DOE Bioenergy Technologies Office (BETO) 2023 Project Peer Review

Advancing Algal Productivity through Innovation in Cultivation Operation and Strain Traits (ADAPT-COST)

4 April 2023

Advanced Algal Systems – Cultivation and Strain Development

Kenneth F. Reardon
Colorado State University

Colorado State University | Arizona State University | Los Alamos National Laboratory | QBI Quantitative BioSciences, Inc. | QUALITAS HEALTH

Project Overview

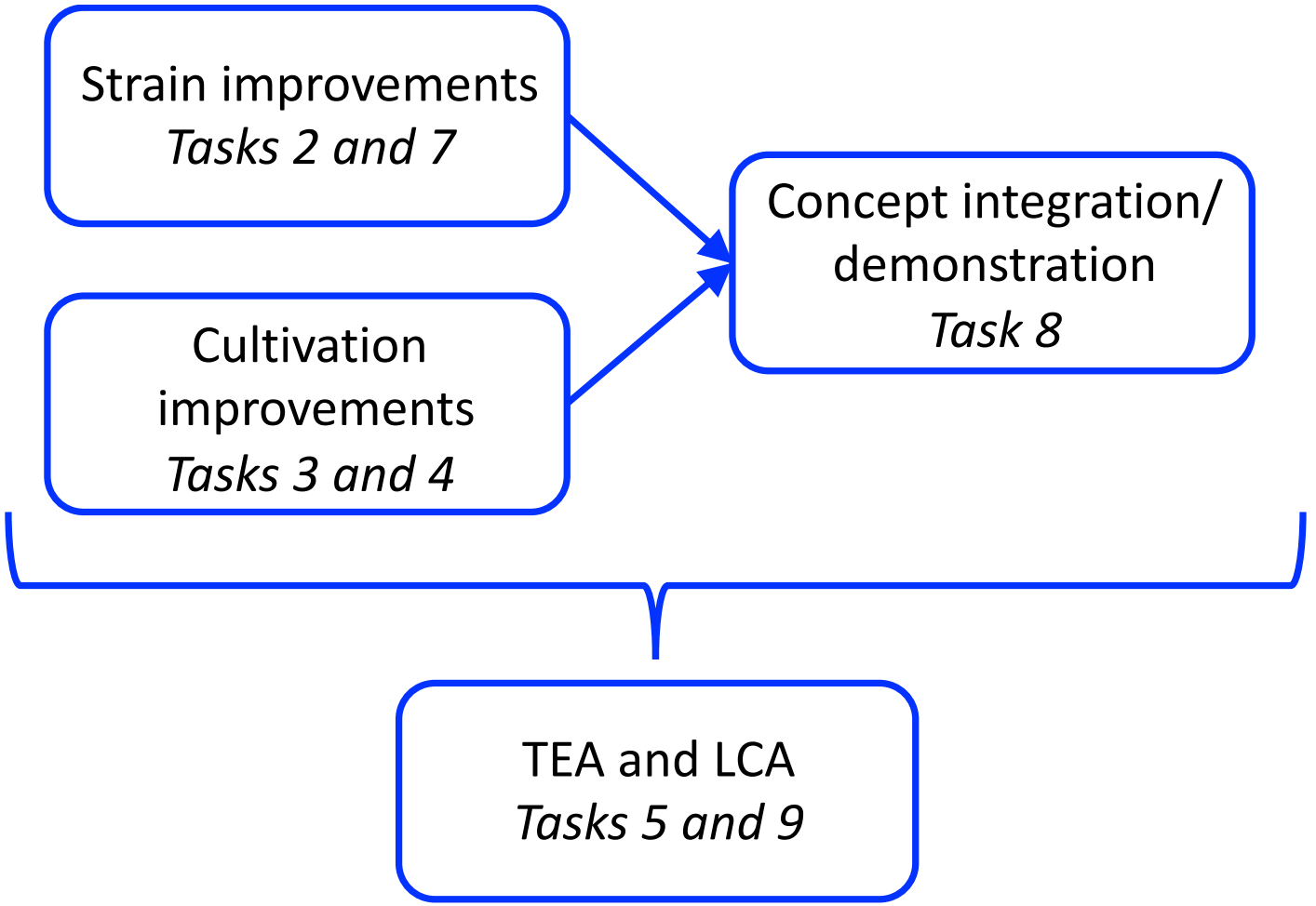
- The productivity of algal cultivations limits economic and environmental sustainability
- The technical goals of the project are to improve productivity and biomass quality of *Nannochloropsis oceanica* CCAP84910 in the spring season through:
  - Genetic modifications to enhance photon and carbon use efficiencies;
  - Development of sensors and strategies for effective cultivation operation;
  - Integration and deployment of strain and operational improvements;
  - Assessing progress toward economic and environmental metrics.
- Laboratory advancements will be demonstrated at pilot scale in large outdoor cultivation systems.

Colorado State University | Arizona State University | Los Alamos National Laboratory | QBI Quantitative BioSciences, Inc. | QUALITAS HEALTH

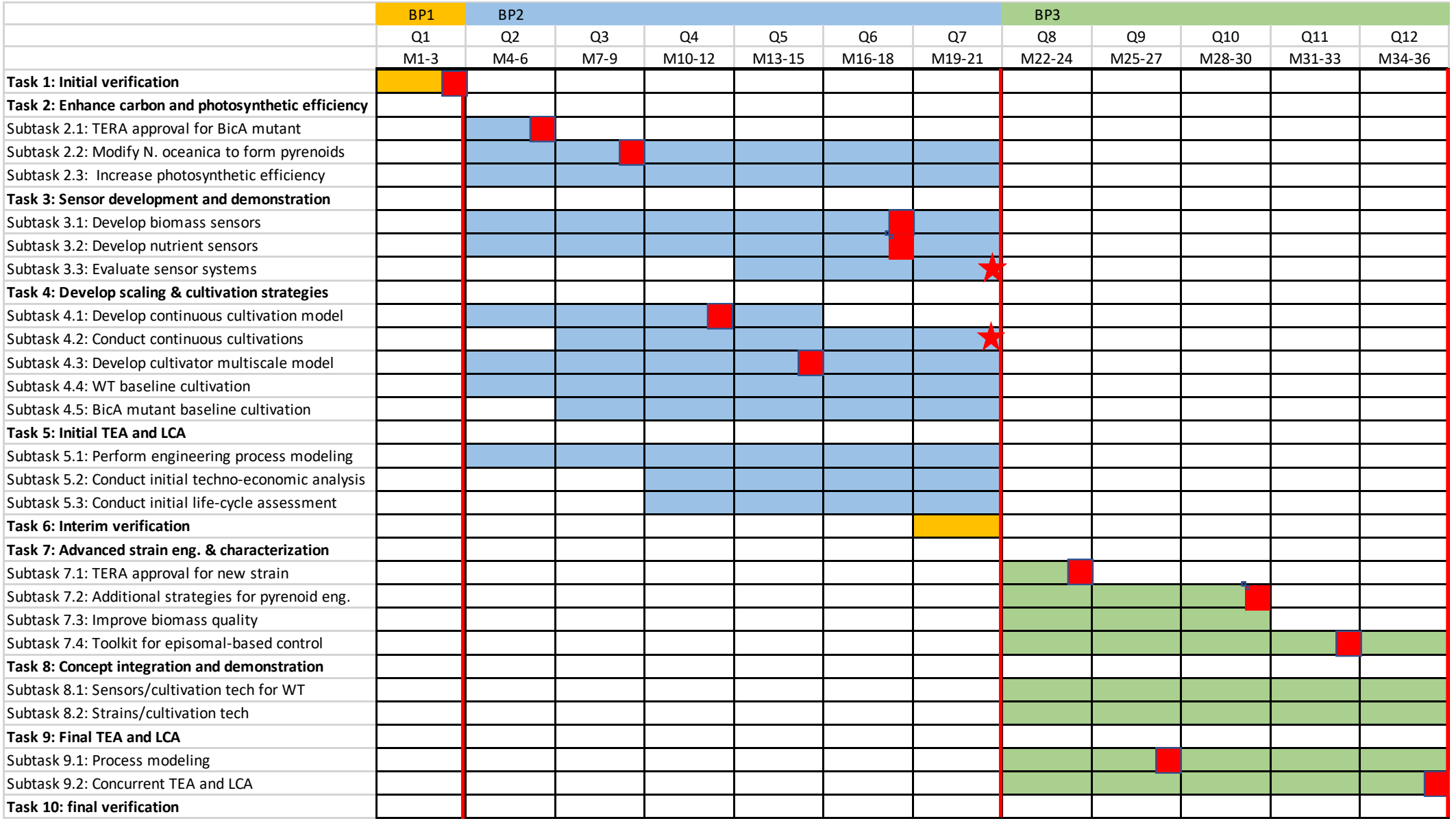
Project Overview

- Project award: October 2021
- Initial verification completed: December 2021
- Subcontracting issues delayed full start until January 2023

1 – Approach


Strain improvements
Tasks 2 and 7
Cultivation improvements
Tasks 3 and 4
Concept integration/ demonstration
Task 8
TEA and LCA
Tasks 5 and 9

1 – Approach

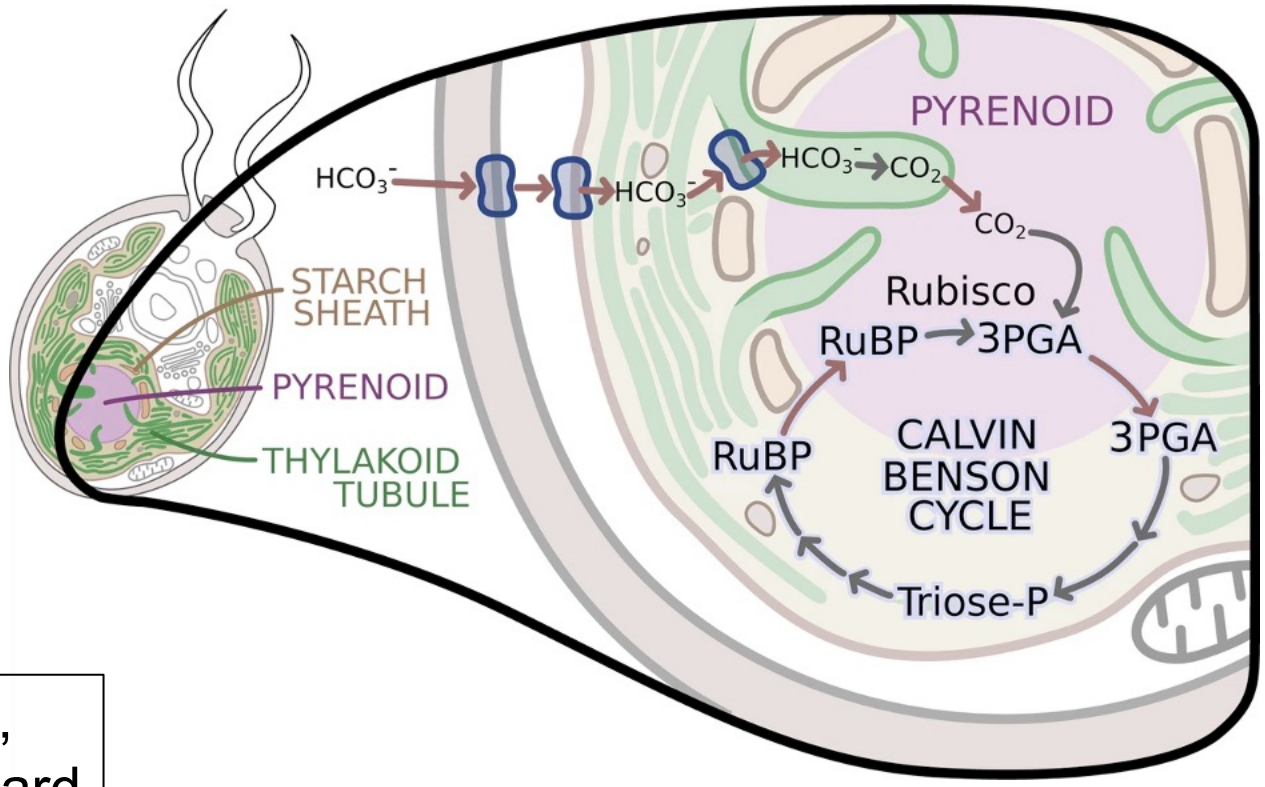

BP1
BP2
BP3
Q1
Q2
Q3
Q4
Q5
Q6
Q7
Q8
Q9
Q10
Q11
Q12
M1-3
M4-6
M7-9
M10-12
M13-15
M16-18
M19-21
M22-24
M25-27
M28-30
M31-33
M34-36
Task 1: Initial verification
Task 2: Enhance carbon and photosynthetic efficiency
Subtask 2.1: TERA approval for BicA mutant
Subtask 2.2: Modify N. oceanica to form pyrenoids
Subtask 2.3: Increase photosynthetic efficiency
Task 3: Sensor development and demonstration
Subtask 3.1: Develop biomass sensors
Subtask 3.2: Develop nutrient sensors
Subtask 3.3: Evaluate sensor systems
Task 4: Develop scaling & cultivation strategies
Subtask 4.1: Develop continuous cultivation model
Subtask 4.2: Conduct continuous cultivations
Subtask 4.3: Develop cultivator multiscale model
Subtask 4.4: WT baseline cultivation
Subtask 4.5: BicA mutant baseline cultivation
Task 5: Initial TEA and LCA
Subtask 5.1: Perform engineering process modeling
Subtask 5.2: Conduct initial techno-economic analysis
Subtask 5.3: Conduct initial life-cycle assessment
Task 6: Interim verification
Task 7: Advanced strain eng. & characterization
Subtask 7.1: TERA approval for new strain
Subtask 7.2: Additional strategies for pyrenoid eng.
Subtask 7.3: Improve biomass quality
Subtask 7.4: Toolkit for episomal-based control
Task 8: Concept integration and demonstration
Subtask 8.1: Sensors/cultivation tech for WT
Subtask 8.2: Strains/cultivation tech
Task 9: Final TEA and LCA
Subtask 9.1: Process modeling
Subtask 9.2: Concurrent TEA and LCA
Task 10: final verification

1 – Approach: Strain improvements

Increase productivity by improving CO_2 fixation

A. Modify *N. oceanica* to form pyrenoids
   - Incorporate genetic elements for formation of pyrenoids

- RuBisCO is relatively inefficient at carbon fixation
- The pyrenoid increases the concentration of CO_2 near RuBisCO, improving carbon fixation at low/ambient CO_2 levels
- Pyrenoids are found in many algae, but not *Nannochloropsis*



High risk, High reward

1 – Approach: Strain improvements

Increase productivity and biomass quality

A. Modify *N. oceanica* to form pyrenoids

B. Increase photosynthetic efficiency
   - Knock out several proteins involved in unproductive photosynthetic pathways

C. Improve biomass quality
   - Alter expression of target gene

Colorado State University | Arizona State University | Los Alamos National Laboratory | QBI Quantitative BioSciences, Inc. | Qualitas Health

1 – Approach: Strain improvements

A. Modify *N. oceanica* to form pyrenoids
   - Milestone 2.2.1: Heterologous expression of one transgene to confer pyrenoid function confirmed
   - Milestone 7.2.1: Experiments conducted to assess levels of biomass productivity or carbon use efficiency by a transgenic *N. oceanica* at lab scale or expression of one or more pyrenoid proteins

B. Increase photosynthetic efficiency
   - Milestone 2.3.1: Creation of 4 mutants associated with photosynthesis and characterization of their growth rate, biomass productivity, and photophysiology

C. Improve biomass quality
   - Milestone 7.3.1: Experiments conducted to show 10% increase in lipid content per unit weight in lab conditions mimicking those found at Mesa, AZ

1 – Approach: Operational improvements

Increase productivity by improving monitoring cells

A. Develop and implement biomass sensors
   - Optical probe placed in pond to make continuous measurements
   - Optical density measurements relate biomass concentration
   - Optical color measurements to indicate algae health

1 – Approach: Operational improvements

Increase productivity by monitoring nutrient levels

A. Develop and implement biomass sensors

B. Develop and implement nutrient sensors
   - Sensors exist for nitrate and phosphate; will be developed for urea and iron
   - Sensors are based on engineered *E. coli* strains

1 – Approach: Operational improvements

Increase productivity by optimizing cultivation operation

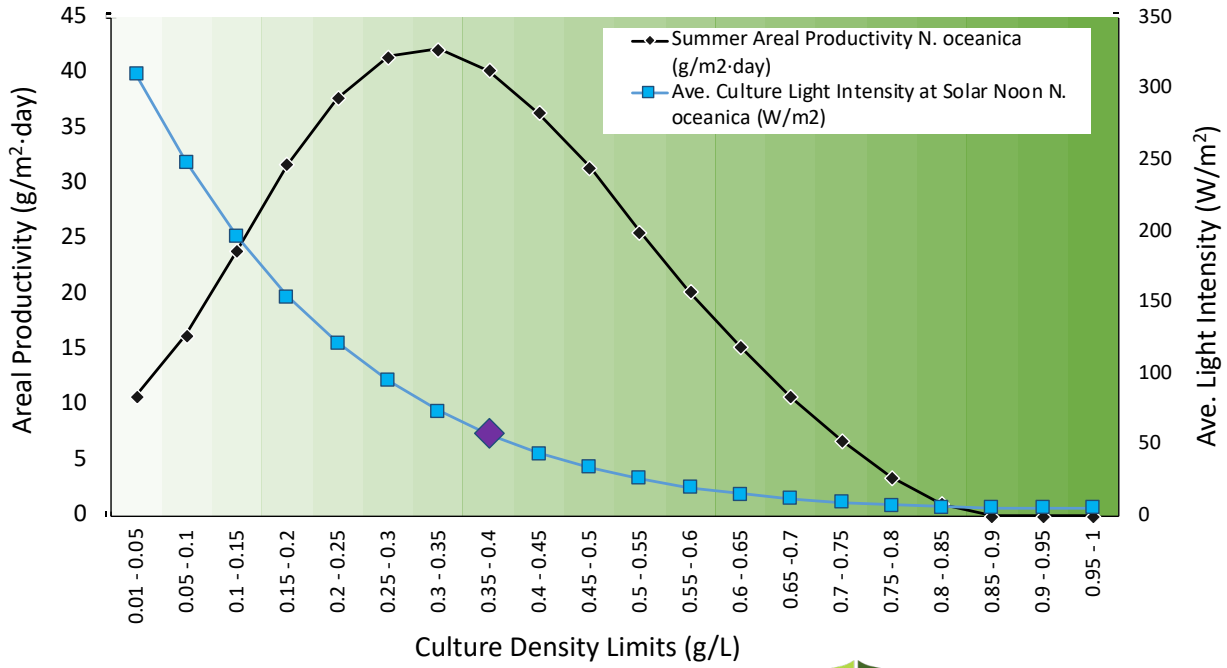
A. Develop and implement biomass sensors

B. Develop and implement nutrient sensors

C. Develop and evaluate continuous cultivation

- Continuous phototrophic cultivation has potential for higher biomass productivity than repeated-batch cultivation because cells grow faster at concentrations at which light penetrates further

Model prediction



1 – Approach: Operational improvements

Increase productivity at larger scales by improving prediction

A. Develop and implement biomass sensors
B. Develop and implement nutrient sensors
C. Develop and evaluate continuous cultivation
D. Develop multi-scale raceway pond models
   - Relate small pond productivity to much larger systems
   - Use 3D computational fluid dynamics simulations, coupled with dimensional analysis, to model flow, mass transport, cell distribution, and light intensity distribution; builds on prior results

Colorado State University | Arizona State University | Los Alamos National Laboratory | QBI Quantitative BioSciences, Inc. | Qualitas Health

1 – Approach: Operational improvements

A. Develop and implement biomass sensors
   - Milestone 3.1.1: Prototype optical sensor can consistently characterize biomass concentration of *N. oceanica* cultivation samples with less than 10% error compared to optical density measurements on a commercial spectrophotometer.

B. Develop and implement nutrient sensors
   - Milestone 3.2.1: Capability to detect 100 ppb or lower of urea and iron in laboratory experiments using the nutrient sensor platform.

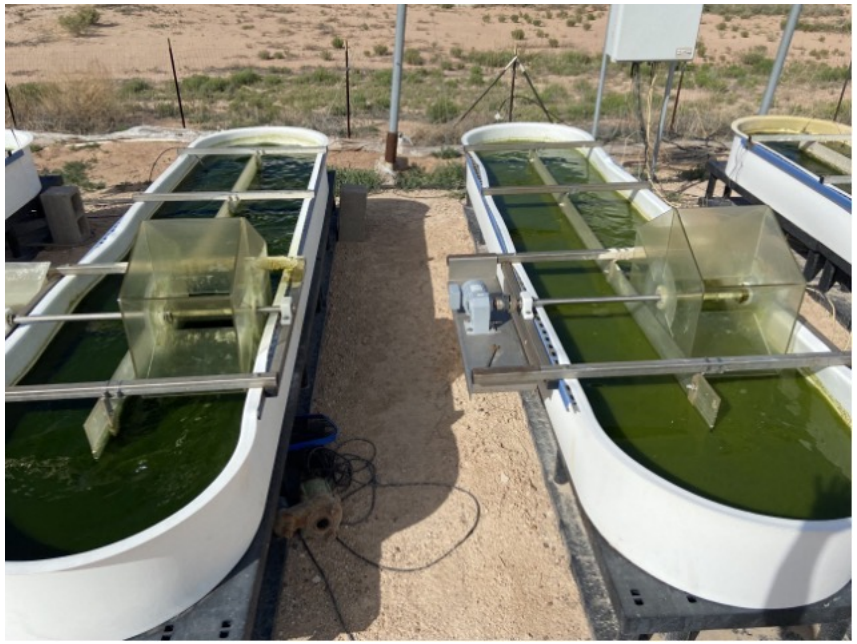
C. Develop and evaluate continuous cultivation
   - Milestone 4.1.1: Development of continuous cultivation model that predicts *N. oceanica* productivity as a function of cell density at one simulated location, validated within 20% by indoor 100-L mini-pond cultivations.

D. Develop multi-scale raceway pond models
   - Milestone 4.3.1: Demonstrate that 2- and 4-day biomass growth rates in a custom programmed benchtop photobioreactor are within 10% of growth rates at the same time points in a 100-L mini-pond.

1 – Approach: Integration and demonstration

Test developments outdoors

A. Field trials of WT *N. oceanica* with operation improvements
   - Deploy sensors and continuous cultivation approach in 330-L mini-ponds
   - Biomass sensors inform input flow rates
   - Nutrient sensors inform on-demand feeding



Colorado State University | Arizona State University | Los Alamos National Laboratory | QBI Quantitative BioSciences, Inc. | QUALITAS HEALTH

1 – Approach: Integration and demonstration

Test developments outdoors

A. Field trials of WT *N. oceanica* with operation improvements

B. Field trials of improved strains with operation improvements
   - Deploy sensors and continuous cultivation approach
   - Obtain TERA approvals for mutants
   - Budget period 1 cultivations use previously-developed BicA *N. oceanica* mutant
   - Budget period 2 cultivations use mutant from Task 2

1 – Approach: Integration and demonstration

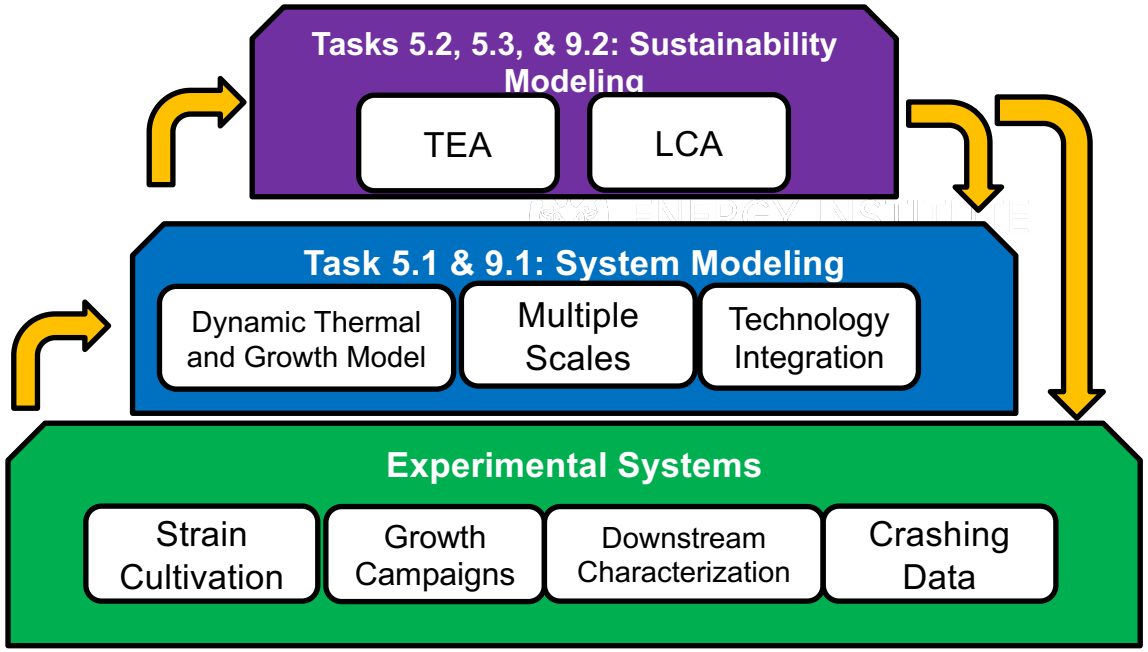
A. Field trials of WT *N. oceanica* with operation improvements
   - Milestone 8.1.1: Completion of one outdoor spring cultivation of wild-type *N. oceanica* using novel cultivation operation technology

B. Field trials of improved strains with operation improvements

1 – Approach: Techno-economic analysis and life-cycle assessment

Determine sustainability of developed improvements

A. Perform engineering process modeling
B. Conduct techno-economic analysis
C. Conduct life-cycle assessment



1 – Approach: Techno-economic analysis and life-cycle assessment

A. Perform engineering process modeling
   - Milestone 5.1.1: Complete preliminary mass and energy balance for the baseline pathway

B. Conduct techno-economic analysis

C. Conduct life-cycle assessment
   - Milestone 9.2.1: Biorefining pathway that meets economic $2.50 per gallon of gasoline equivalent) and environmental goals (<45 g-carbon dioxide equivalent per MJ) defined

1 – Approach: Go/No-Go milestones

✓ **BP 1 Go/No-Go**: Process information and data supporting the technology readiness level of the overall process, the unit operations within the process, and the original application. Technical metrics are based on preliminary/previous data and represent a meaningful baseline and set of targets.

- **BP 2 Go/No-Go**: Laboratory-demonstrated 10% improvement arising from either cultivation or genetic improvements or their combination. Sensors for iron and urea demonstrate the capability to detect 100 parts per billion or lower in indoor mini-pond *N. oceanica* cultivations. The optical sensor can consistently characterize biomass optical density of indoor mini-pond *N. oceanica* culture samples with less than 10% error compared with optical density measurements using a commercial spectrophotometer. Initial technoeconomic analysis and life-cycle assessment completed. Interim targets for biomass productivity and quality will be achieved.

1 – Approach: Go/No-Go milestones

- **End-of-Project Goal**: The end-of-project goal is >20% increase in areal biomass productivity and biomass quality yielding >85 gallons of gasoline equivalent/ton from Nannochloropsis oceanica. These metrics will be achieved both indoors under conditions resembling spring in Mesa, AZ (minimum targets) and outdoors in spring in Mesa, Arizona and Imperial, TX (stretch targets). The baseline for Nannochloropsis oceanica spring season biomass productivity is 15 g $m^{-2} \cdot d^{-1}$ in 730-L outdoor ponds at Imperial, TX. In addition, toolkits for determining optimal cultivation operation and algal strain engineering will be generated.

1 – Approach

Potential challenges facing the technical approach

- Difficulties with strain improvement approaches
- Difficulties with sensor development
- Culture contamination

1 – Approach

Risk analysis and mitigation strategies

- **Risk:** Pyrenoid strain improvement strategy is slow or unsuccessful
- **Mitigation:** Alternative strategies to strain improvement are built into plan

- **Risk:** Culture contamination
- **Mitigation:** Use strategies developed at QH and AzCATI to identify and overcome contamination; anecdotal evidence that continuous cultivation reduces contamination

2 – Progress and Outcomes

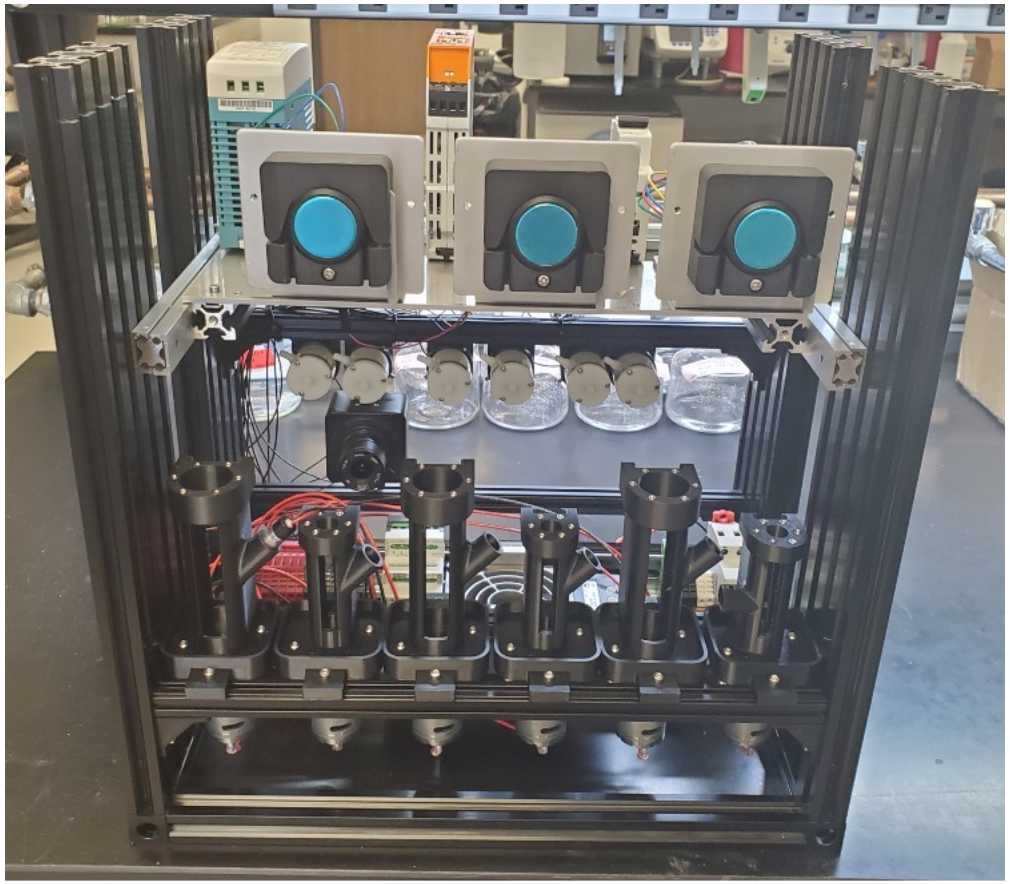
Task 2: Strain improvement

- TERA for previously developed mutant in preparation; submission in mid-April
- Research staff hired
- Engineering targets for enhancing CO_2 fixation have been identified and work is underway

2 – Progress and Outcomes

Task 3: Sensor development and demonstration

- QBISCI has created a new variant of their nutrient sensor known as the cQUBE
- The cQUBE is a low cost “lab in a box” instrument
- The aluminum extrusion scaffold of the cQUBE which allows easy field installation and servicing
- The cQUBE can precisely set up and monitor chemical reactions using low-cost optical systems
- First field tests in April

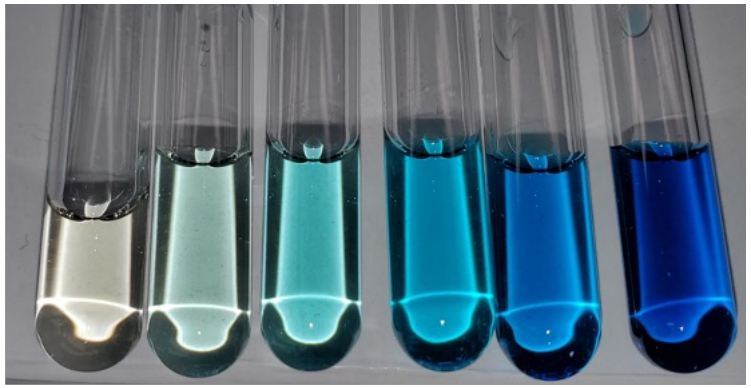


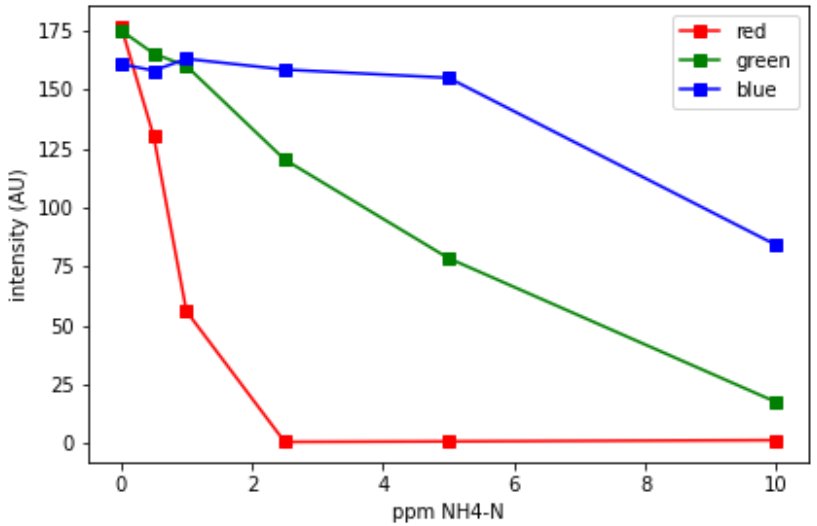
Colorado State University | Arizona State University | Los Alamos National Laboratory | QBI Quantitative BioSciences, Inc. | Qualitas Health

2 – Progress and Outcomes

Task 3: Sensor development and demonstration

- The cQUBE optics allow us to quickly follow the progress of colorimetric reactions.
- Example →: colorimetric test for free ammonia; samples from an algae pond at the QBISCI facility in Modesto CA
- By monitoring the reaction progress in multiple color channels, a precise estimate for the ammonia present can be generated.



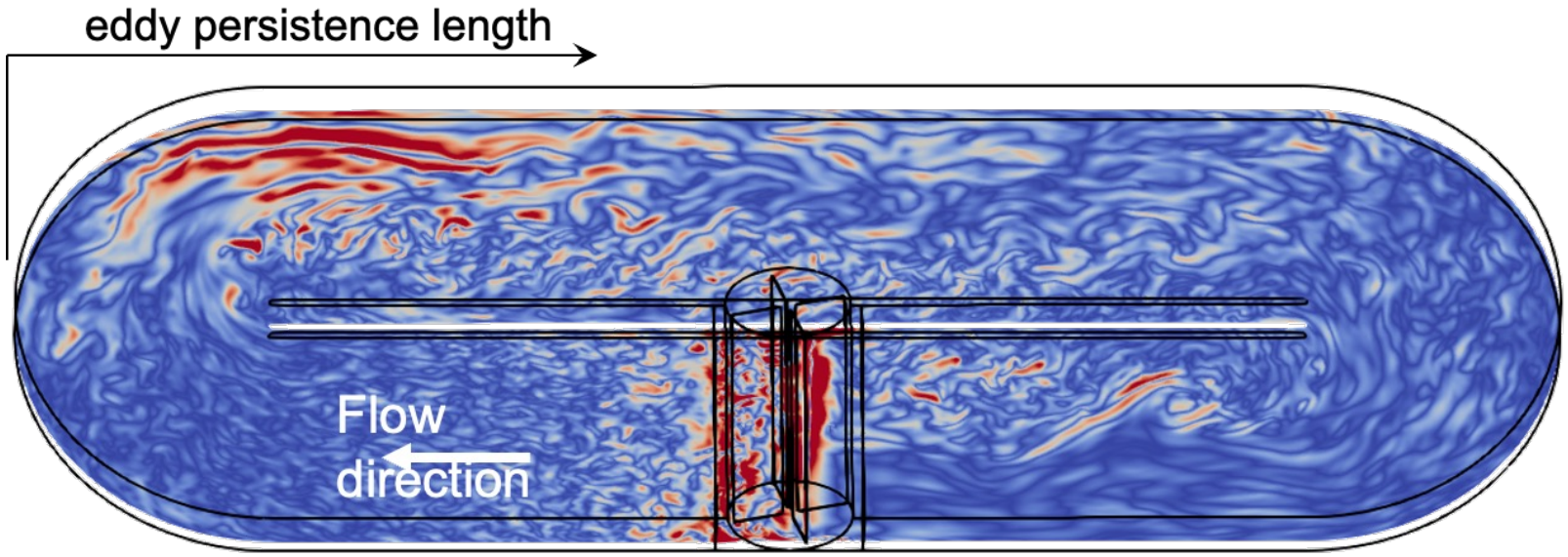


Colorado State University
Arizona State University
Los Alamos NATIONAL LABORATORY
QBI Quantitative BioSciences, Inc.
QUALITAS HEALTH

2 – Progress and Outcomes

Task 4.3: Multiscale model development

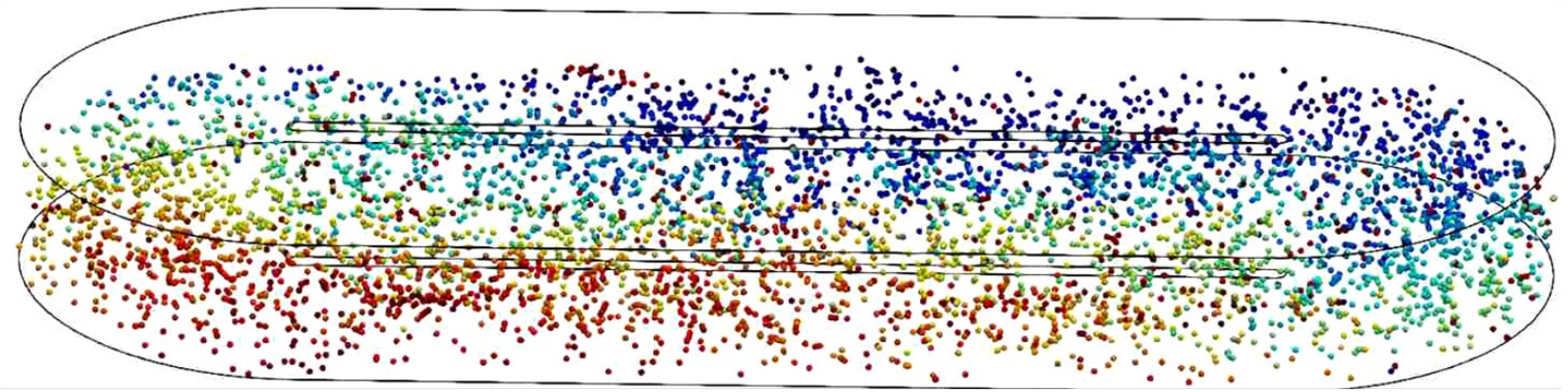
- Quantify mixing behavior
- Cell trajectory data converted to light intensity history
- Construct controller and program LED array for 1-L bioreactor with 100-L pond light dynamics



2 – Progress and Outcomes

Task 4.3: Multiscale model development

Particle tracking: Random initial locations of more than 10,000 cells in turbulent flow field



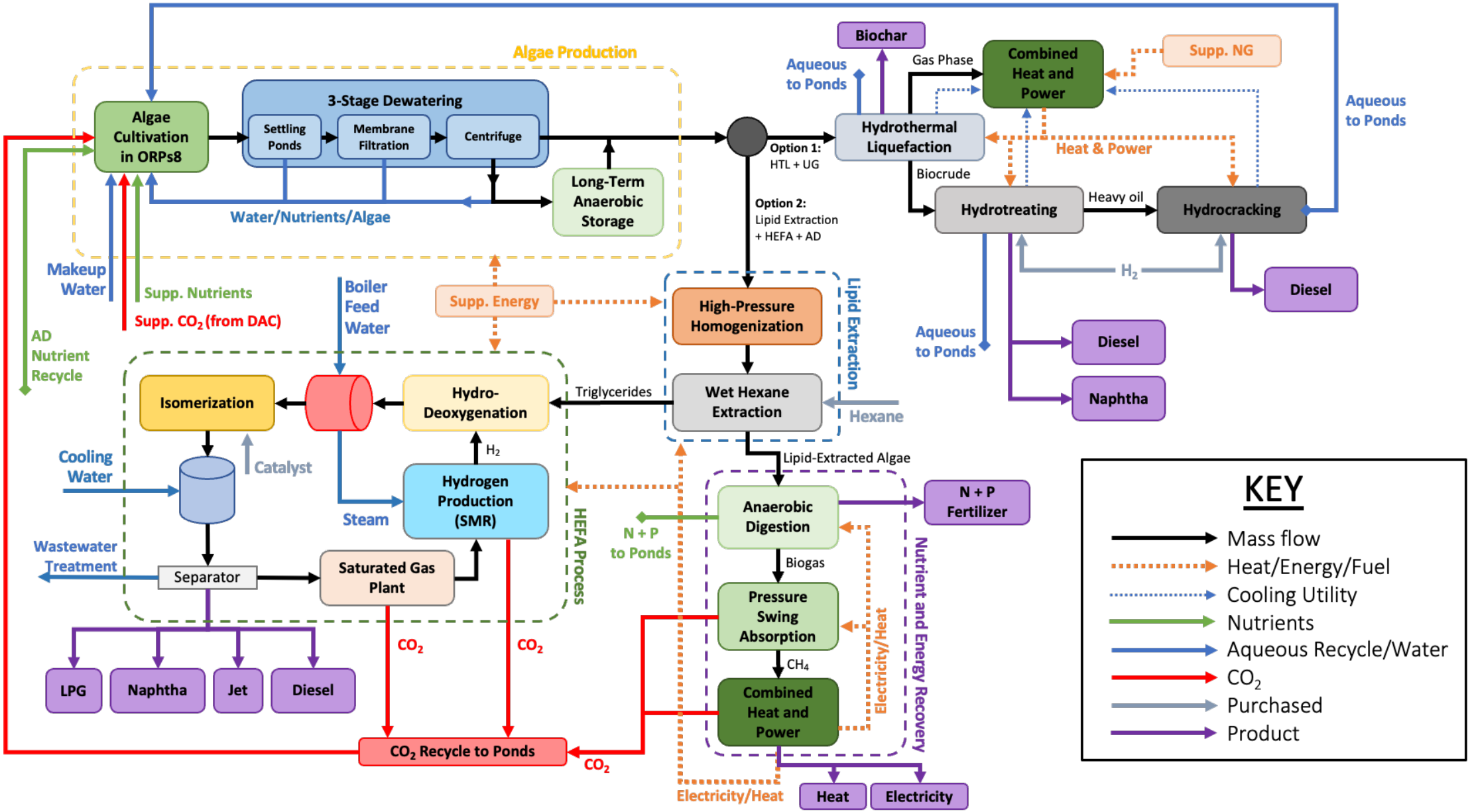
Colorado State University · Arizona State University · Los Alamos National Laboratory · QBI Quantitative BioSciences, Inc. · Qualitas Health

2 – Progress and Outcomes

Task 5: Techno-economic analysis and life-cycle assessment

- Completed initial process flow diagram
- Initial integration of TEA and LCA model components

Task 5: Process Flow Diagram



Task 5: Model Summary

- Spatiotemporal Growth Model
  - Estimates algal productivity with hourly resolution
    - Growth in open raceway ponds
    - Continuous or batch cultivation
    - 3-stage dewatering process
    - Long term anaerobic storage
- Multiple Conversion Pathways
  - Conversion to biocrude through hydrothermal liquefaction
    - Fuel upgrading with hydrotreating and hydrocracking
  - Conversion to sustainable aviation fuel (SAF)
    - Lipid extraction with solvent extraction
    - Hydrotreated esters and fatty acids (HEFA) process model
    - Energy/nutrient recovery from AD of lipid extracted algae
    - Combined heat and power
- Integrated techno-economic and life cycle assessments
  - Discounted cash flow rate of return
  - Minimum product selling prices (biomass, lipids, fuels)

3 – Impact

- Technology impacts
  - Innovation in strain photon/CO_2 utilization and composition
  - Novel cultivation management strategy
  - Development of real-time, continuous, quantitative sensing
  - Delivery of 2 toolkits for pond management and strain engineering
- Demonstration of pathway that meets DOE goals
  - 85 GGE $tonne^{-1}$
  - \$2.50 GGE^{-1}
  - <45 g-CO_2e MJ^{-1}
- Valuable engagement with industry
  - QBI for nutrient sensor development and commercialization
  - QH for large-scale cultivation know-how
- Dissemination
  - Publications, patent applications planned

Colorado State University | Arizona State University | Los Alamos National Laboratory | QBI Quantitative BioSciences, Inc. | Qualitas Health

Summary

- Combining strain and operational improvements to achieve productivity goal
- Concurrent TEA and LCA provide feedback to experimental work
- Multi-pronged approach to strain development mitigates risks
- Delayed start but now at speed

Project Team

Colorado State University
David Dandy, Graham Peers, Jason Quinn, Ken Reardon, Tessema Kassaw, Brandon Rohnke

Arizona Center for Algae Technology and Innovation, Arizona State University
John McGowan

Los Alamos National Laboratory
Shawn Starkenburg

Qualitas Health
Jakob Nalley

Quantitative Biosciences
Natalie Cookson, Michael Ferry

Quad Chart Overview

Timeline

- *Start: 10/01/2021*
- *End: 09/30/2025*

	FY22 Costed	Total Award
DOE Funding	*$978,432*	*$3,199,990*
Project Cost Share *	$801,143	

Project Goal: *Improve the productivity and biomass quality of N. oceanica in the spring season to achieve >20% increase in areal biomass productivity and biomass quality that yields >85 GGE/ton of biomass.*

End of Project Milestone: *>20% increase in areal biomass productivity and biomass quality yielding >85 gal of gasoline equivalent/ton from N. oceanica. The baseline for N. oceanica spring season biomass productivity is 15 g $m^{-2} \cdot d^{-1}$ in 730-L outdoor ponds at Imperial, TX. In addition, toolkits for determining optimal cultivation operation and algal strain engineering will be generated.*

Funding Mechanism

DE-FOA-0002423; Feedstock Technologies and Algae FOA

Topic Area: Topic Area 2: Algae Productivity Exceeding Expectations (APEX)

Subtopic 2a: Improvements in Productivity with Traditional CO2 Supply

TRL at Project Start: 3

TRL at Project End: 6

Project Partners

- AzCATI, ASU
- LANL

* Quantitative Biosciences
* Qualitas Health

Colorado State University | Arizona State University | Los Alamos National Laboratory | QBI Quantitative BioSciences, Inc. | Qualitas Health

Additional Slides

Milestones

Phase	Milestone	Description	Date
1	G/NG #1		M3
2		TERA application approved for the current *N. oceanica* BicA mutant	M6
2	M 2.2.1	Heterologous expression of one transgene to confer pyrenoid functionality confirmed by PCR and/or qPCR.	M9
2	M 4.1.1	Development of continuous cultivation model that predicts *N. oceanica* productivity as a function of cell density at one simulated location, validated within 20% by indoor 100-L mini-pond cultivations.	M12
2	M 4.3.1	Demonstrate that light intensity history obtained from the mini-pond yields the same photophysical behavior in the benchtop photobioreactor.	M15
2		Prototype optical sensor can characterize biomass and algae health of samples with less than 10% error compared to a spectrophotometer.	M18
2		Demonstration of capability to detect 100 ppb or lower of urea and Fe in laboratory.	
2			
3		TERA approved for the improved *N. oceanica* strain developed in Task 2.	M24
3			
3	M 7.3.1	Experiments to show 10% content in lipid content per unit weight (compared to WT) in lab conditions mimicking those at AzCATI.	M30
3	M 7.4.1	Delivery of episomal vectors to Addgene including at least one episome for control of gene expression and 20 containing distinct promoter/terminators.	M33
3		Biorefining pathway that meets economic \$2.50 GGE^{-1}) and environmental goals (<45 g-CO_2e MJ^{-1}) defined	

Task 2: Enhance carbon fixation and photosynthetic efficiency in *N. oceanica*

Subtask 2.1- Obtain TERA approval for previously developed mutant

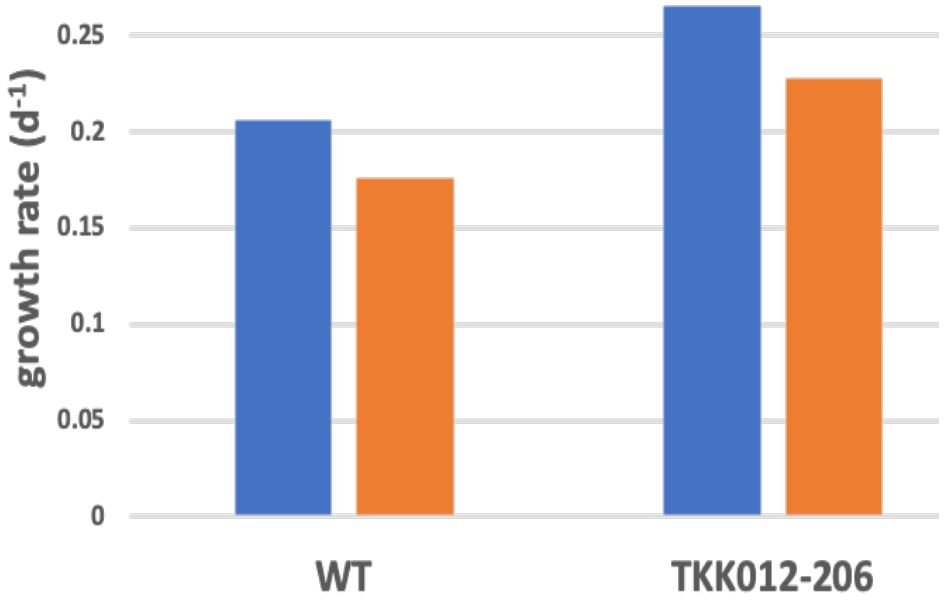
Colorado State University
Arizona State University
Los Alamos NATIONAL LABORATORY
QBI Quantitative BioSciences, Inc.
QUALITAS HEALTH

Subtask 2.1: Summary

Applying experience with obtaining TSCA Environmental Release Application (TERA) permits from previous DOE BETO projects, a TERA will be prepared and submitted to the EPA for the current *N. oceanica* mutant.

Subtask 2.1: Background

- Previously developed BicA mutant shows higher productivity in high bicarbonate lab conditions but remains untested at scale & outdoors (USPTO App. No. 63/275,850).



Colorado State University
Arizona State University
Los Alamos NATIONAL LABORATORY
QBI Quantitative BioSciences, Inc.
QUALITAS HEALTH

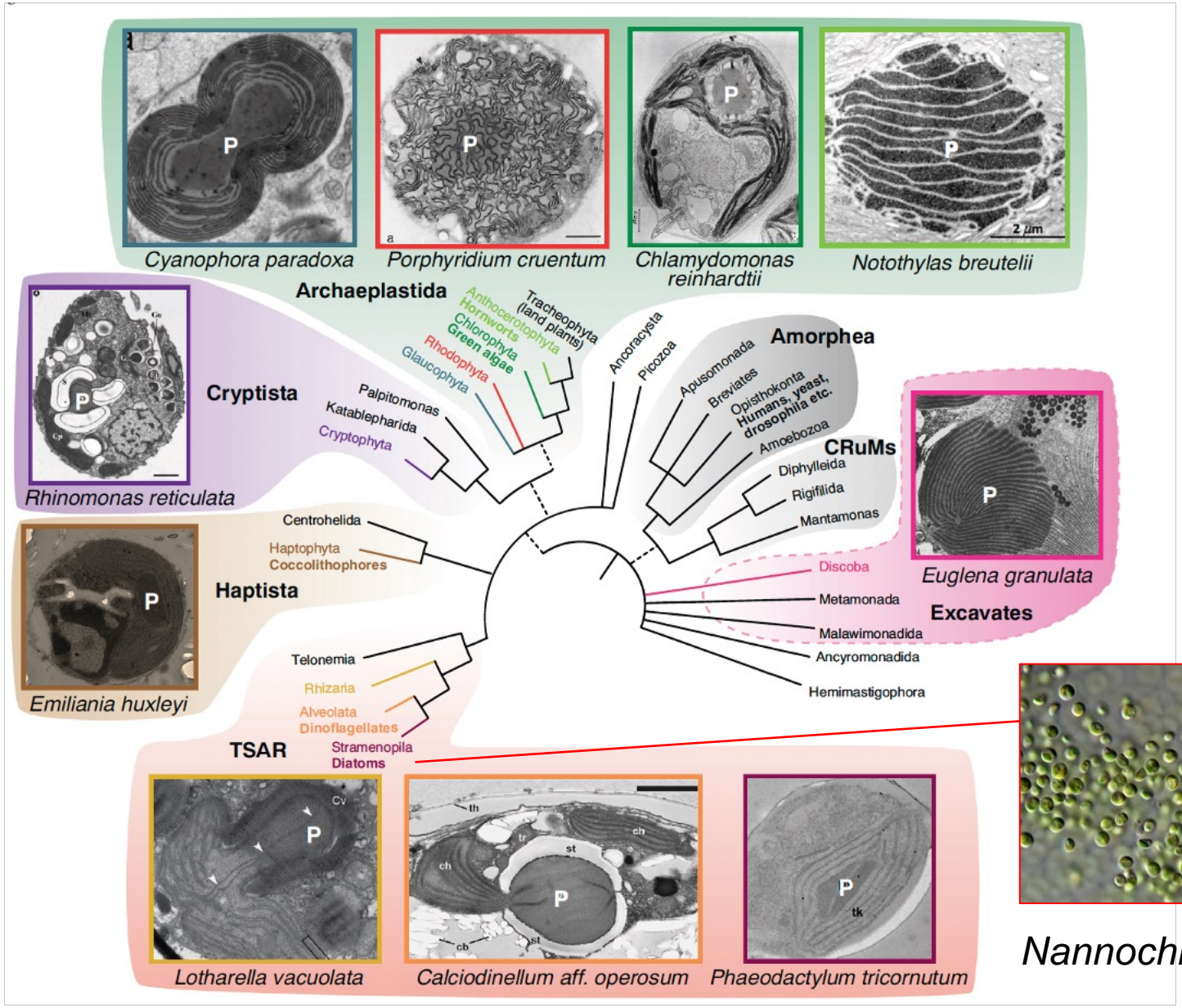
Subtask 2.2 - Modify N. oceanica *to form pyrenoids*

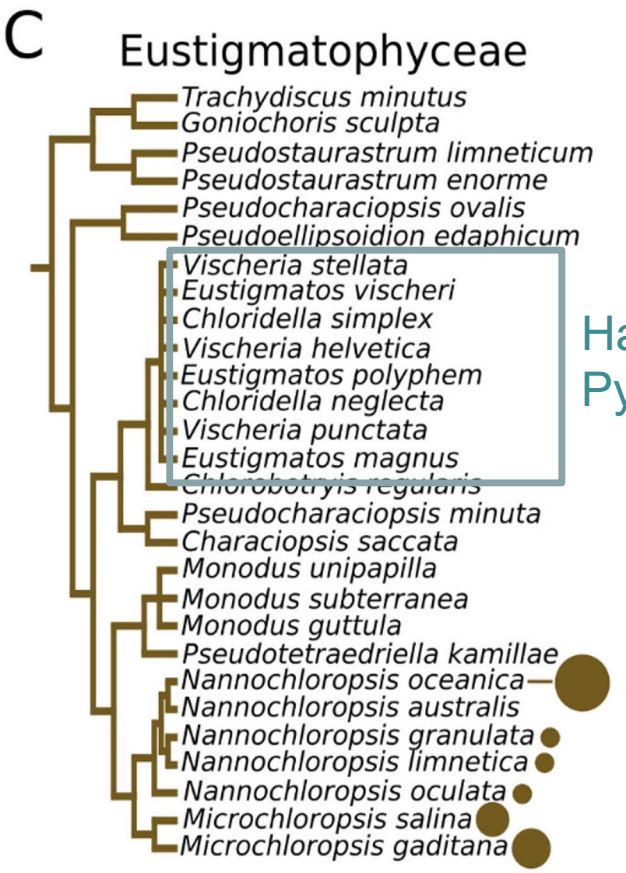
Colorado State University
Arizona State University
Los Alamos NATIONAL LABORATORY
QBI Quantitative BioSciences, Inc.
QUALITAS HEALTH

Subtask 2.2: Summary

As a high-risk/high-reward effort, the genetic elements for formation of pyrenoids will be incorporated.

Pyrenoids have evolved many times across large phylogenetic distances and share similar properties

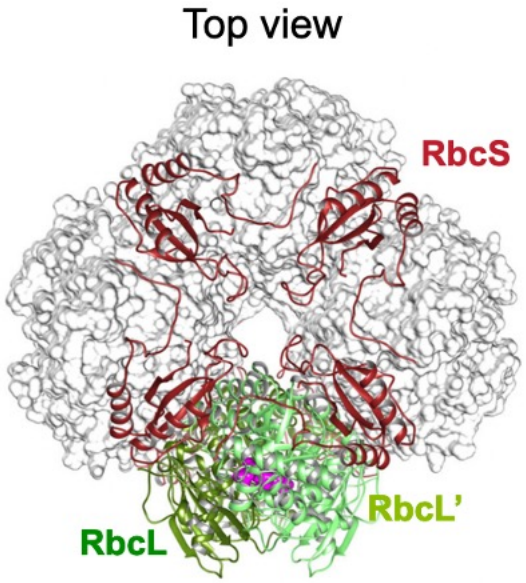




Hanschen & Starkenburg, Algal Research 2020

BBA - Molecular Cell Research 1868 (2021)

RuBisCO



A hybrid RuBisCO protein will be engineered consisting of native *Nannochloropsis* RbcL and an RbcS from a 'near neighbor' that forms pyrenoids

Motifs of the RuBisCO small subunit (RbcS)

		HELIX A	HELIX B
'Green-type'	*Chlamydomonas*	PPLTDEQIAAQVDYIVANGWI	DPMQVLREIVACTKAFP
	Spinacia	PPLTTDQLARQVDYLLNNKWV	DPAQVLNELEECKKEYP
'Red-type'	*Heterosigma*	PDLTDAQIIKQIDYCLSRGWS	DSSAILYEVNECRRLNP
	Nannochloropsis	PDLTNEQILGQLKYLCSKGWA	DASVIMYELDQCRAAHP

Species RbcS	Helix A Aliphatic Index	Helix B Aliphatic Index
Chlamydomonas		
Heterosigma	97.62	91.76
Spinacia	106.67	68.82
Nannochloropsis		

Colorado State University · Arizona State University · Los Alamos National Laboratory · QBI Quantitative BioSciences, Inc. · Qualitas Health

Subtask 2.3 – Increase photosynthetic efficiency

Colorado State University
Arizona State University
Los Alamos NATIONAL LABORATORY
QBI Quantitative BioSciences, Inc.
QUALITAS HEALTH

Subtask 2.3: Summary

To increase photosynthetic efficiency, genes for several proteins involved in photosynthesis will be knocked out.

Subtask 2.3: Background

- Photosynthetic efficiency in mass culture is affected by not only by adequate CO_2 delivery to the CBB cycle, but also by inefficient light energy conversions to chemical energy.
- Research by the Peers group, and others, have identified processes that lead to inefficiencies in mass culture, including light harvesting.

Subtask 2.3: Approach

- CRISPR/Cas9-mediated gene knockout of genes related to photosynthesis
- We will target independent pathways known to influence photosynthetic efficiency in other algae and cyanobacteria
- Photosynthetic physiology: Gas exchange through Membrane Inlet Mass Spectrometry; light energy partitioning through Pulse Amplitude Modulated Fluorometry. Carbon accumulation via TOC. Growth by cell concentration (flow cytometry, microscopy) and/or OD

Task 3: Sensor development and demonstration

Colorado State University
Arizona State University
Los Alamos NATIONAL LABORATORY
QBI Quantitative BioSciences, Inc.
QUALITAS HEALTH

Subtask 3.1 – Develop biomass sensors

Colorado State University
Arizona State University
Los Alamos NATIONAL LABORATORY
QBI Quantitative BioSciences, Inc.
QUALITAS HEALTH

Subtask 3.1: Summary

- Develop continuous biomass sensors for outdoor algae ponds
- Engineer biomass sensors to be low-cost
- Incorporate optical color measurements to indicate algae health

Subtask 3.1: Biomass sensor risks and mitigation

Risk	Mitigation
Daily temperature shifts cause sensor drift	• Compensate with temperature measurements • Active temperature control of optical sensing elements
Optical interference from sunlight	• Design optical system to minimize sunlight on optical sensors
Algae pigment fluorescence causes interference in biomass measurement	• Shift biomass wavelength to avoid fluorescence • Optical filter to block fluorescence signal
Biofouling of optical windows	• Daily cleaning of optical windows

Subtask 3.2 – Develop nutrient sensors

Colorado State University
Arizona State University
Los Alamos NATIONAL LABORATORY
QBI Quantitative BioSciences, Inc.
QUALITAS HEALTH

Subtask 3.2: Summary

Sensors will be developed for urea and iron. These will be integrated into a microfluidic sensor chip and integrated into a real-time sensor platform. The resulting platform will be used to collect calibration data to develop computational tools to correlate fluorescence signals with nutrient levels. A machine learning algorithm will be trained using induction data from a range of experiments.

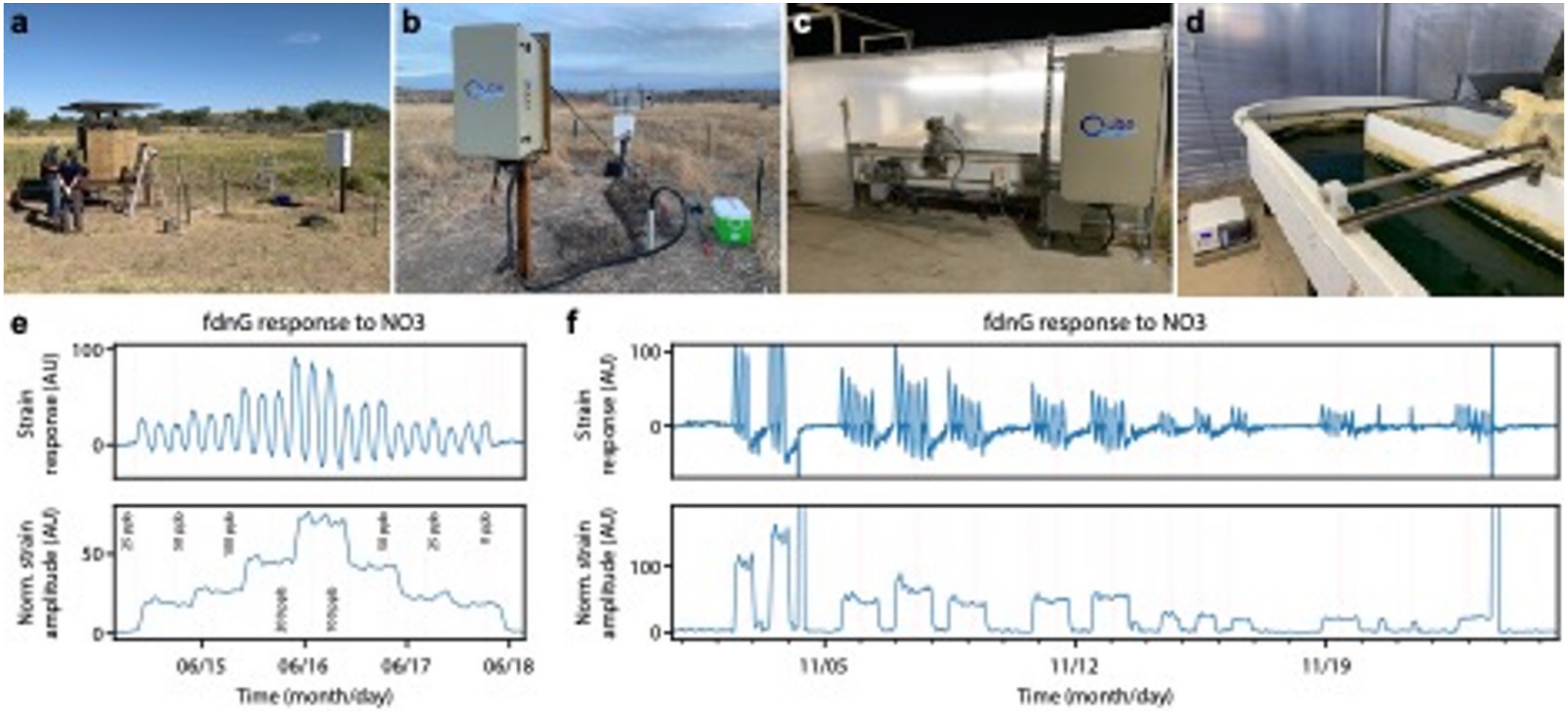
Subtask 3.2: Background

- QBI is a biotechnology small business focused on the develop of water quality technologies (treatment and sensing)
- Our laboratory is in San Diego, CA where we develop biosensors for water contaminants
- We also have a facility in Modesto, CA, where we are partnered with the Fiscalini Dairy
- We are developing a facility that uses algae for wastewater remediation, biogas purification, and animal feed production

Colorado State University | Arizona State University | Los Alamos National Laboratory | QBI Quantitative BioSciences, Inc. | QUALITAS HEALTH

Subtask 3.2: Background

QBISCI has deployed the Qube at numerous outdoor field sites and has validated the ability to sample from outdoor algae ponds



Subtask 3.3 – Evaluate sensor systems in mini-ponds

Colorado State University
Arizona State University
Los Alamos NATIONAL LABORATORY
QBI Quantitative BioSciences, Inc.
QUALITAS HEALTH

Subtask 3.3: Summary

The biomass and nutrient sensors will be tested in 100-L mini-pond indoor cultivations of WT *N. oceanica* under a range of light and nutrient conditions.

Subtask 3.3: Approach

- Cultivate WT *N. oceanica* in indoor 100-L mini-pond cultivations under a range of light and nutrient conditions in QH medium
- Monitor with in-line sensors and validate with off-line measurements:
  - Biomass: cuvette-based UV-Vis spectrophotometer
  - Nutrients (urea, iron): standard methods
- Improve sensors as necessary to achieve accuracy over 2-week cultivations

Task 4: Development of scaling and cultivation strategies

Colorado State University
Arizona State University
Los Alamos NATIONAL LABORATORY
QBI Quantitative BioSciences, Inc.
QUALITAS HEALTH

Subtask 4.1 – Develop continuous cultivation model

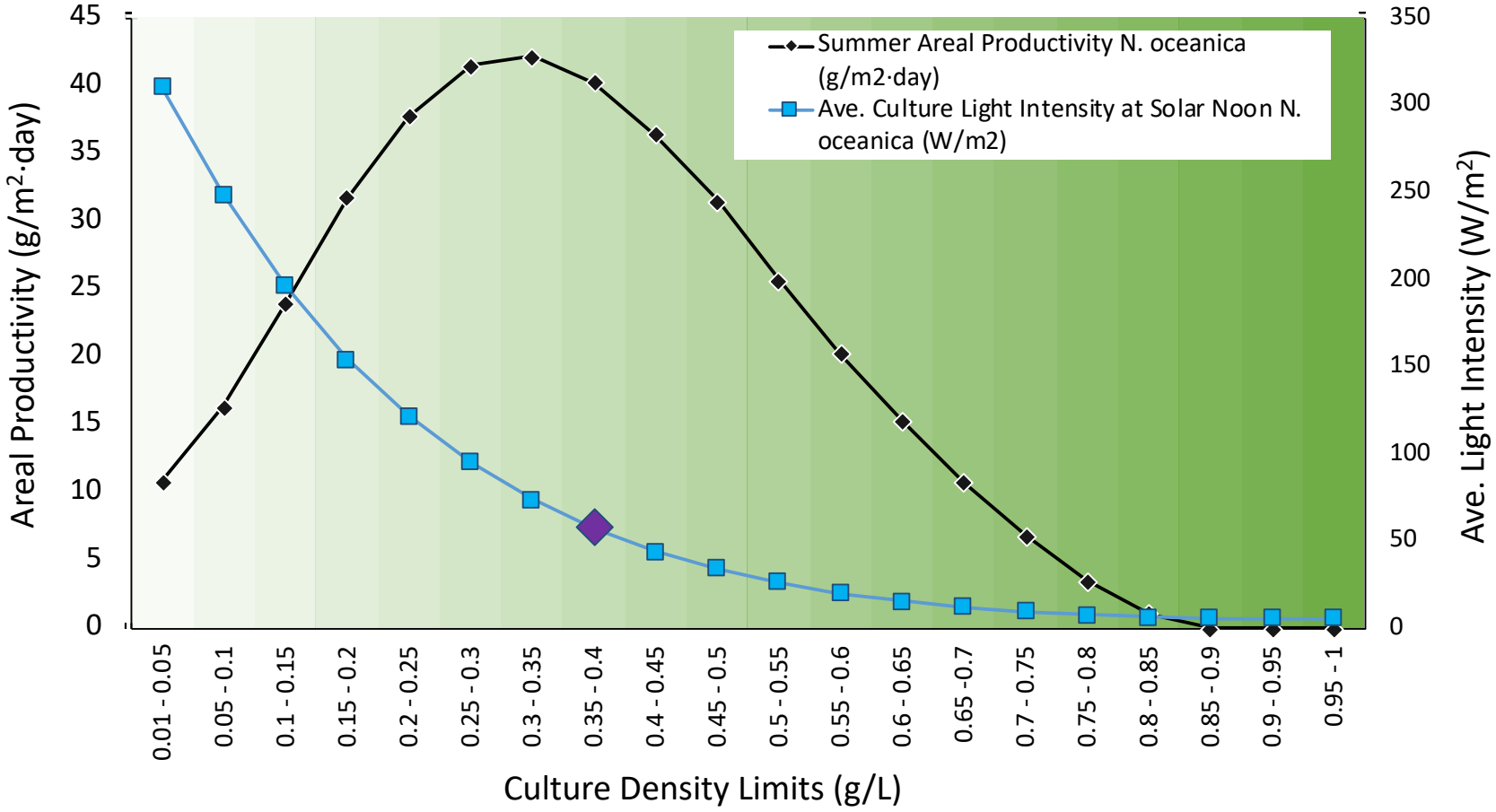
Colorado State University
Arizona State University
Los Alamos NATIONAL LABORATORY
QBI Quantitative BioSciences, Inc.
QUALITAS HEALTH

Subtask 4.1: Summary

A TEA-based model will be developed to predict the constant-concentration (turbidostat), continuous cultivation operating conditions that provide maximal biomass productivity.

Subtask 4.1: Background

- Continuous phototrophic cultivation enables higher biomass productivity than semi-batch
  - Faster growth owing to higher light intensities (less shading)
  - Reports of fewer pond crashes with more frequent harvesting



Productivity vs. cell density (*N. oceanica*, summer, Imperial, CA). To operate the algae pond within the culture density limits, the pond is harvested at the upper density and only a portion of the volume is removed until the lower limit is achieved. Higher densities lead to self-shading, lower average light intensity, and slower growth. The purple diamond is the saturation light intensity. In the range 0.3–0.35 g/L, the intensity at noon is higher than the saturation level but the culture is at the optimal intensity long enough to achieve the maximal productivity of **40 g/m²·day**. Typically, ponds are operated at 0.5–0.8 g/L, resulting in significant self-shading.

Colorado State University · Arizona State University · Los Alamos National Laboratory · QBI Quantitative BioSciences, Inc. · Qualitas Health

Subtask 4.1: Approach

- Refine growth model for use in continuous cultivation
- Determine optimal operating conditions for *N. oceanica* for any location and season, including diurnal cycles
- Evaluate influence of near-continuous operation

Subtask 4.2 – Evaluate continuous cultivation model in mini-ponds

Colorado State University
Arizona State University
Los Alamos NATIONAL LABORATORY
QBI Quantitative BioSciences, Inc.
QUALITAS HEALTH

Subtask 4.2: Summary

Experiments will be conducted in 100-L indoor, illuminated mini-ponds to obtain data (e.g., light attenuation) for the cultivation operating model and to test the model predictions of growth rate at different constant biomass concentrations, under different light intensities and diurnal cycles.

Subtask 4.2: Approach

- Cultivate WT *N. oceanica* in indoor 100-L mini-pond *continuous* cultivations under a range of light and nutrient conditions in QH medium
- Use biomass sensors to control cell concentration at targeted levels
- Biomass concentration (AFDW) and composition determined with standard off-line methods.
- Test model predictions of growth rate and productivity

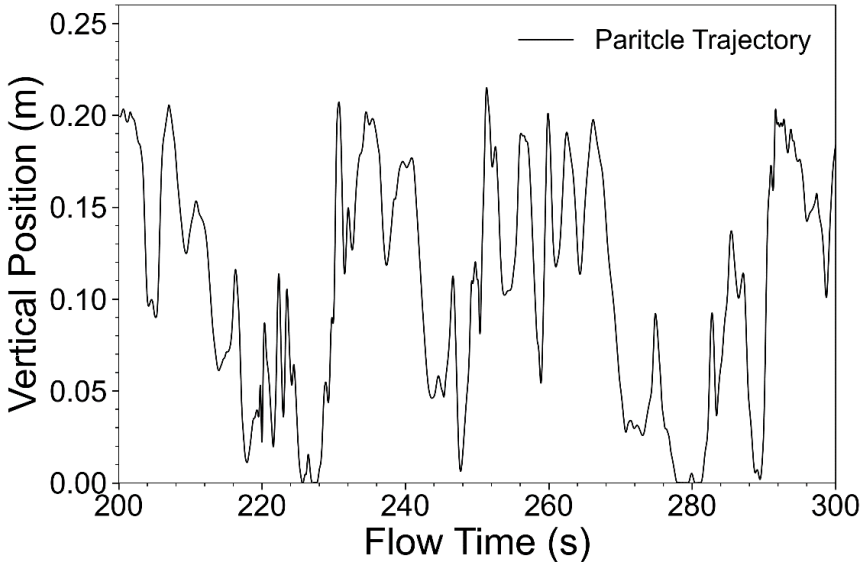
Subtask 4.3 – Cultivator multiscale model development

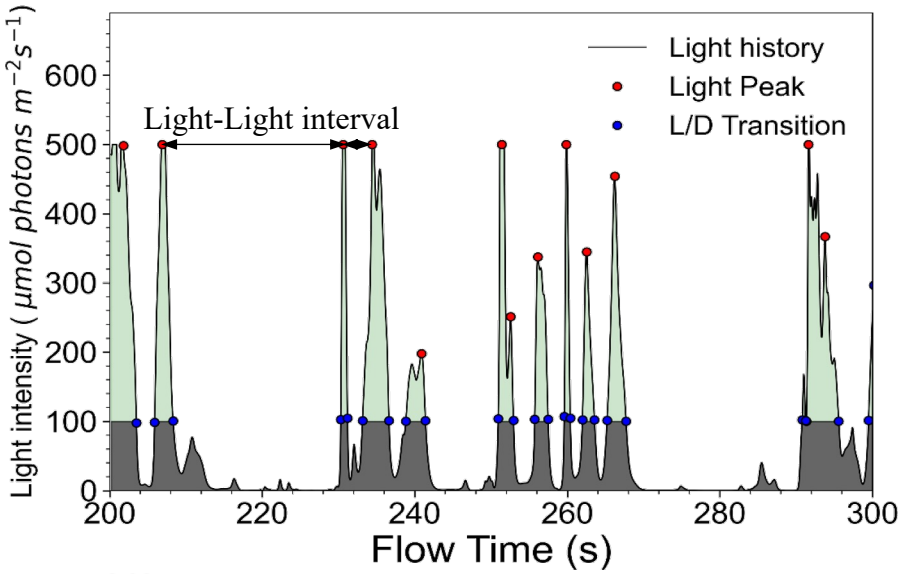
Subtask 4.3: Summary

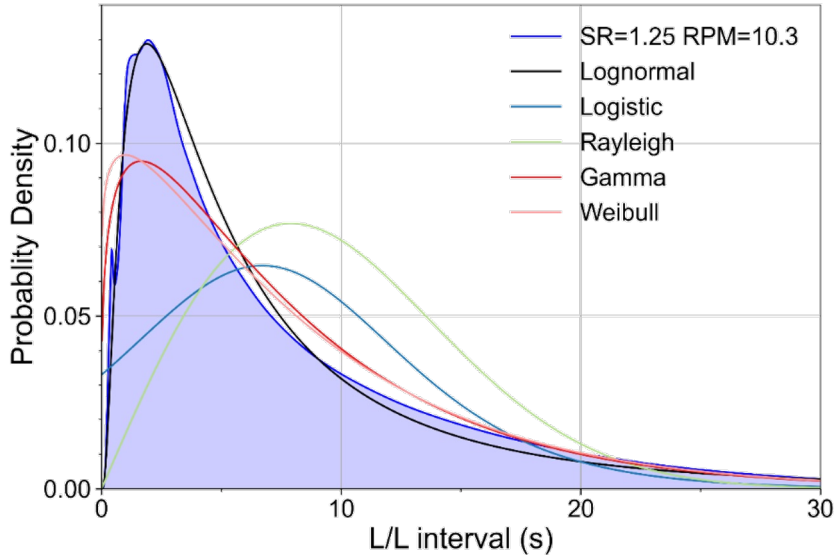
3D computational fluid dynamics simulations, coupled with dimensional analysis, will be used to model liquid flow, energy and mass transport, cell distribution, and light intensity distribution; the first goal will be to compare the photosynthetic behavior of cells at the 1 L scale to the cultivations in Subtasks 3.3 and 4.2.

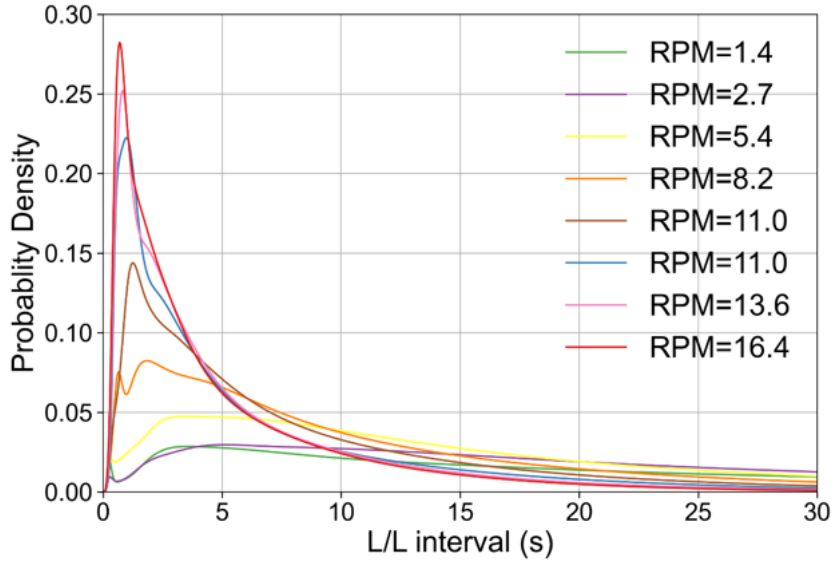
Task 4.3: Multiscale model development – Progress and outcomes

Vertical position is converted to light intensity using the Beer-Lambert law





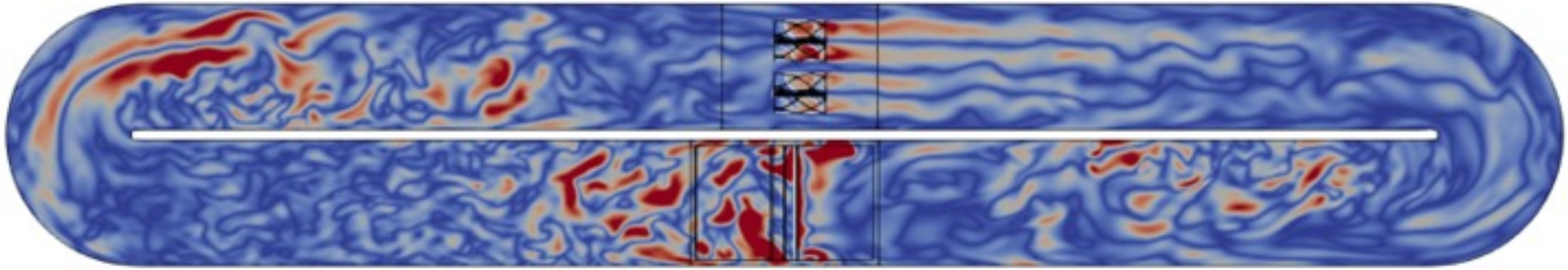




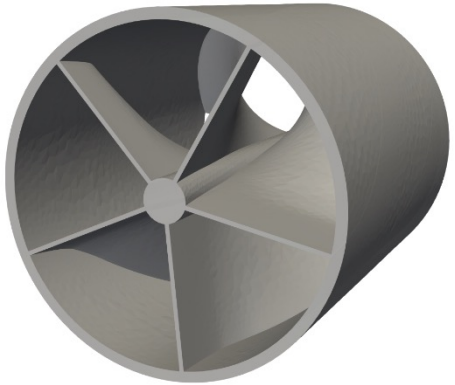
Task 4.3: Multiscale model development – Progress and outcomes

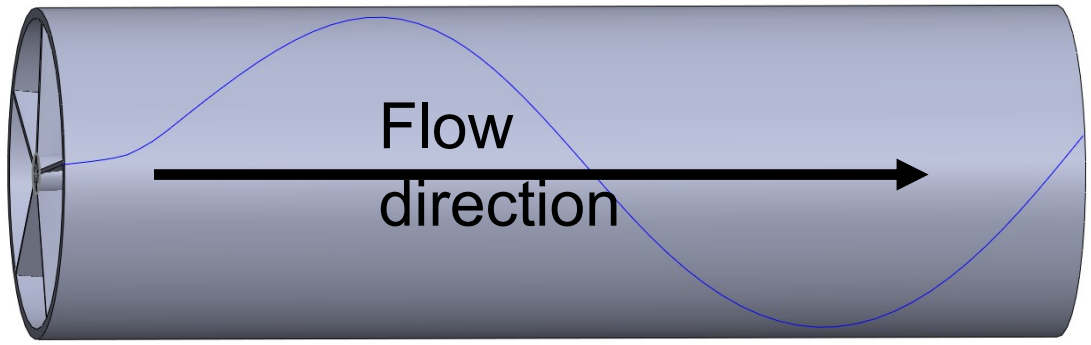
Stationary vortex generators have been included to extend eddy persistence length in straight section

generator pair



paddle wheel





Colorado State University

Arizona State University

Los Alamos NATIONAL LABORATORY

QBI Quantitative BioSciences, Inc.

QUALITAS HEALTH

Subtask 4.4 – Characterize baseline WT cultivation

Colorado State University
Arizona State University
Los Alamos NATIONAL LABORATORY
QBI Quantitative BioSciences, Inc.
QUALITAS HEALTH

Subtask 4.4: Summary

Baseline productivity and biomass quality data for WT *N. oceanica* will be obtained from an outdoor 730-L mini-pond.

Subtask 4.4: Approach

- Baseline productivity and biomass quality will be collected at QH TX farm
- 1000-L (operational depth 9”/730-L) mini-ponds will be deployed in triplicate to validate baseline data
- Daily measurements of AFWD, OD750, PAM, and microscopy, and weekly measurements of TN, PO_4, alkalinity, salinity and fatty acid profile

Colorado State University
Arizona State University
Los Alamos NATIONAL LABORATORY
QBI Quantitative BioSciences, Inc.
QUALITAS HEALTH

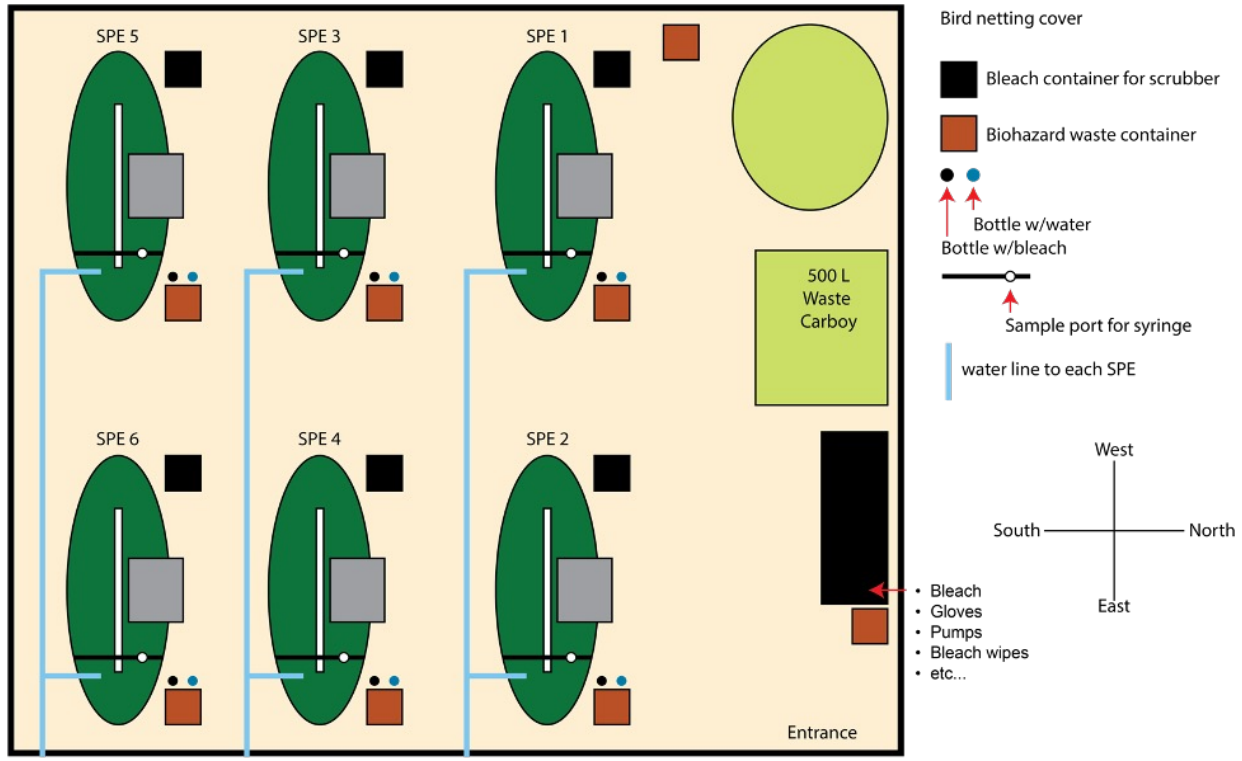
Subtask 4.5 – Characterize baseline BicA mutant cultivation

Colorado State University
Arizona State University
Los Alamos NATIONAL LABORATORY
QBI Quantitative BioSciences, Inc.
QUALITAS HEALTH

Subtask 4.5: Summary

WT *N. oceanica* and the BicA mutant will be cultivated in the spring outdoors in 4.2-m^2 raceways at a depth of 20 cm (830 L).

AzCATI Fieldsite Experimental Design

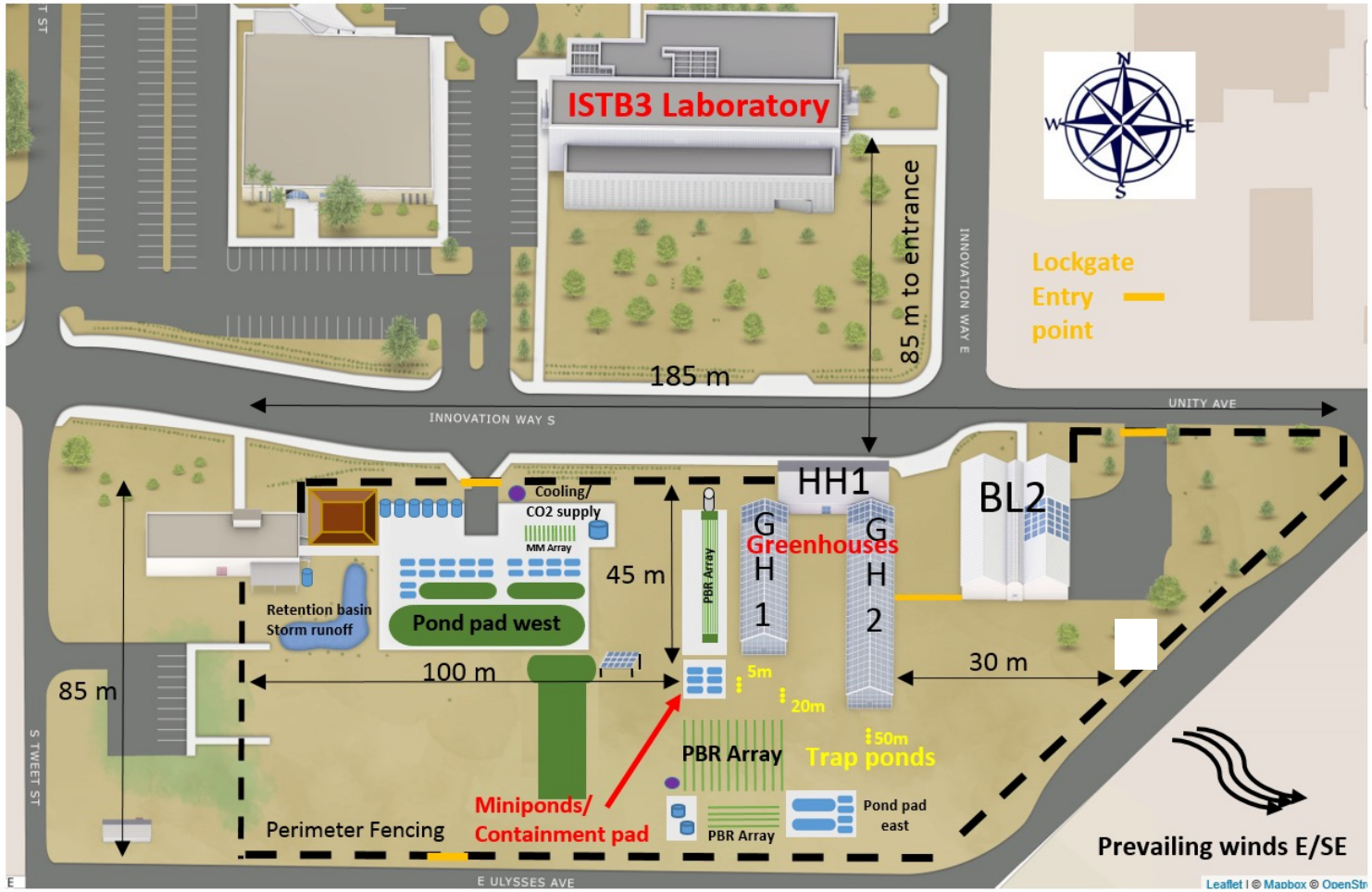


- Single pond inoculation from indoor seed train straight to outdoor ponds
- Rapid split to replicate ponds
- Trap ponds with same medium as used for cultivation arrayed around pond N-S-E-W (70L) and along prevailing winds (2x15L) – 14 total
- To minimize cross contamination between ponds while sampling:
  - sample ports with luerlocks – no human contact with pond needed
  - dedicated hoses/pumps for daily additions of water and weekly harvest/resets
  - biohazard waste containers, buckets with bleach water for scrubber when needed
  - 2x1-liter containers with bleach and water for sterilizing sample syringes and sodium hypochlorite bleach wipes on-site in real-time
  - Cultures collected on pad in storage container – bleached and disposed of per ASU biosafety protocols.

AzCATI Fieldsite Experimental Design (cont.)

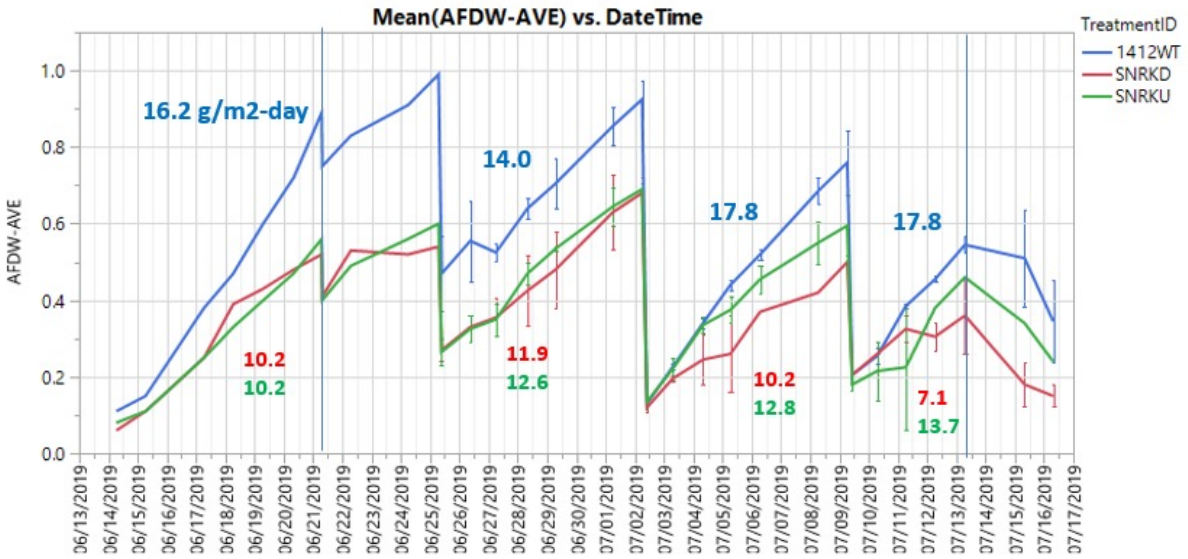
Measurement	Grab sample amount	Grab sample source	Frequency of Sampling	Place of Sampling
$OD_{750/680}$	150 mL	Same grab sample as AFDW	Daily	Containment miniponds
DW/AFDW	150 mL	Same grab sample as $OD_{750/680}$	Daily	Containment miniponds
Nutrient levels in Media (N:P)	15 mL (supernatant)	Same grab sample as DNA/ microscopy	Daily	Containment miniponds
Microscopic Observations (culture health)	15 mL (supernatant)	Same grab sample as DNA/ nutrients	Daily	Containment miniponds
Algal DNA analysis (ponds)	15 mL (pellet)	Same grab sample as Nutrients/microscopy	Daily	Containment miniponds
Proximate analysis (total FAME, Protein, and Carboydrate; ASH)	1 L	N/A	1 -3 week/ T0 and TF	Containment miniponds
Trap pond samples (AzCATI) Flow/DNA	50 mL	N/A	3 x week (M, W, F)	Containment miniponds
Trap pond samples (LANL) Flow/DNA	50 mL	N/A	3 x week (M, W, F)	Containment miniponds
In-situ YSI 5200 sensors (pH, Pond water temperature, salinity, % oxygen saturation)	N/A	N/A	15 mins	Containment miniponds
Environmental (RH, Air Temp, wind speed, wind direction, total irradiance (W/m^2), PAR	N/A	N/A	Hourly	Weather station
Precipitation	N/A	N/A	Hourly	Phx/Gateway Airport

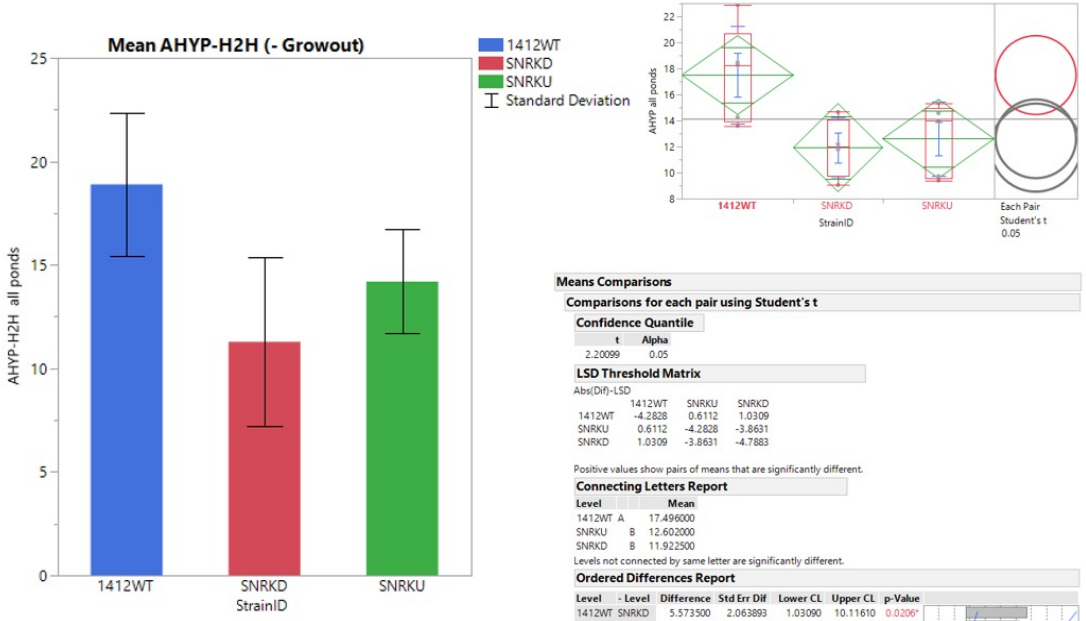
AzCATI On-site Containment Practices



Secure location, controlled access
Dedicated containment area for all outdoor operations
Additional containment requested by ASU Biosafety (bird netting)
GM algae transported to fieldsite in secondary containment

AzCATI experience with GM Outdoor, Open Release Trials





- AzCATI conducted an outdoor, open pond trial with *C. sorokiniana (DOE1412)* GM strains under PACE
- Summer run, 2019, 30 days – terminated due to culture crashing in all strains
- No significant “wandering” of GM strain as indicated by lack of detection DOE 1412 in trap ponds arrayed around site
- WT outperformed GM lines (different result than what was observed indoors or in GH at partner site)
- Major issue with that trial was difficulty in tracking transgenic – strains did NOT have an easy marker for detection/confirmation of GM.

Task 5: Initial technoeconomic and life-cycle analyses

Task 5: Summary

Sustainability modeling is a critical tool for technology assessment. Here, experimental data will be used to validate engineering process modeling work and support the evaluation of different scenarios including a baseline, conservative and optimistic. Data feedback to experimental systems will be done through identifying performance targets to meet economic goals. The work will include a direct comparison to conventional systems to understand the potential of the advancements made in this study.

Task 5 Subtasks

Subtask 5.1: Perform engineering process modeling

Subtask Summary: The foundation of the sustainability modeling work is a modular engineering process model. Existing algal models previously developed will be leveraged with improvements made based on the needs of the project. Specifically, the downstream processing will include the recovery of high-value products. The baseline model will include unit-process operations that represent the growth, harvesting, high-product recovery, fuel production, and transportation and distribution of the final fuel product.

Subtask 5.2: Conduct initial techno-economic analysis

Subtask Summary: The architecture of the economic modeling will be structured to integrate with the engineering system model. This task seeks to estimate the minimum fuel selling price of a large-scale system over the life of the facility. A Discounted Cost Flow Rate of Return (DCFROR) analysis will be leveraged, outlining the economics for each year of the project. Inputs will include capital investment, loan repayment (separated into principal and interest), operation costs, annual depreciation, annual sales, net revenue, taxable income and annual cash flows. The baseline assessment will include DOE BETO standard economic assumptions (nth plant, 10% internal rate of return, etc.).

Subtask 5.3: Conduct initial life-cycle assessment

Life cycle methodology will be used to determine the net energy ratio of the system. Net energy ratio is a holistic method for assessing the life cycle energy of the system. The net energy ratio will focus on the evaluation of the operational changes and the corresponding required improvement such that the system is energy positive.

Task 7: Advanced strain engineering and characterization

Colorado State University
Arizona State University
Los Alamos NATIONAL LABORATORY
QBI Quantitative BioSciences, Inc.
QUALITAS HEALTH

Subtask 7.1- Obtain TERA approval for new variant

Colorado State University
Arizona State University
Los Alamos NATIONAL LABORATORY
QBI Quantitative BioSciences, Inc.
QUALITAS HEALTH

Subtask 7.1: Summary

Applying experience with obtaining TERA permits from previous DOE BETO projects, an application will be prepared and submitted to the EPA for approval of the improved *N. oceanica* strain developed in Task 2.

Subtask 7.2 – Implement additional strategies for pyrenoid engineering

Colorado State University
Arizona State University
Los Alamos NATIONAL LABORATORY
QBI Quantitative BioSciences, Inc.
QUALITAS HEALTH

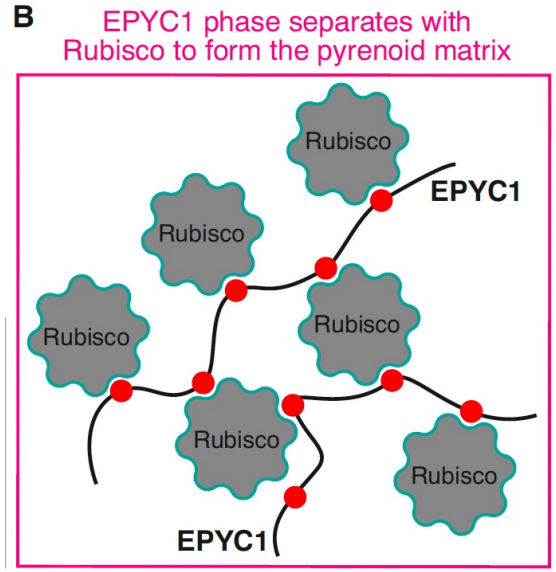
Subtask 7.2: Summary

To determine the advantages of a pyrenoid-based Carbon Concentration Mechanism (CCM), growth and lipid production will be measured under bicarbonate-limited and bicarbonate-replete conditions.

If our initial attempts at engineering a pyrenoid are unsuccessful or result in modest improvements, subsequent genetic additions of key proteins known to be associated with pyrenoid formation will be completed to increase the likelihood of assembling a functional pyrenoid-based CCM.

First Target: EPYC1

Appears to be conserved in pyrenoid-forming Heterokonts



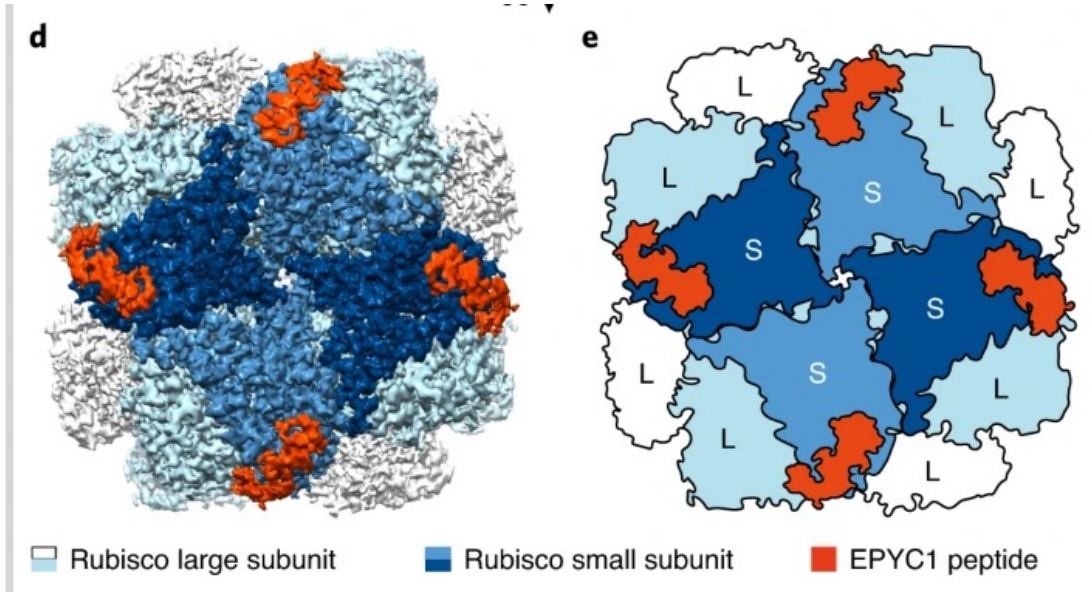
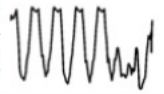
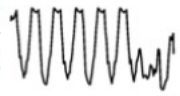


Table S6. Analysis of pyrenoid positive and pyrenoid negative algae for proteins with EPYC1-like physicochemical properties

Species (Phylum)	Pyrenoid	Number of proteins with… …>=3 repeats with a 40-80aa repeat length…	…and a pI >8…	…and an oscillating disorder profile…	…and no transmembrane domains	Uniprot or phytozome protein ID	Protein characteristics Length	Repeat length	Repeat copy #	pI	Consensus repeat sequence from Xstream	Disorder profile*
Chlamydomonas reinhardtii (Chlorophyta)	Y	18	8	1	1	Cre10.g436550 (EPYC1)	318	61	3.84	11.8	VTPSRSALPSN WKQELESLRSS SPAPASSAPAP ARSSSASWRDA APASSAPARSS SASKKA	
Thalassiosira pseudonana (Heterokontophyta)	Y	4	1	1	1	B8CF53_THAPS	376	53	6.21	9.1	LSSKPSSAPFVR SEKPSSAPSDS PSASVAPTLETS FSPSSSGQPSP MTSESPS	
Phaeodactylum tricornutum (Heterokontophyta)	Y	12	1	1	1	B7GDW7_PHATC	380	46	7.17	9.9	TGPSMTGPSDS DDRRLRSPSST GPSLTGPSMTG PSATGPSMTGP SM	
Emiliania huxleyi (Haptophyta)	Y	99	10	2	2	R1G412_EMIHU	353	70	4.10	12.1	PYLPISPARLAR GSTSPHLSPSLP ISPHISRTARSR FHIAPSLPISPHI SPTAPHGFHEA PHLPISPHLS	
						R1D601_EMIHU	255	60	3.70	10.1	WTAADDALVKA GQEAGESWVDI AKRLPGRSADS VKSRSNRLKRQ PDTSVKHEPVK RELVR	
Ostreococcus tauri (Chlorophyta)	Y/N†	3	3	2	2	A0A096PAN3_OSTTA	407	63	5.02	11.1	MAASKLGSKNA STRPTVGSTLD ASALTPPSLRFT TENNIHSVPTAF GVADRPASRRV LRREDA	
						A0A090M8K8_OSTTA	470	63	6.02	11.2	MAASKLGSKNA STRPTVGSTLD ASALTPPSLRFT TENNIHSVPTAF GVADRPASRRV LRREDA	
Micromonas pusilla (Chlorophyta)	Y	6	0	0	0							
Chlorella variabilis (Chlorophyta)	Y	3	2	1	0							
Chlorella prototheocoides (Chlorophyta)	N	1	0	0	0							
Cyanidioschyzon merolae (Rhodophyta)	N	0	0	0	0							
Galdieria sulphuraria (Rhodophyta)	N	2	0	0	0							
Nannochloropsis gaditana (Heterokontophyta)	N	3	0	0	0							

*Disordered profiles are a plot of disorder propensity (y axis; 0-1; 0 = ordered, 1 = disordered) against amino acid number (x-axis; 0-437). All profiles are on the same scale. †TEM images of *Ostreococcus tauri* show a singular starch deposit typical of a pyrenoid, however a Rubisco matrix has yet to be confirmed (71).

Colorado State University · Arizona State University · Los Alamos National Laboratory · QBI Quantitative BioSciences, Inc. · Qualitas Health

Subtask 7.3 – Improve biomass quality using transgenic approaches

Colorado State University
Arizona State University
Los Alamos NATIONAL LABORATORY
QBI Quantitative BioSciences, Inc.
QUALITAS HEALTH

Subtask 7.4 – Develop toolkit for episomal-based control of gene expression

Colorado State University
Arizona State University
Los Alamos NATIONAL LABORATORY
QBI Quantitative BioSciences, Inc.
QUALITAS HEALTH

Task 8: Concept integration and demonstration

Subtask 8.1 – Evaluate sensors and cultivation technologies with WT strain

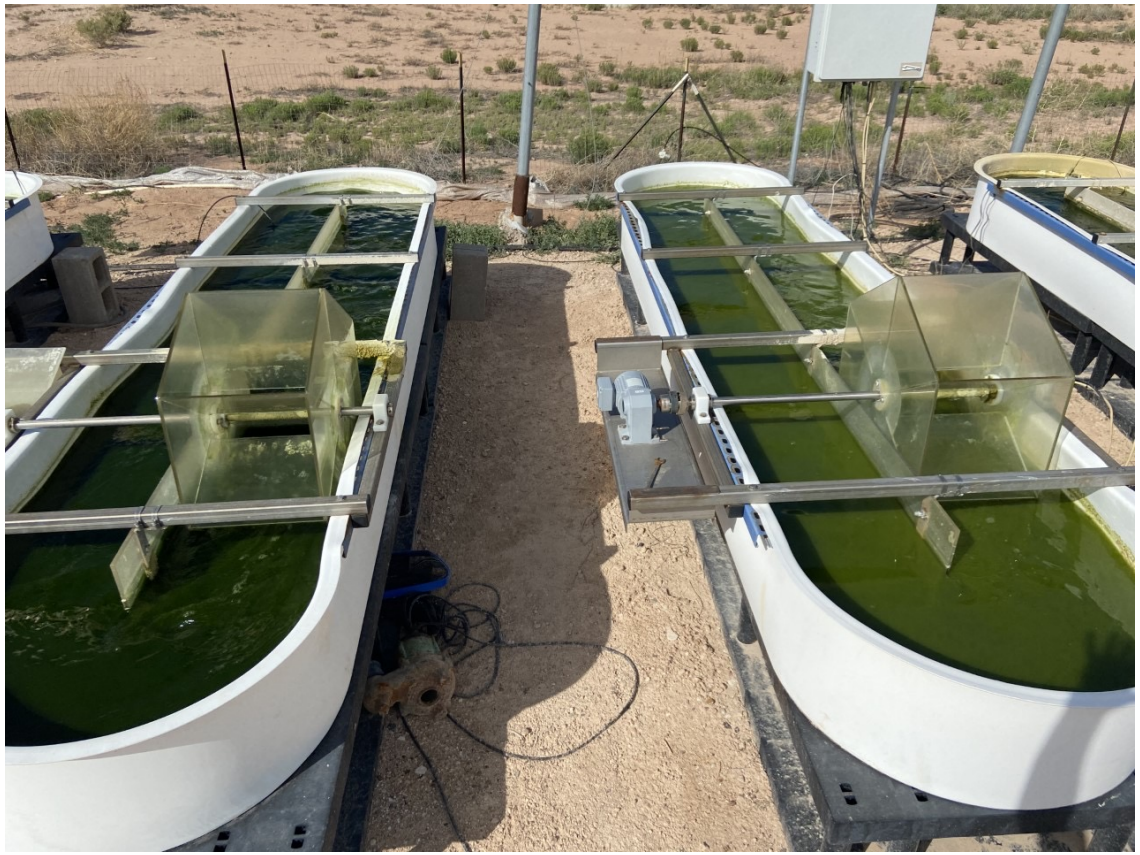
Colorado State University
Arizona State University
Los Alamos NATIONAL LABORATORY
QBI Quantitative BioSciences, Inc.
QUALITAS HEALTH

Subtask 8.1: Summary

Cultivations of WT *N. oceanica* will be conducted in the spring. Biomass productivity and quality data will be obtained from an outdoor 730-L mini-pond.

Subtask 8.1: Approach

- Same operation parameters and sampling regime as Subtask 4.4
- Sensors will be deployed in triplicate ponds, while still maintaining triplicate control ponds for comparison
- Higher resolution at the biomass density/growth rate level will inform more real-time harvesting decisions to maintain optimal growth and achieve higher productivity
- On-demand feeding will be evaluated with nutrient sensor to investigate whether a crop protection benefit, and thus productivity, benefit can be achieved versus maintaining replete nutrient conditions in the controls



Colorado State University | Arizona State University | Los Alamos National Laboratory | QBI Quantitative BioSciences, Inc. | Qualitas Health

Subtask 8.2 – Evaluate strains, sensors, and cultivation technologies

Colorado State University
Arizona State University
Los Alamos NATIONAL LABORATORY
QBI Quantitative BioSciences, Inc.
QUALITAS HEALTH

Subtask 8.2: Summary

Cultivations of the WT, BicA mutant, and Task 2 *N. oceanica* mutants will be performed in the spring. Cultivations will be performed in standard 4.2-m^2 raceways at a depth of 20 cm (830 L).

Subtask 8.2: Approach

- Each sensor system will be deployed at locations guided by the simulations of Subtask 4.3 and measurements compared to off-line assay
  - AzCATI has significant experience in deploying novel sensor tech
- Ponds will be operated in the conditions predicted for optimal productivity from Subtask 4.1
- Performance of the WT and previous mutant will be compared to values determined in Subtask 4.5 to evaluate the influence of the operating conditions
- The performance of the previous mutant and selected Task 2 mutant will be compared to values of the WT

Qube deployment at AzCATI (other DOE project)

Colorado State University | Arizona State University | Los Alamos National Laboratory | QBI Quantitative BioSciences, Inc. | QUALITAS HEALTH

Task 9: Final technoeconomic and life-cycle analyses

Task 9: Summary

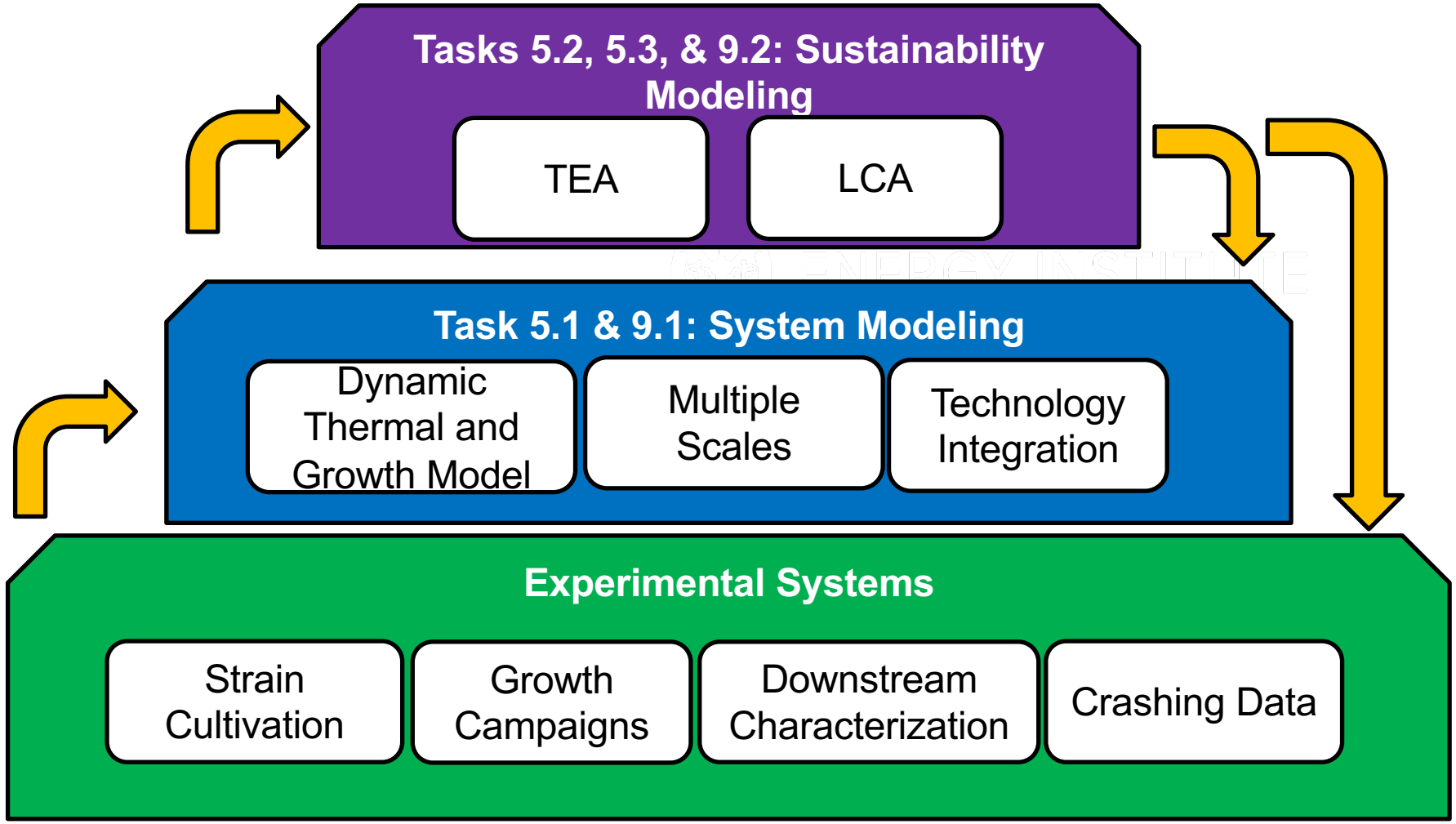
The foundational engineering process model will continually be updated with experimental data. Concurrently, alternative processing scenarios based on experimental work will be modeled. The exact unit process operations will be defined by the results from the experimental work. At a minimum, multiple fuel pathways including the production of heavy marine grade and aviation fuel pathways will be modeled.

Task 9 Subtasks

Subtask 9.1: Process modeling (M22-M36)
Subtask Summary: The process modeling is the core of the sustainability work. Modularity will support the evaluation of alternative processing pathways. Additionally, an existing growth model will continue to be used and validated with the most recent growth data. This work will enable the evaluation of the system at geographically diverse locations.

Subtask 9.2: Concurrent techno-economic and life cycle assessment modeling (M22-M36)
Subtask Summary: The modeling work competed in the previous budget period will be expanded to include multiple scenarios. The work will include defining a pathway that meets the DOE goal of achieving a minimum fuel selling price of $3 GGE-1 while meeting the renewable fuel standard. The effort will include the evaluation of alternative scenarios including carbon economy with results from LCA used with TEA modeling and a carbon tax to understand the economic viability under this policy scenario.
LCA will be performed to determine whether energy and emissions are consistent with that required to produce an advanced biofuel product based on the renewable fuel standard. Life cycle modeling will use standard life cycle assessment methods demonstrated by Quinn through previous work. The engineering process model will be coupled with life cycle inventory data to understand the net energy ratio and global warming potential of the process. Nine additional LCA capacities will be integrated based on TRACI 2.1 methods and corresponding impact categories.

Task 9: Approach



Colorado State University

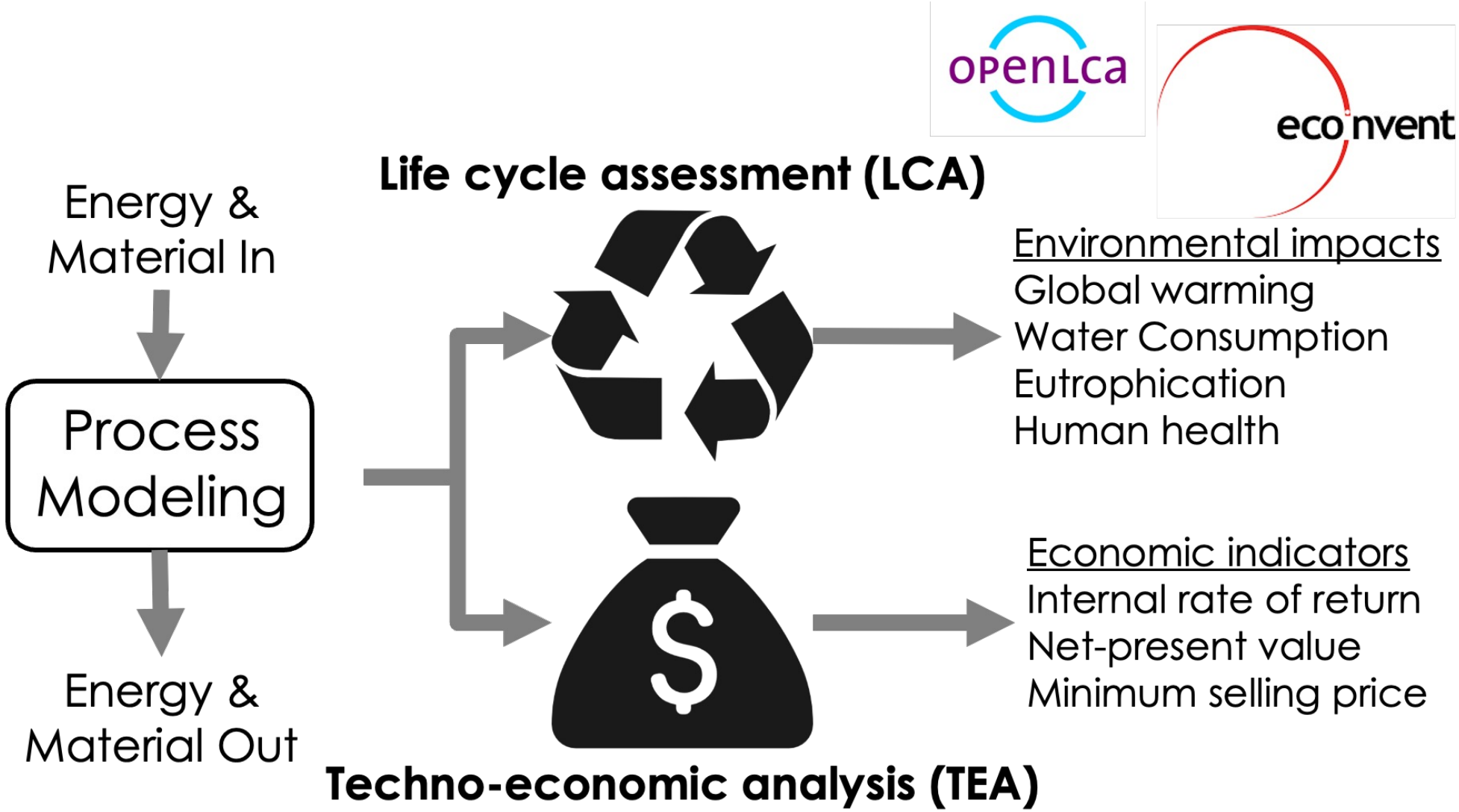
Arizona State University

Los Alamos NATIONAL LABORATORY

QBI Quantitative BioSciences, Inc.

QUALITAS HEALTH

Task 9: Approach



Colorado State University · Arizona State University · Los Alamos National Laboratory · QBI Quantitative BioSciences, Inc. · Qualitas Health