass yield and 5X increase in oil yield, validated in the lab
- **Application of the tool box** for transferring improved traits to GMO new production strains will require additional time and effort beyond NAABB



Algae have evolved numerous strategies for producing lipids. \*

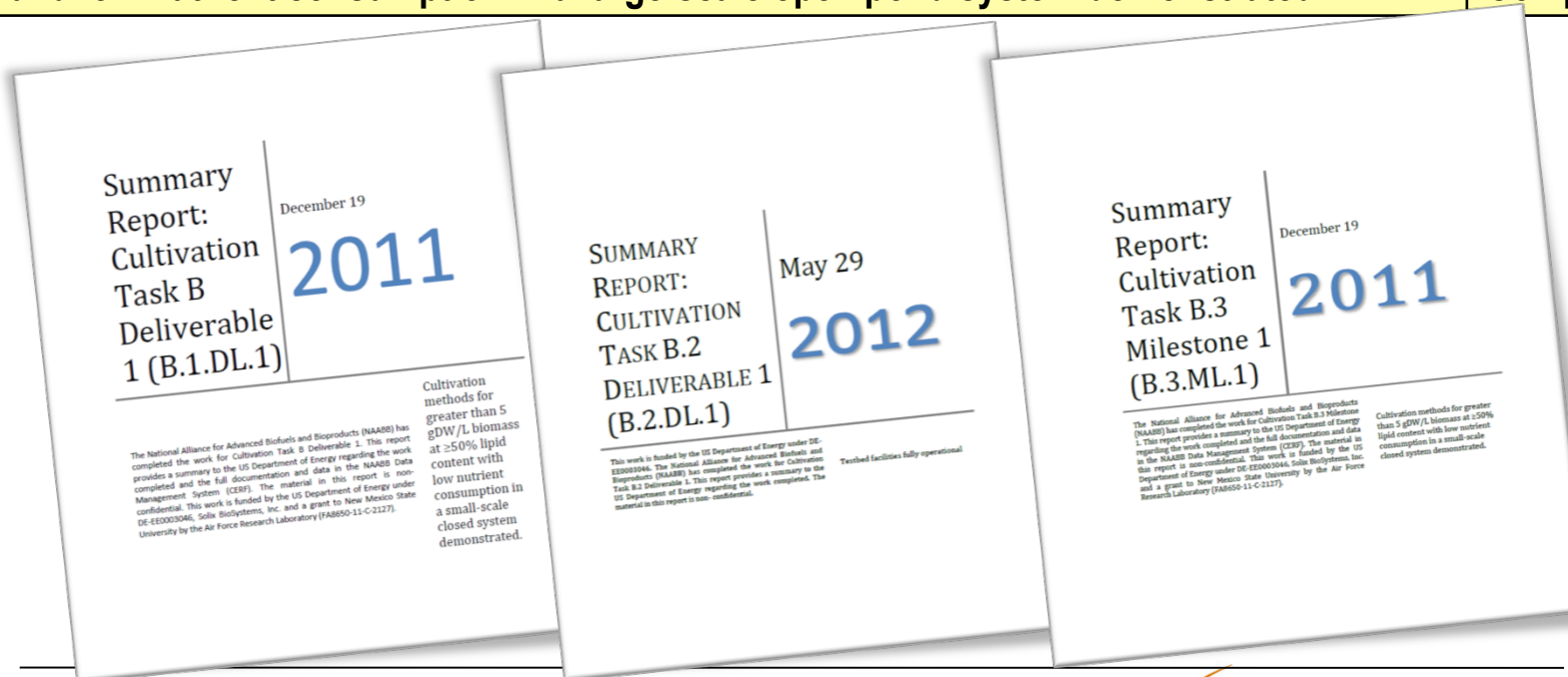
\* From Goodenough lab (Washington University, St. Louis)

# Cultivation Task Framework



# Cultivation Milestones and Deliverables

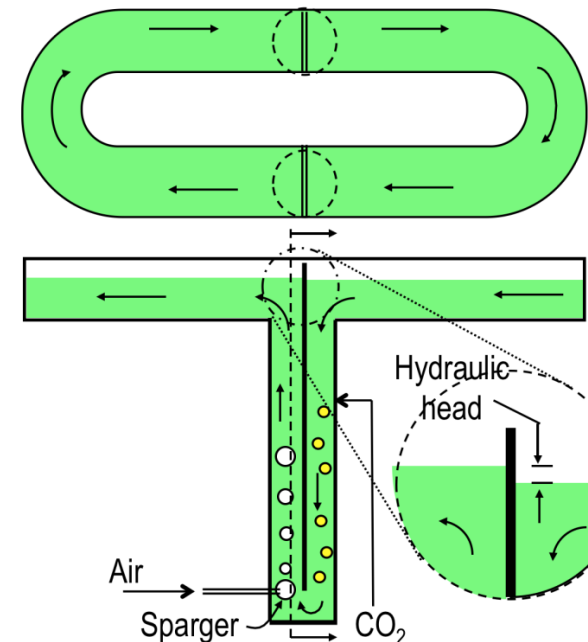
| Milestones (M), Decision Points (GN) and Deliverables (DL)  | Time (mo)<br>Status |
|---|---------------------|
| B.1.DL.1: Cultivation methods for greater than 5 gdw/L/day biomass at ≥50% lipid content with low nutrient consumption in a small-scale closed system demonstrated (report)     | 18<br>Complete      |
| B.2.DL.1: Test bed Facilities fully operational   | 24<br>Complete      |
| B.3.ML.1: Cultivation methods for approaching target growth rates and lipid yield with best strain and low nutrient consumption in a large-scale open pond system demonstrated. | 36<br>Complete      |





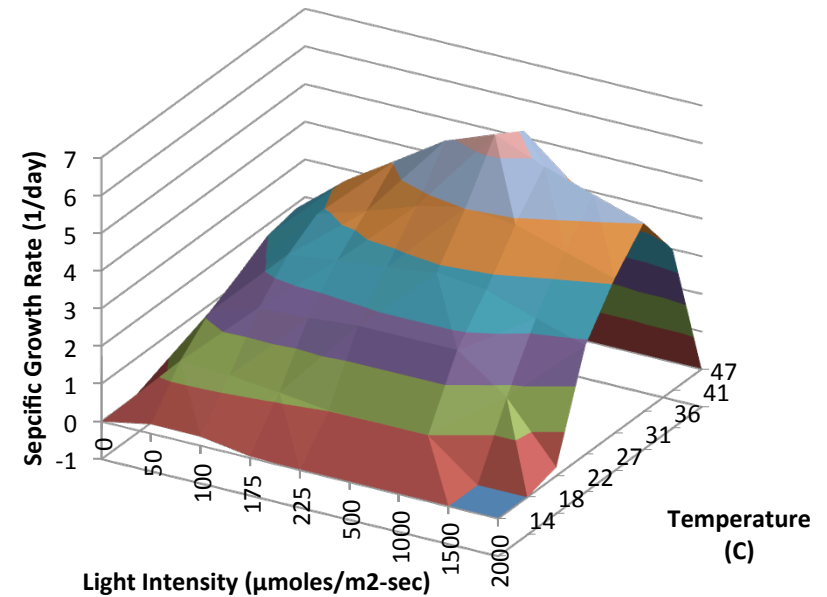
# Cultivation Highlights

- Optical sensors (NIR) were developed to quantify algal populations and estimate oil accumulation
- Ash accumulation from wind blown sources in ponds can be substantial (18% mass) and interferes with downstream processing
- The optimal CO<sub>2</sub> concentration for algal (*Nannochloropsis*) growth in ponds was 0.5%
- Algal growth and lipid production on sterile municipal waste effluent was shown to be similar to that on proscribed growth media.
- Bacterial contaminants in ponds were identified but were stabilized (10% of total cellular population) by growth with algae (*Nannochloropsis*).
- **Airlift pond mixing systems are five times more energy efficient** than direct infusion of CO<sub>2</sub> and mixing with paddle wheels



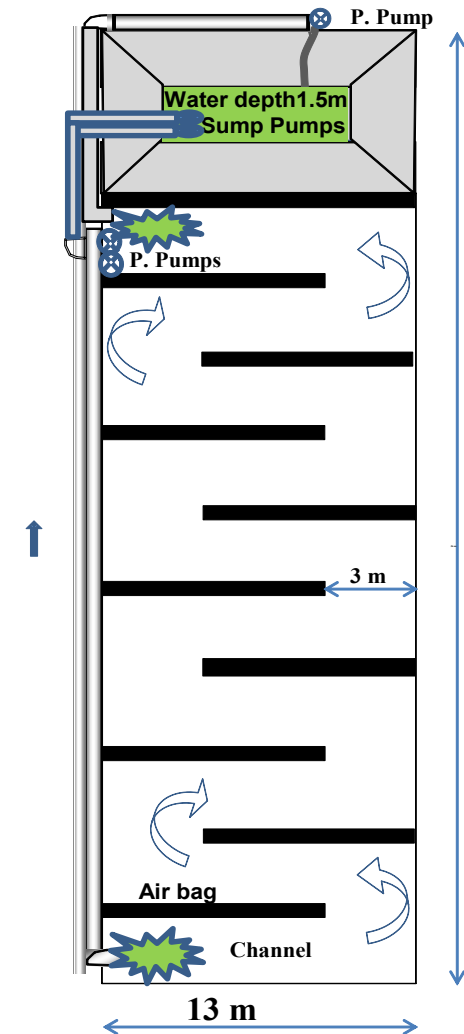
# Cultivation Highlights

- A biomass growth model was developed which in conjunction with the Biomass Assessment Tool (BAT) was able to predict biomass productivities of promising microalgae strains in hypothetical outdoor ponds at any geographic location
- In some instances, mixed algal species cultures produced significantly more biomass than some monoculture systems but not for all monoculture systems.



# Cultivation Highlights

- The ARID raceway system has been shown to be a **cost-effective** means for maintaining stable culture temperatures and enhancing biomass yields.
  - CAPEX for ARID raceway was 8% higher than open ponds.
  - But, OPEX for ARID system is 45% lower than open pond, and production is 19.5% higher than open pond.
- Demonstrated that growth media salt concentrations and pH can be manipulated to reduce biocontaminant loads
- A commercial photobioreactor system that mimics a pond environment has been developed and a start-up company, *Phenometrics*, initiated



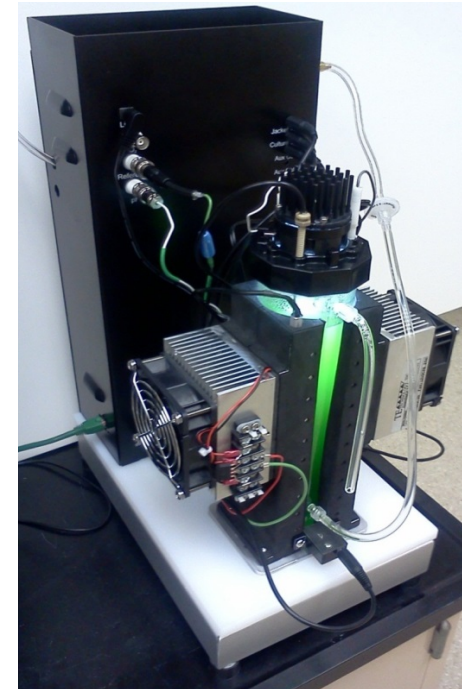
# Cultivation Summary

## Cultivation:

- Developed new commercial photobioreactors that mimic pond environment and a start-up company, *Phenometrics*, to commercialize
- Developed and tested models to predict algal growth throughout the world
- Developed advanced NIR biosensors for cell numbers and oil content for real-time pond production assessment
- Developed low energy algae mixing and gas injection systems
- Developed more cost-effective ARID raceway systems
- Developed optimized growth media for algal cultivation

**Milestone 1:** Produced algal biomass at Pecos TX, open pond facility at a maximum rate of 17-25 gdw/m<sup>2</sup>/day with a lipid yield of 20 to 25%

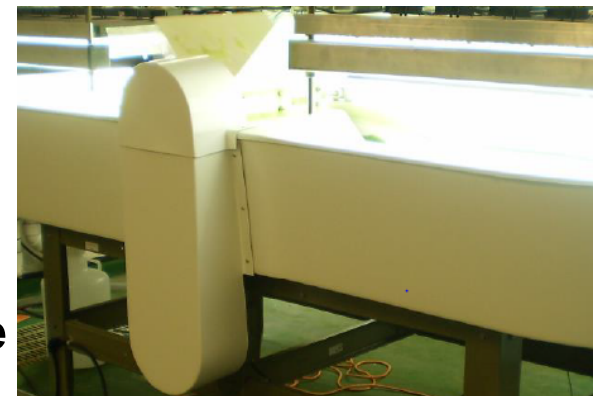
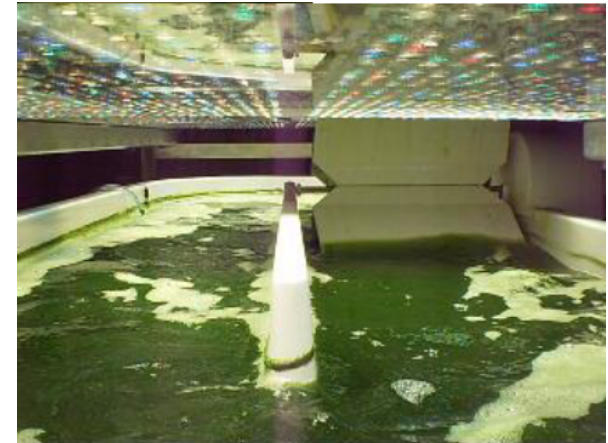
**Deliverables 1 and 2:** Yields exceeding 5 gdw/L and 50 % (g/g) lipid were achieved at the Solix PBR System in Las Cruces, NM.



# Cultivation Synopsis

## Impact

- **Developed laboratory photobioreactor (Phenometrics) and Environmental Simulated Culturing System** to predict algal performance at any outdoor location, any time of year.
- **Benchmarked new strains in 5 outdoor facilities** (1) aerial productivity and lipid content doesn't match lab data; (2) ARID system improves winter (cold temperature) productivity.
- **Lowered cost of media and validated cultivation with non-potable waters.**
- **Developed improved ARID raceway system with increased yield and reduced costs relative to open ponds.**
- **Produced biomass needed for downstream processing.**



Environmental Simulated Culture Ponds allow full control over solar radiance and water temperature to mimic conditions at different times, seasons and locations.

# Questions?

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