DOE Bioenergy Technologies Office (BETO)
2019 Project Peer Review

High-Throughput Directed Evolution of Microalgae and Phototrophic Consortia for Improved Biomass Yields

March 2019
Advanced Algal Systems

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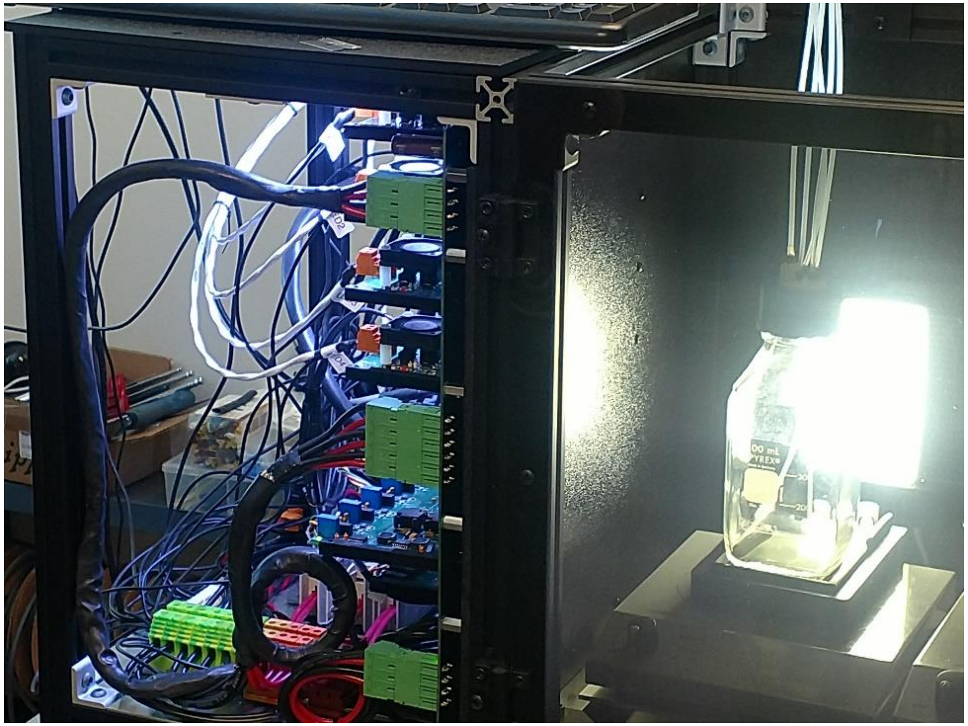
Collaboration with Pacific Northwest National Laboratory, Colorado State University and Global Algae Innovations

Goal Statement

- Use directed evolution approaches as a tool for the improvement of photoautotrophic microorganism biomass yields.
- Leverage strains grown by Global Algae Innovations (GAI) to further improve productivities by enabling improved tolerance to higher pH, O_2 concentrations and temperature.
- Directed evolution has a long and successful history in industrial microbiology but has not been leveraged extensively with microalgae.
- Ultimately select strains/consortia that attain 24 $g/m^2/day$ in the spring growing season in Kauai.

Goal Statement



Stress adaptation foci

- *High Light*
- *High pH*
- *High Oxygen Stress*
- *High Temp*
- *Rapid Growth*

Lee DH, Palsson BO: Adaptive evolution of Escherichia coli K-12 MG1655 during growth on a non-native carbon source, L- 1,2-propanediol. Appl Environ Microbiol 2010, 76:4158-4168.

Hu H, Wood TK: An evolved Escherichia coli strain for producing hydrogen and ethanol from glycerol. Biochem Biophys Res Commun 2010, 391:1033-1038.

Balderas-Hernandez VE, Hernandez-Montalvo V, Bolivar F, Gosset G, Martinez A: Adaptive evolution of Escherichia coli inactivated in the phosphotransferase system operon improves co-utilization of xylose and glucose under anaerobic
conditions. Appl Biochem Biotechnol 2010, 163:485-496.

Wang Y, Manow R, Finan C, Wang J, Garza E, Zhou S: Adaptive evolution of nontransgenic Escherichia coli KC01 for improved ethanol tolerance and homoethanol fermentation from xylose. J Ind Microbiol

Cakar ZP, Seker UO, Tamerler C, Sonderegger M, Sauer U: Evolutionary engineering of multiple-stress resistant Saccharomyces cerevisiae. FEMS Yeast Res 2005, 5:569-578.

Yomano LP, York SW, Ingram LO: Isolation and characterization of ethanol-tolerant mutants of Escherichia coli KO11 for fuel ethanol production. J Ind Microbiol Biotechnol 1998, 20:132-138.

Kwon YD, Kim S, Lee SY, Kim P: Long-term continuous adaptation of Escherichia coli to high succinate stress and transcriptome analysis of the tolerant strain. J Biosci Bioeng 2011, 111:26-30.

Agrawal M, Mao Z, Chen RR: Adaptation yields a highly efficient xylose-fermenting Zymomonas mobilis strain. Biotechnol Bioeng 2010, 108:777-785.

Liu ZL: Genomic adaptation of ethanologenic yeast to biomass conversion inhibitors. Appl Microbiol Biotechnol 2006, 73:27-36.

Quad Chart Overview

Timeline

- Project start date: August 15, 2018
- Project end date: May 14, 2021
- Percent complete: 5%

	Total Costs Pre FY17**	FY 17 Costs	FY 18 Costs $105,504	Total Planned Funding (FY 21-Project End Date) $2,732,421
DOE Funded	**0**	**0**	**$45,213 (43%)** **$15,041 PNNL (14%)**	**$1,919,178 (70%)** **$540,000.00 PNNL (20%)**
Project Cost Share*	**0**	**0**	**$45,250 (43%)**	**$273,243 (10%)**

Barriers addressed

This project specifically addresses BETO MYPP challenges in algal **Biomass Availability/Cost** (Aft-A).

Objective

Use directed evolution to improve biomass yields by improving pH, O_2 and temperature tolerances in high bicarbonate, high pH media (GAI proprietary).

End of Project Goal

Increase biomass yields by ~20% above background strains in GAI high bicarbonate/high pH media. Attain 24 $g/m^2/day$ algal biomass at the GAI facility in Kauai during the spring growing season.

1 - Project Overview

- *GAI has isolated a diatom that is among their superior outdoor strains at the Kauai growth facility. Further improvements in biomass and lipid yield will benefit biofuel applications.*
- *Yields presently suffer putatively due to high O_2 levels during periods of high productivity, increases in pH as bicarbonate is assimilated and high temperatures during part of the day.*
- *Both PNNL and Mines have established expertise in building photobioreactors that can mimic the solar day in terms of light intensities and temperatures, including custom turbidostat technology built at PNNL.*
- *Cells can be cultured with increased O_2/pH/temperature pressures to select more robust strains under "domesticated conditions".*
- *Starting with an already promising strain, the goal is to further improve yields ~20%.*
- *Additional strains (e.g. cyanobacteria, algae) that grow well in GAI media will also be grown with selective pressures to mitigate risk.*
- *Establish organism consortia.*

2 – Approach (Management)

- *Mines is the overall lead collaborating extensively with PNNL, CSU and GAI.*
- *Mines/PNNL are responsible for the directed evolution of GAI-229 (diatom) and Nannochloropsis.*
- *PNNL is responsible for the directed evolution of cyanobacteria and photoautotroph consortia.*
- *GAI is responsible for outdoor growth trials.*
- *CSU is responsible for geographically resolved TEA and LCA.*
- *Weekly teleconference meetings are held to discuss data and research thrusts.*
- *Annual in person meetings held to assess progress and new directions.*

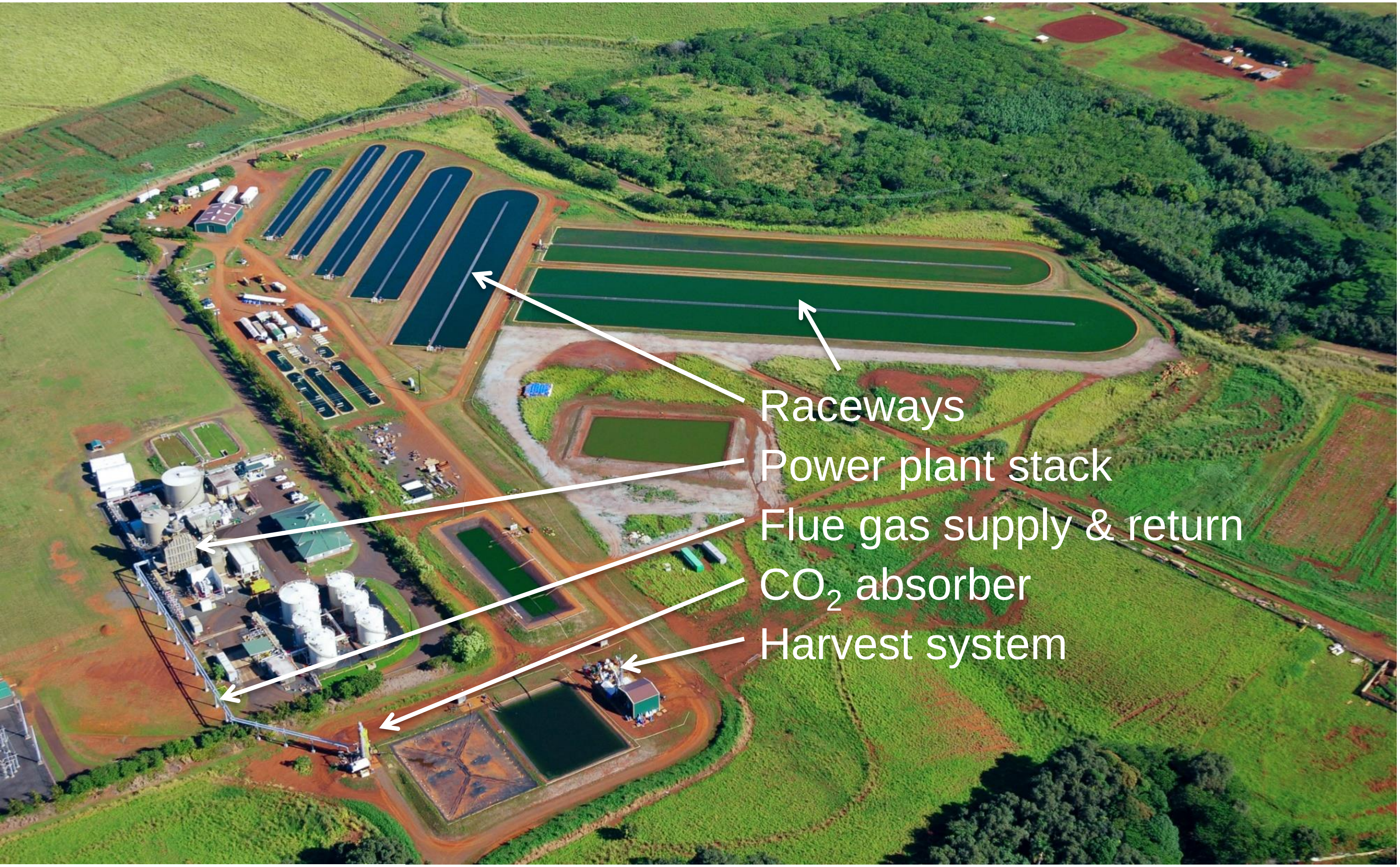
2 – Approach (Technical)

- *Technical approach*
  - *Grow GAI-229 (and mitigation strains) cultured in solar simulating bioreactors (high light).*
  - *Escalate thermal pressures to slightly exceed the highest temperatures recorded at the GAI facility.*
  - *Grow strains at (and slightly above) the highest O_2 levels recorded at the GAI growth facility for extended periods.*
  - *Evolve and probe additional strains for growth in GAI media.*
  - *Assemble and evaluate productivities of photoautotroph consortia.*
  - *Test evolved strains outdoors at the GAI growth facility.*
  - *Develop TEA/LCA model of evolved strains at GAI growth facility.*

- *Potential challenges include*
  - *Inability of strain(s) to adapt to the applied selective pressures.*
  - *Maintaining evolved strains.*

- *Critical success factors*
  - *GAI is already an established algal biomass provider. Improved biomass yields will benefit existing business applications.*

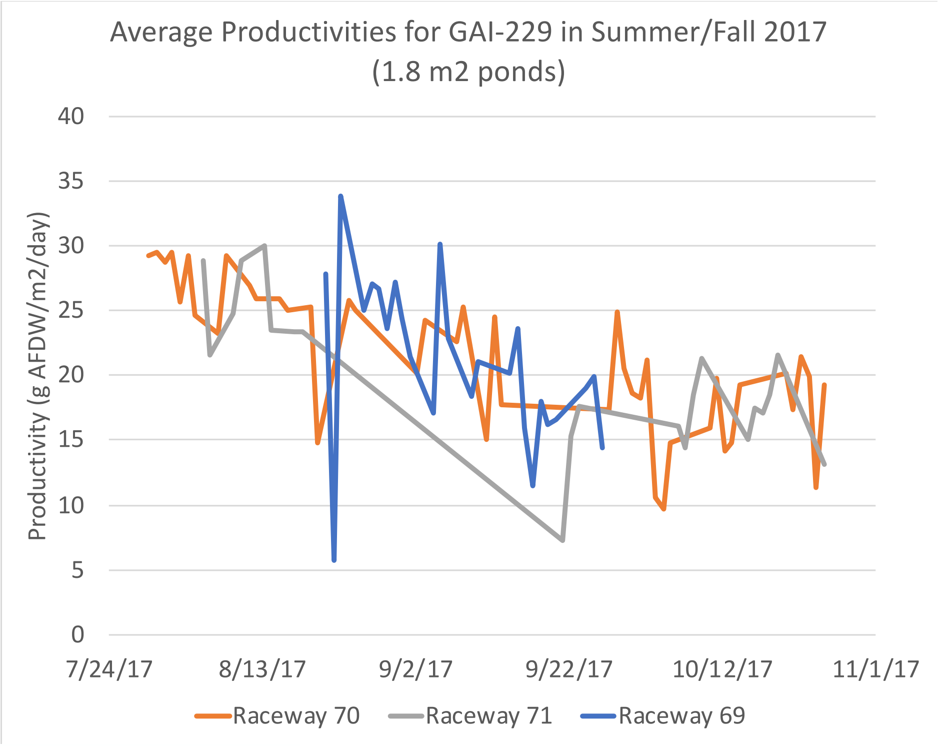
Algae cultivated on CO_2 supplied from power plant flue gas since June 2014



GAI-229

GAI-229 (*Nitzschia sp.*, a diatom) is one of Global Algae's primary production strains that grows well in GAI cultivation conditions. GAI-229 is a great producer of lipid, particularly omega-3 EPA, and is our baseline strain for this project as well as other Global Algae DOE projects.

Summer/Fall from 7/29-10/25 productivity of ~21 $g/m^2/day$.



GLOBAL ALGAE

GAI-301 Kauai isolated coccoid cyanobacterium outdoors.

Ran 12/6-12/22/2018

Productivity reached as high as 12.87 $g/m^2/day$ in December

Further outdoor trials and lab experiments will aid in optimizing. Overall, the first trial was an exciting indicator that this organism is promising

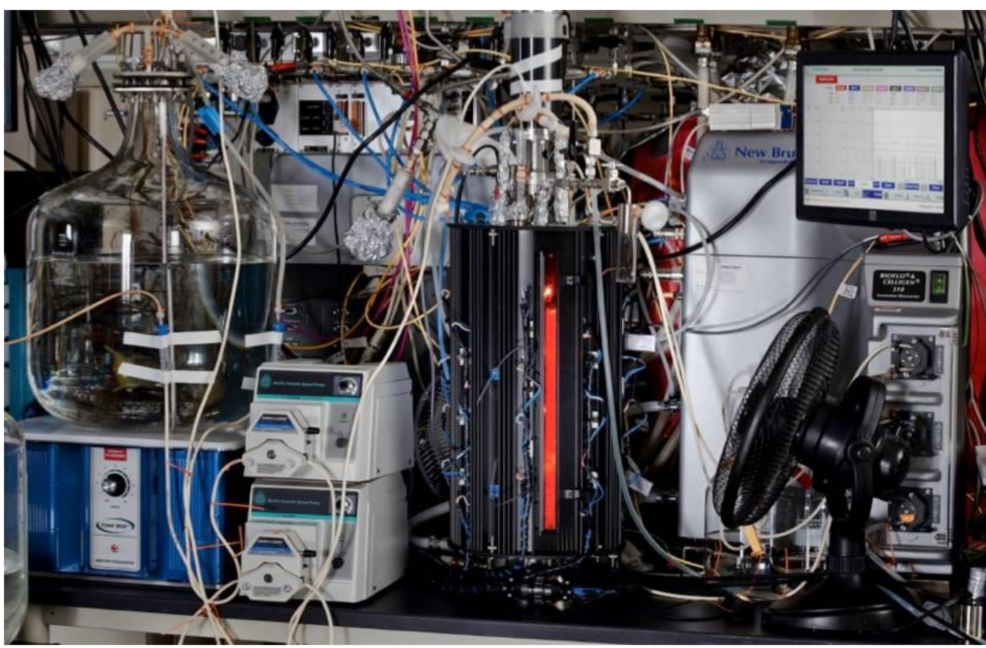
GLOBAL ALGAE

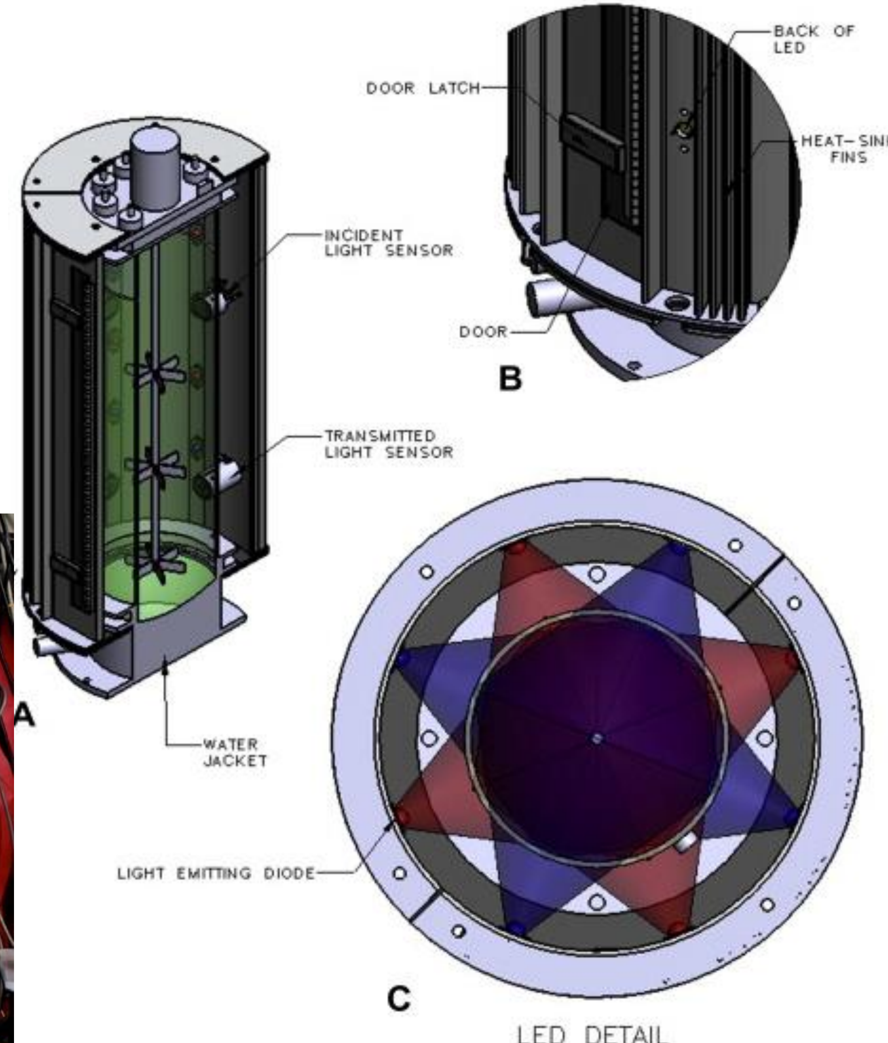
3 – Technical Accomplishments/ Progress/Results

- *Kickoff meeting at GAI algal farm in Kauai in August 2018*
- *Transfer of GAI229 to Mines and PNNL*
- *Successful cultivation of GAI229 in all laboratories*

3 – Technical Accomplishments/ Progress/Results: GAI-229

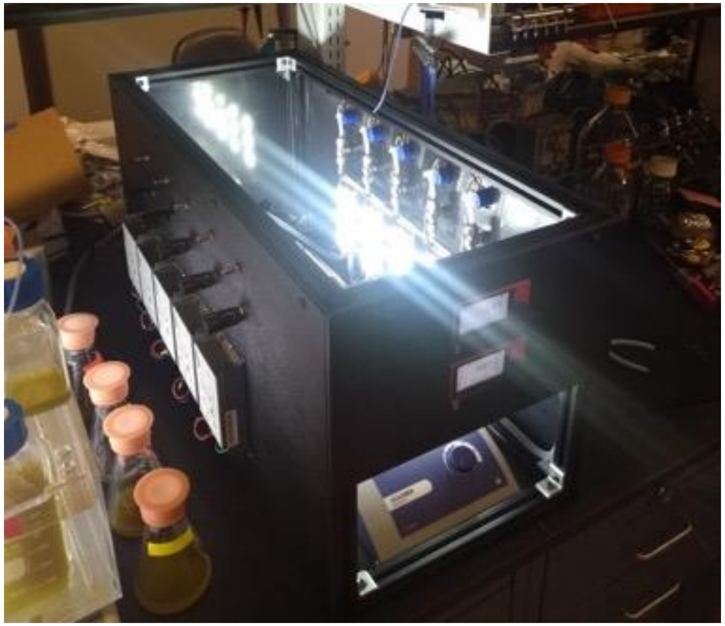
- *Both Mines and PNNL successfully culturing GAI229*
- *1st Milestone of 2 g/L biomass density attained on schedule*
- *Mapping pH optimum of GAI229 during dilute growth in PNNL turbidostat.*





3 – Technical Accomplishments/ Progress/Results: diatom

- *Both Mines and PNNL successfully culturing GAI229*
- *Multiple parallel stress tests with high throughput automated bioreactors*
- *pH, O_2, temperature are the primary pressures*



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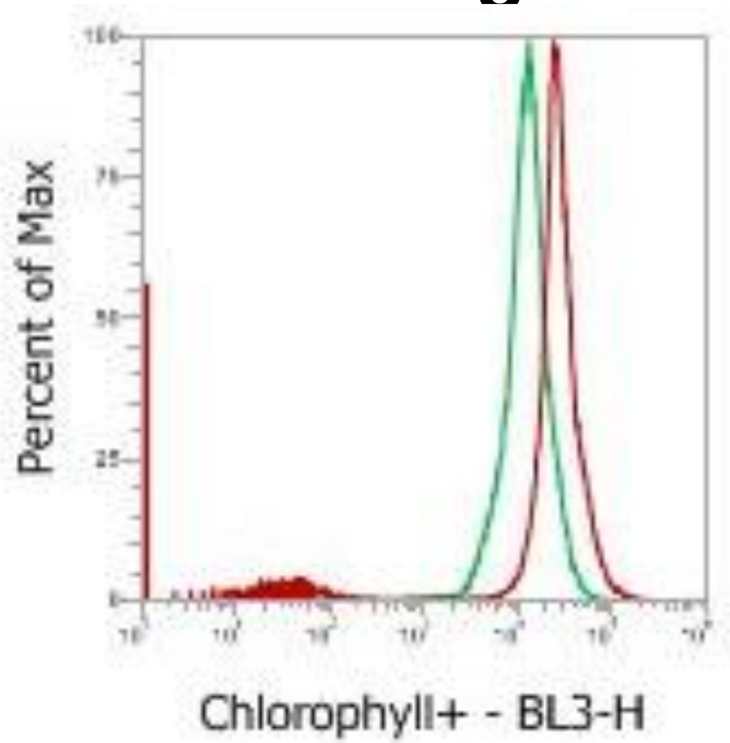
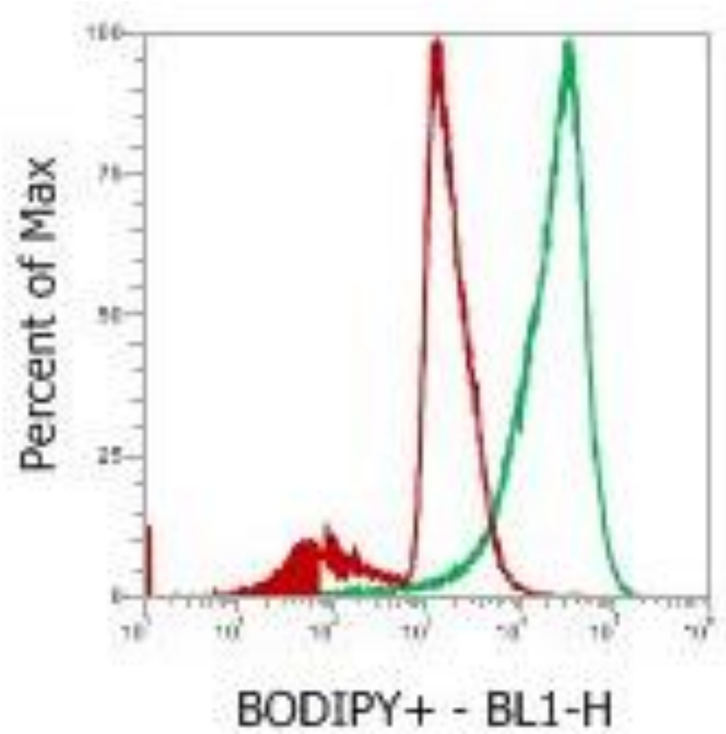


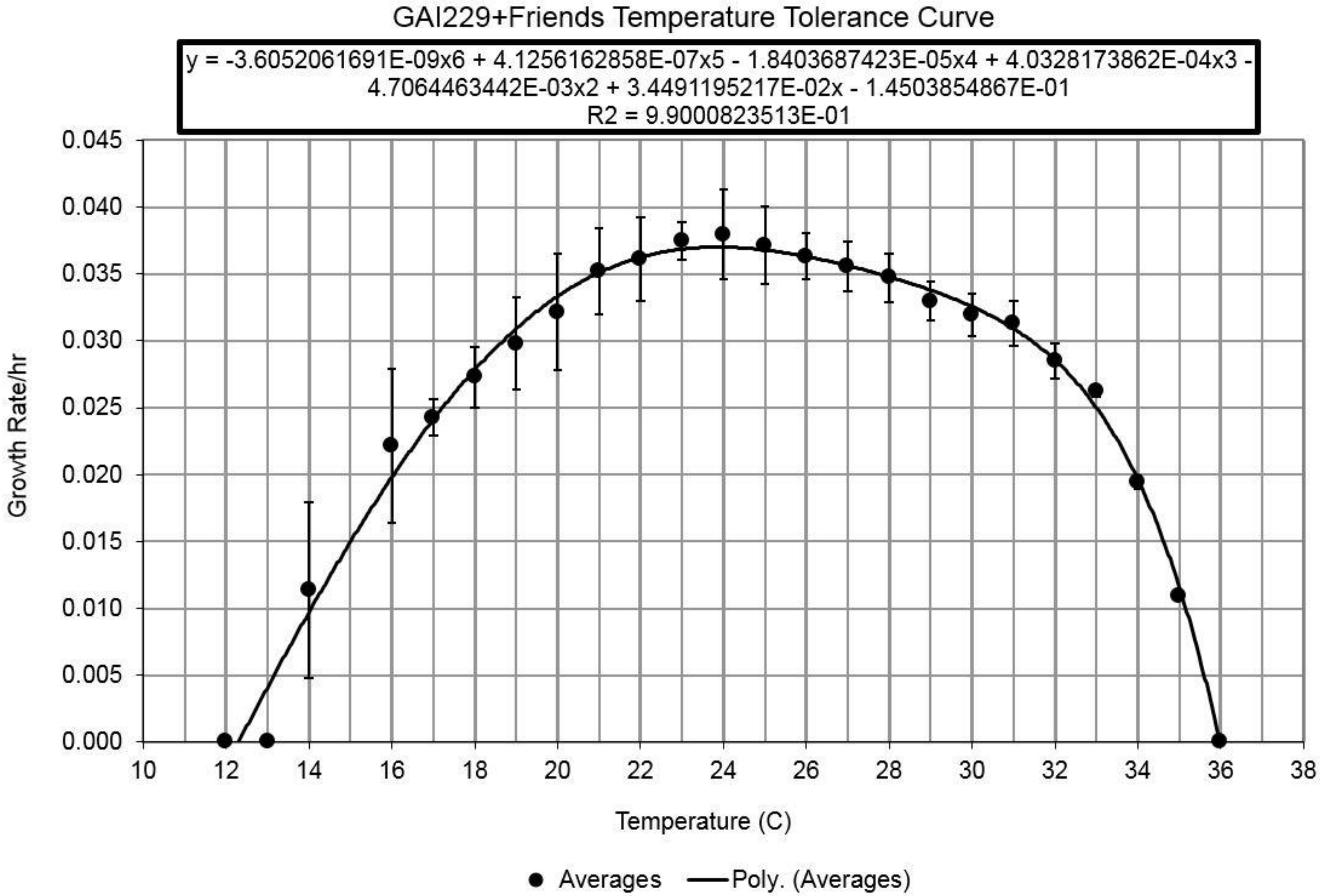


Green = Nutrient limited flask
Red = Nutrient replete bottle without bubbling

Rapid oil/chlorophyll by cell cytometry

3 – Technical Accomplishments/ Progress/Results: diatom

- *Both Mines and PNNL successfully culturing GAI229*
- *1st Milestone of 2 g/L biomass density attained on schedule*
- *Mapping temp/pH/O_2 optima of GAI229 during dilute growth in PNNL turbidostat.*



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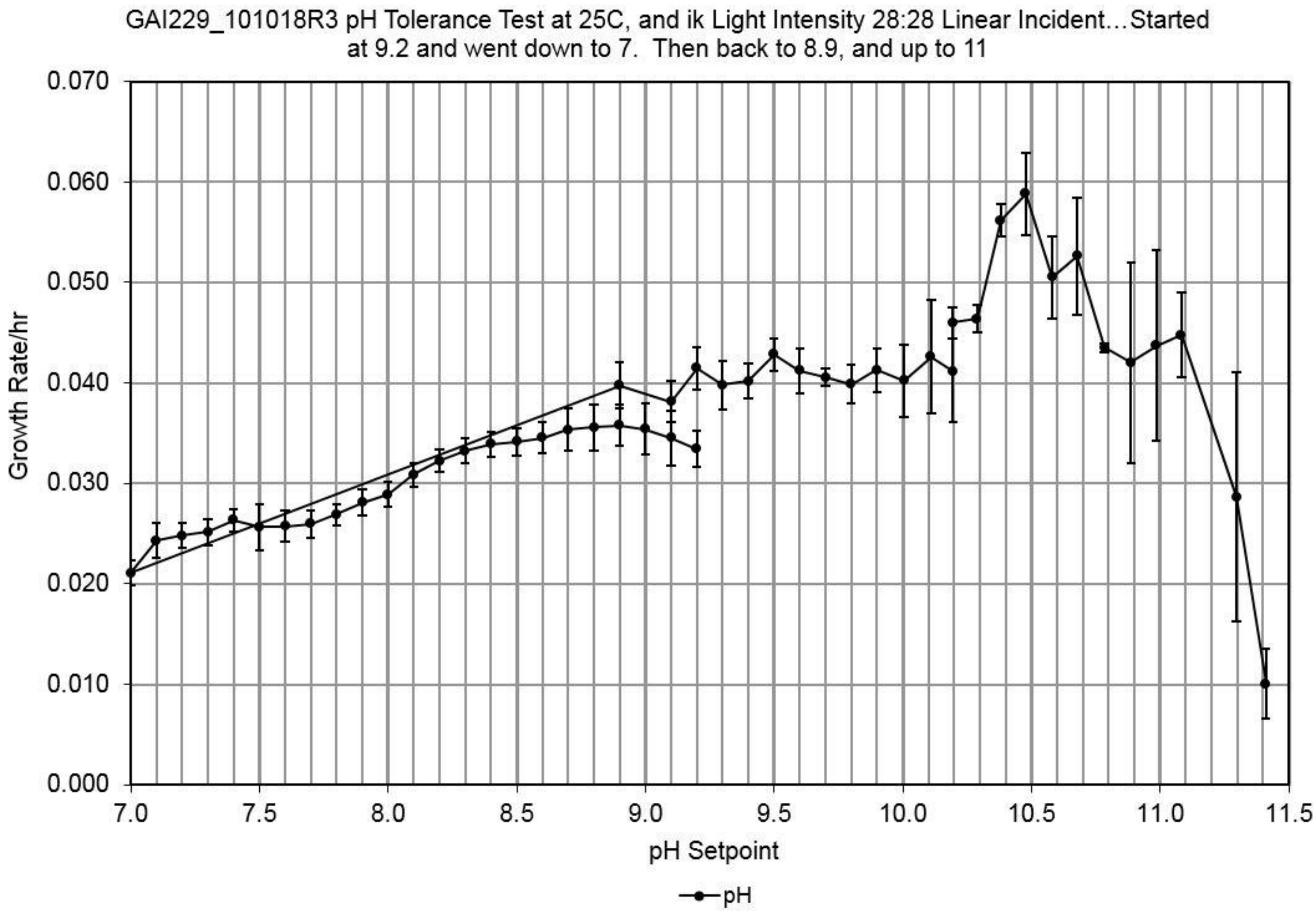
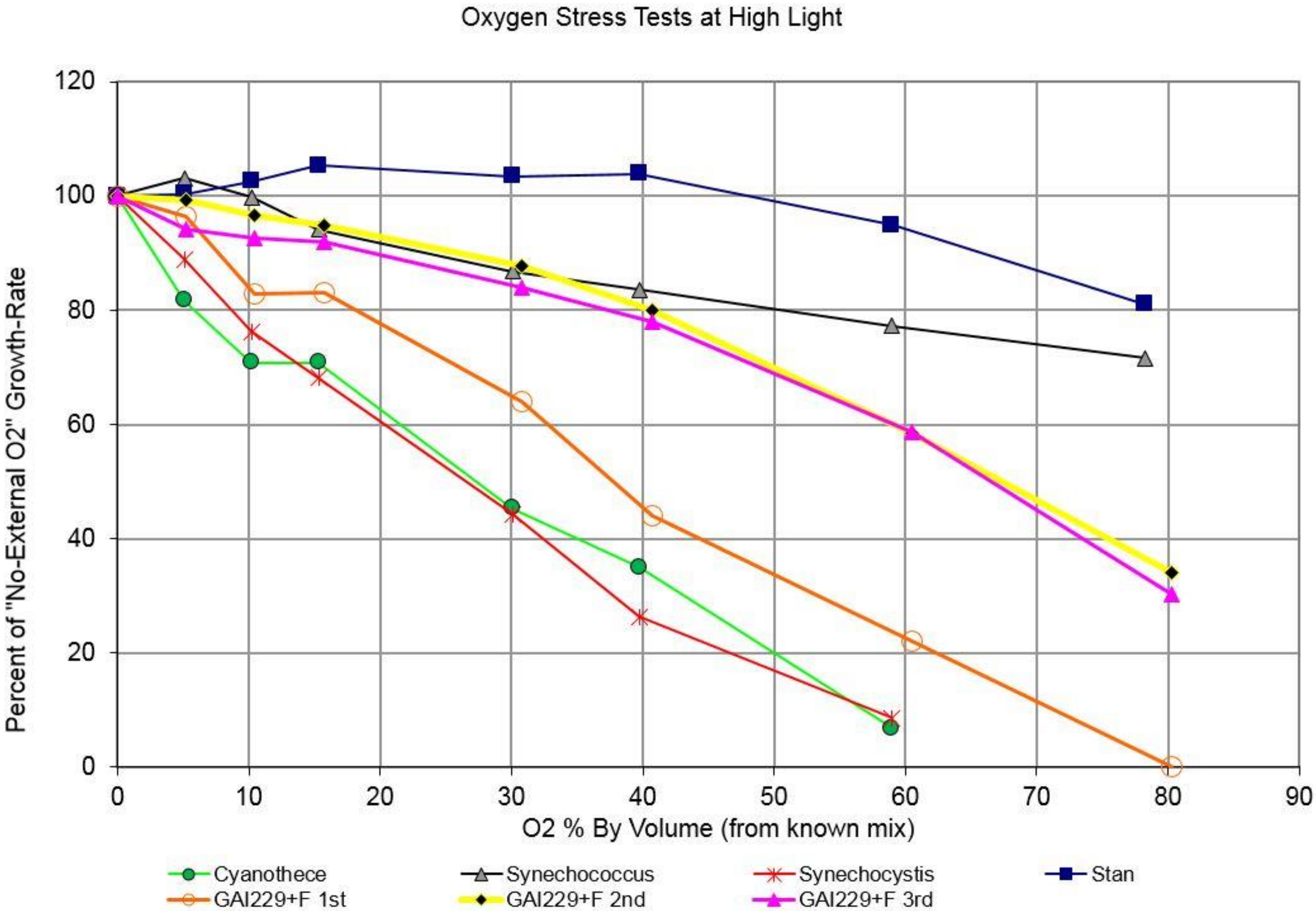


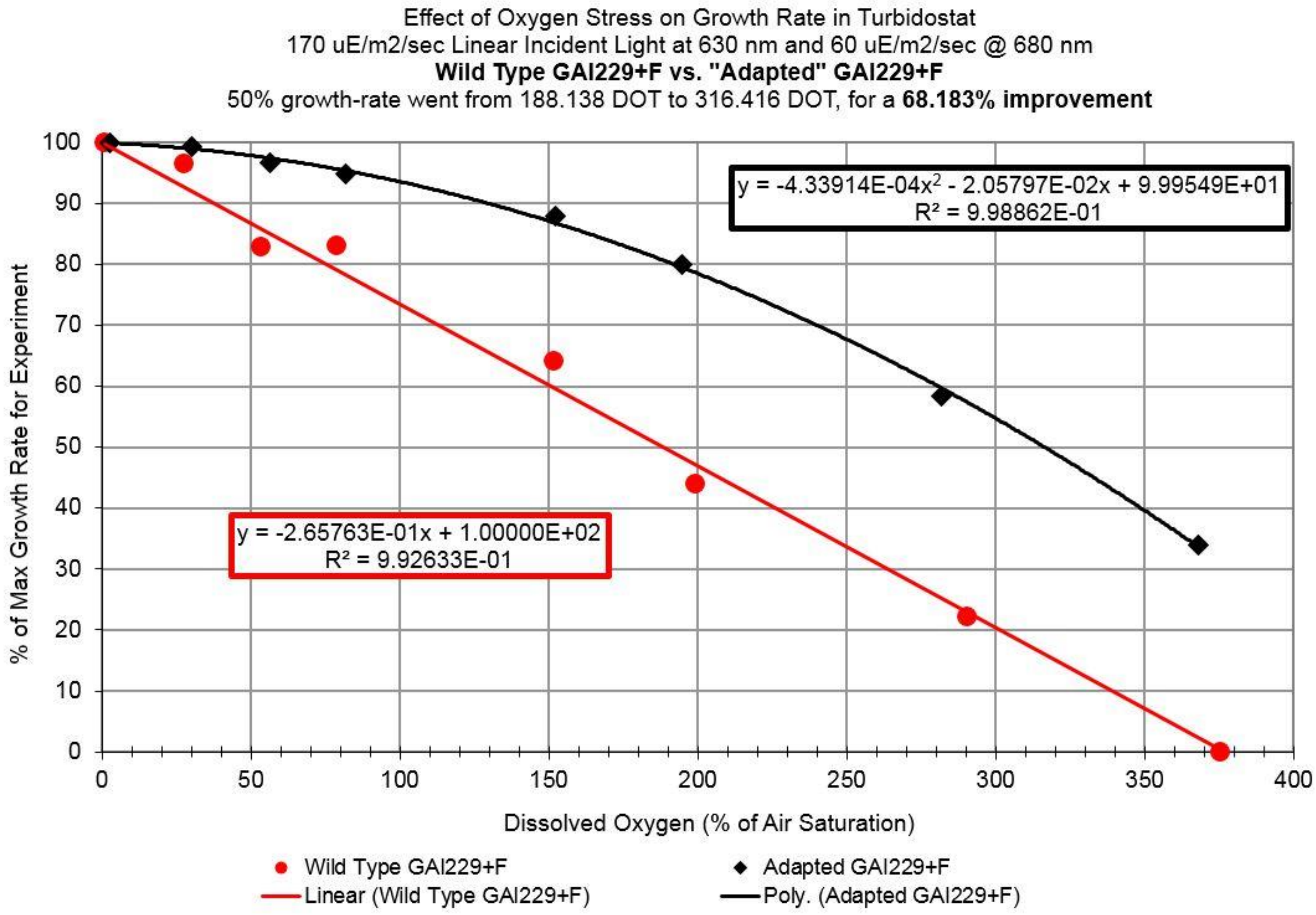
Mapping pH optimum

3 – Technical Accomplishments/ Progress/Results: diatom



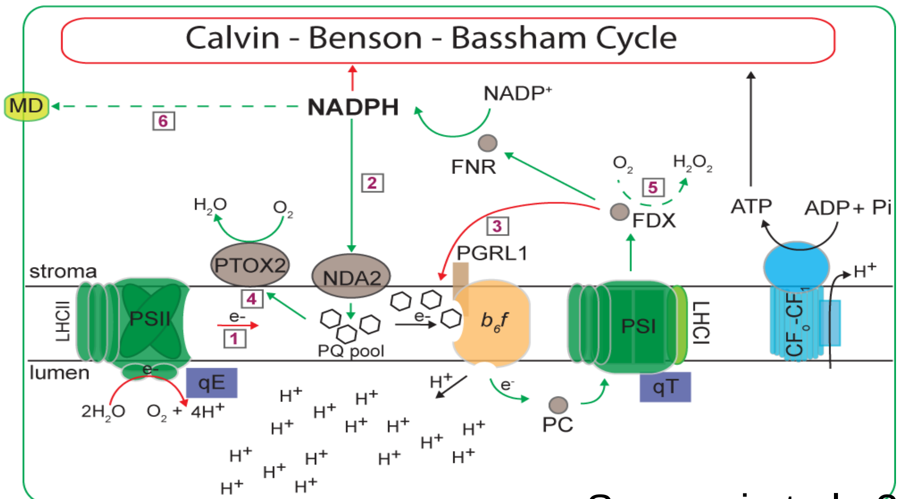
Multiple O_2 stress tests are leading to increased O_2 tolerance

3 – Technical Accomplishments/ Progress/Results: diatom

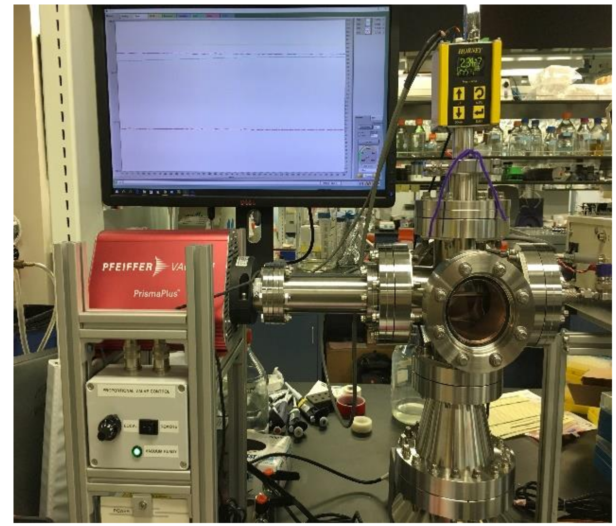


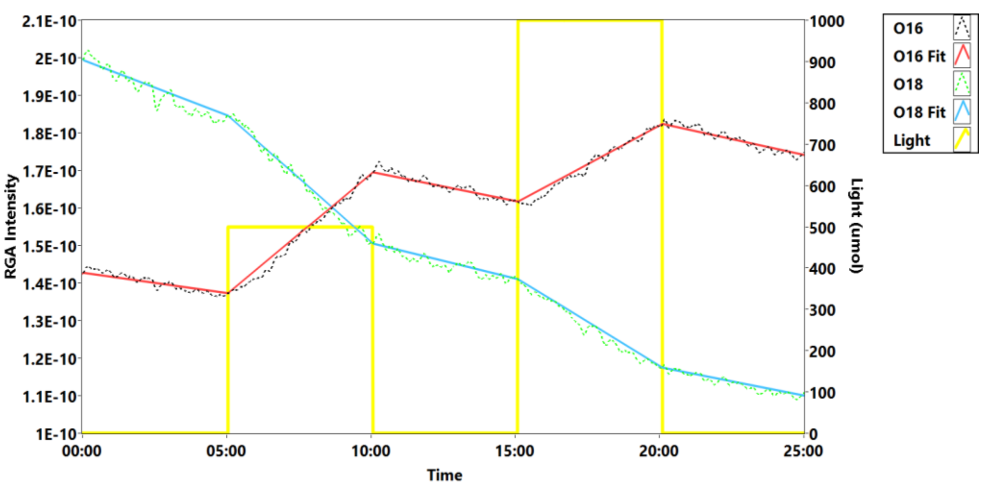
Enhanced O_2 tolerance

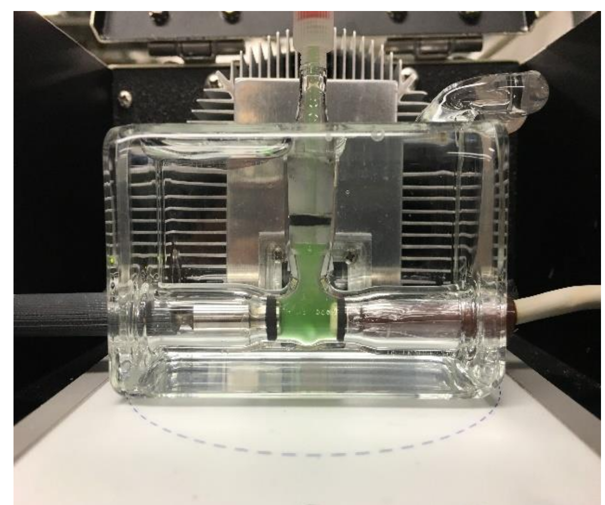
Enhanced O_2 Reduction in the Light



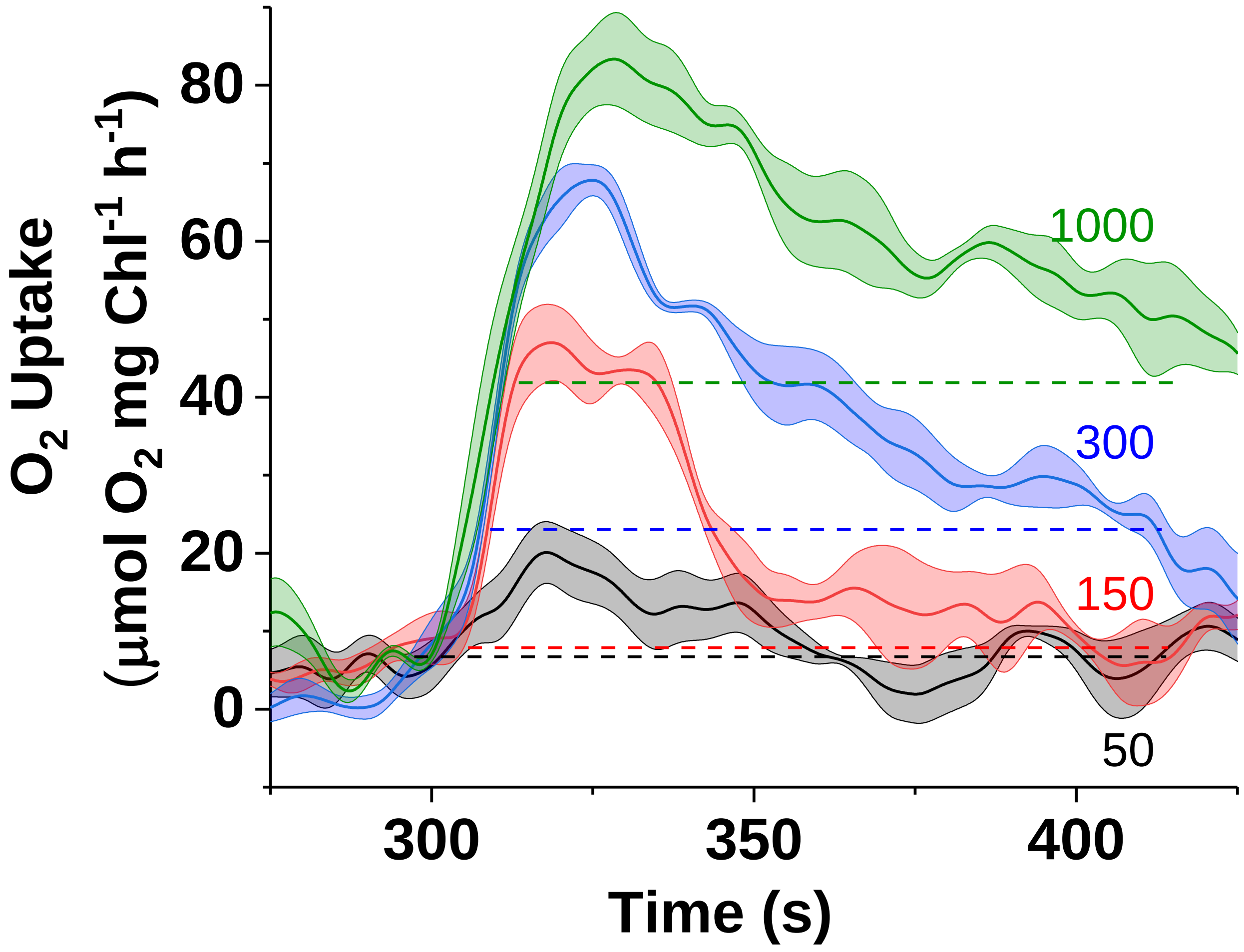
Saroussi et al., 2016





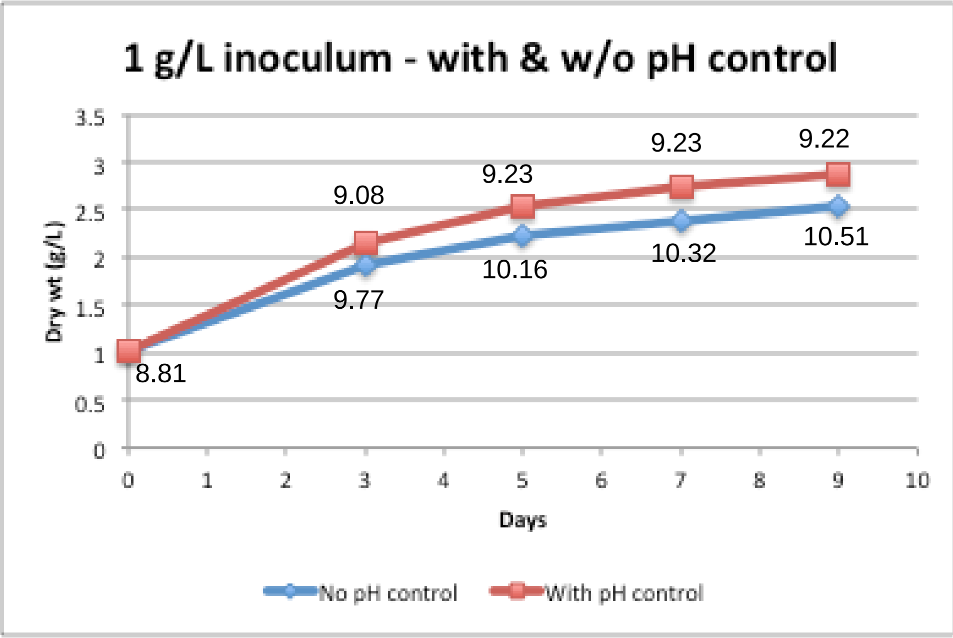


O_2 Reduction in the Light

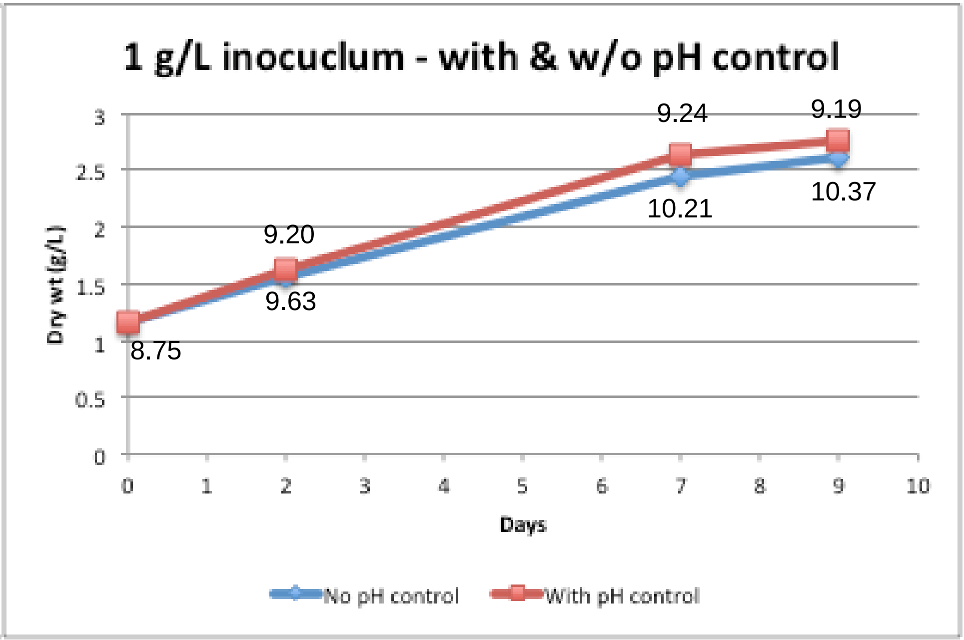


3 – Technical Accomplishments/ Progress/Results: diatom

Trial 1



Trial 2



Trial 1

Dry Wt (Inoculum size with & w/o pH Control low light)

	0	3	5	7	9
No pH control	1.01	1.92	2.22	2.39	2.55
With pH control	1.01	2.14	2.53	2.74	2.86

Trial 2

Dry Wt (Inoculum size with & w/o pH Control low light)

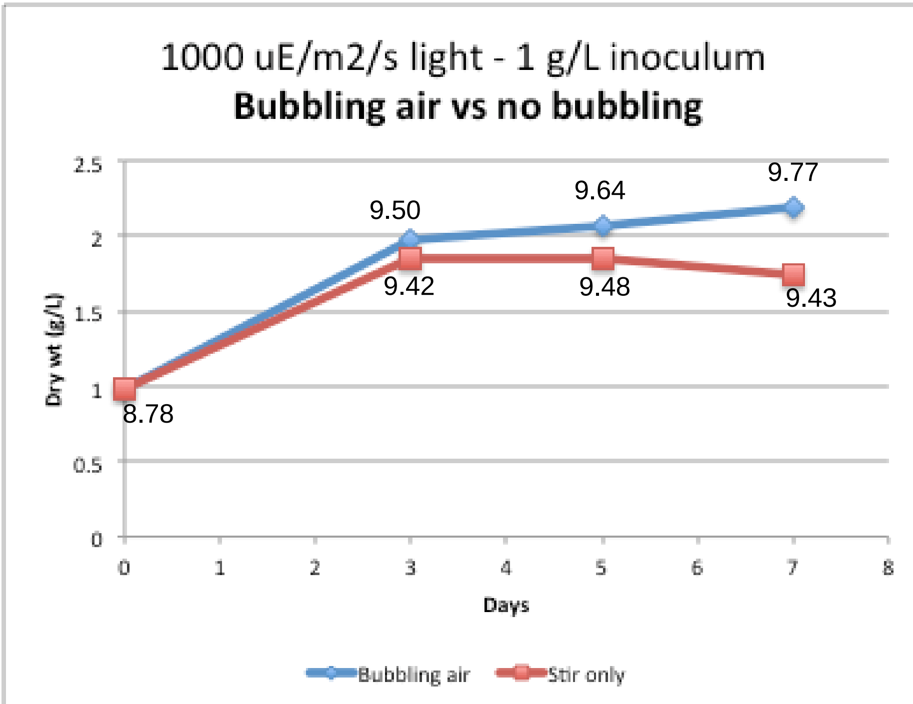
	0	2	5	7
No pH control	1.15	1.56	2.45	2.61
With pH control	1.15	1.62	2.63	2.75

* pH values are indicated at each time point on graph

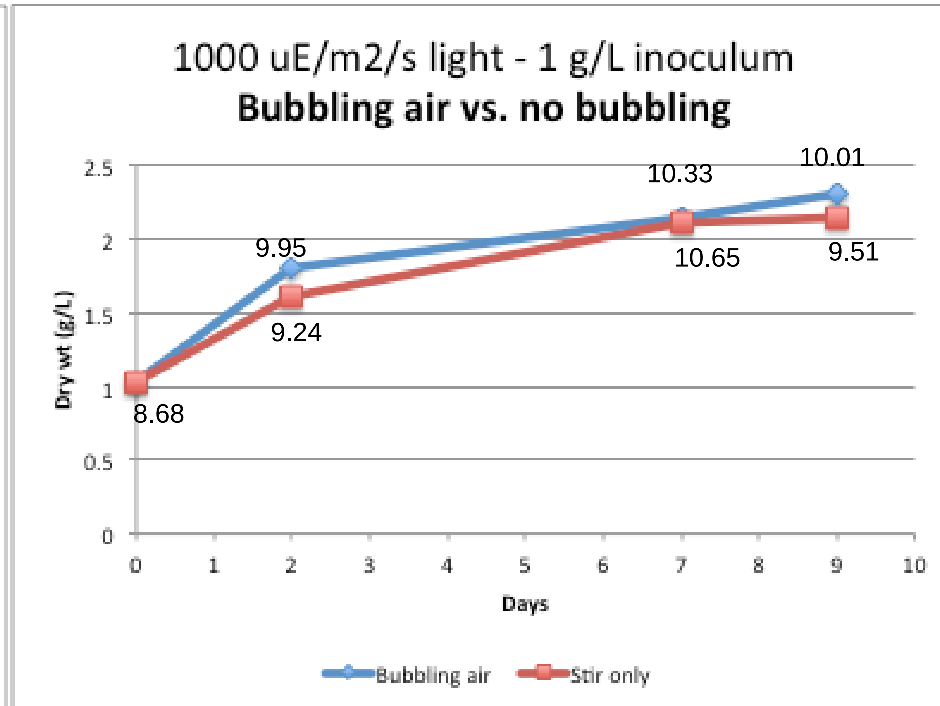
Attaining 2 g/L of biomass

3 – Technical Accomplishments/ Progress/Results: diatom

Trial 1



Trial 2



Trial 1

Dry wt (Manual Light Station)

	0	3	5	7
Bubbling air	0.98	1.97	2.07	2.19
Stir only	0.98	1.86	1.86	1.74

Trial 2

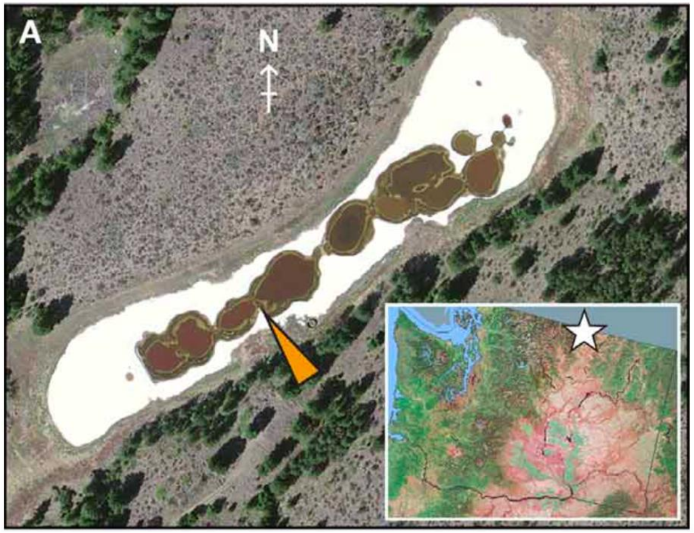
Dry wt (Manual Light Station)

	0	2	5	7
Bubbling air	1.03	1.81	2.14	2.31
Stir only	1.03	1.61	2.11	2.14

Attaining 2 g/L of biomass

* pH values are indicated at each time point on graph

3 – Technical Accomplishments/ Progress/Results: cyanobacteria



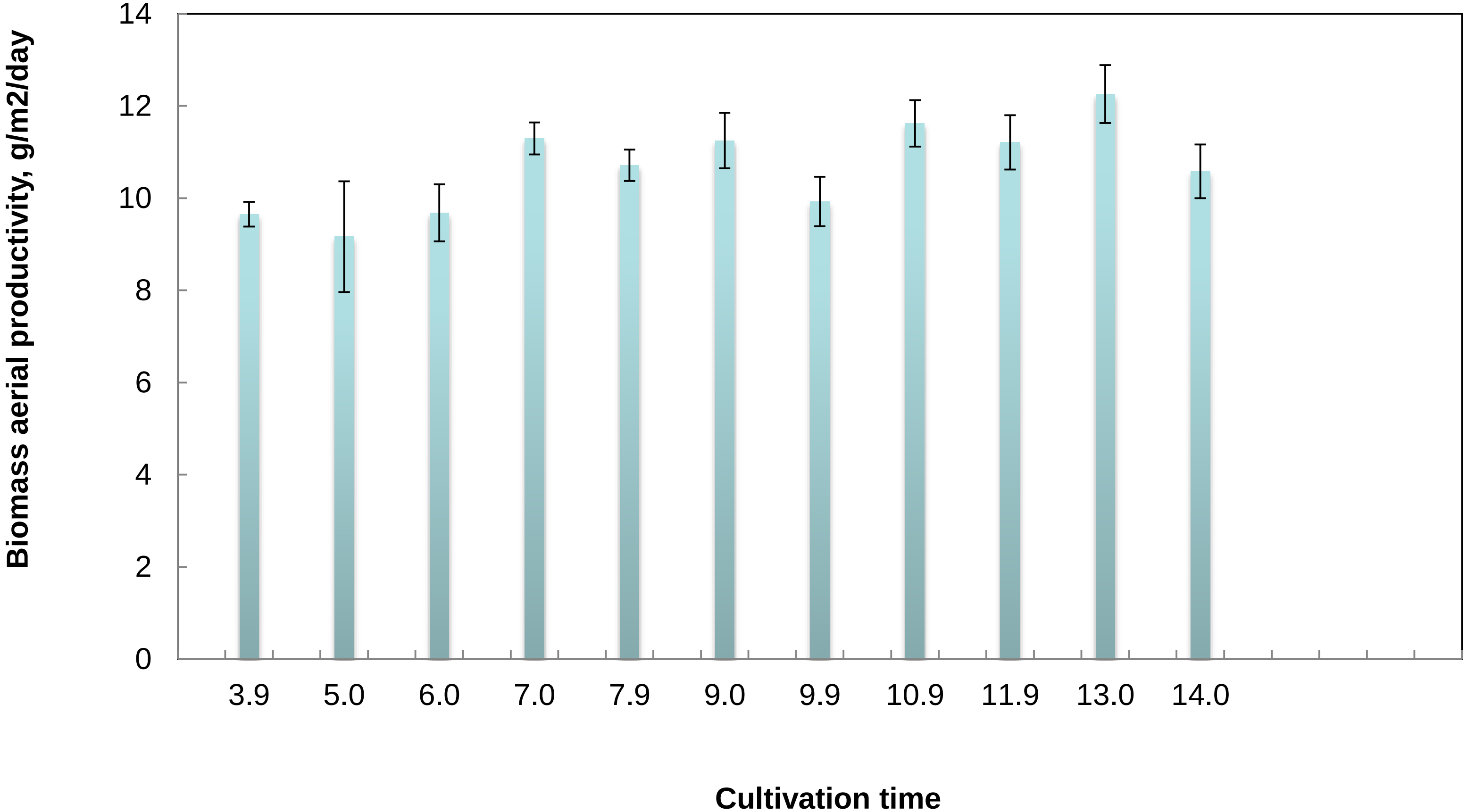
The Hot Lake microbial mat is a source for photosynthetic communities of exceptional productivity and stress resistance

- Hot Lake on July 7, 2011
- heliothermal hypersaline lake in north-central Washington
- contains extreme (>1 M) concentrations of salts
- exhibits an inverse thermal gradient, sometimes >50°C

Isolation of promising cyanbacterium

Lindemann et al. (2013) **Frontiers in Microbiology.** Vol 4(1) doi: 10.3389/fmicb.2013.00323

3 – Technical Accomplishments/ Progress/Results: cyanobacteria



C. stanieri HL-69 biomass areal productivity when grown in Global Algae medium

Isolation of promising cyanbacterium

3 – Technical Accomplishments/ Progress/Results: cyanobacteria

- *C. Stanieri* HL - 69 was able to promptly acclimate to high bicarbonate medium, slight growth rate reduction was observed at 45 g/L.
- The batch cultures of adapted *C. stanieri* HL-69 reached high OD_{640nm} > 4 when grown in Global Algae bicarbonate medium
- This concentration corresponded to biomass concentration of up to 1.5 g/L
- There was no significant variation in optical density and biomass concentration between the four biological replicates
- The cultures quickly reached steady state when switched to semi-continuous growth mode with dilution rate D = 0.5 $days^{-1}$
- The biomass density varied from OD_{640nm} ~ 1.55-1.7 (right after addition of fresh medium) to OD_{640nm} ~ 3-3.4 (right before addition of fresh medium). This concentrations corresponded to biomass densities of ~0.45-0.55 g/L and 1.6-1.7 g/L, respectively
- The volumetric and aerial biomass productivities reached about 0.5-0.7 g/L/day and 9.5-12 $g/m^2/day$
- The achieved biomass densities and productivities were observed at relatively low light intensity of ~85 µmol m^{-2} s^{-1}

4 – Relevance

- *The overarching goal is to use directed evolution to improve algal biomass yields at the GAI algal growth facility. Specifically, we are targeting productivities of 24 g/m^2/day during the spring season.*
- *Low biomass yields remain an impediment to the development of algal biofuels.*
- *Increasing areal biomass yields is essential to realizing BETO targets of 22 g/m^2/day during the spring growing season and 20 g/m^2/day on an annualized basis.*
- *Directed evolution has a proven track record in a variety of biotechnology applications and should be particularly useful to the bioenergy industry.*
- *Increasing biomass yields will lower the cost of biomass feedstock.*
- *GAI is already an algal biomass provider – advances in productivity will benefit their existing technologies.*

5 – Future Work

- *Use selective pressures to adapt GAI to the highest pH/O_2/temperature levels where cultures maintain robust growth.*
- *Attain necessary permits to grow* Cyanobacterium stanieri *strain HL69 at GAI facility in high bicarbonate medium*.
- *Evaluate whether species of Nannochloropsis can adapt to GAI high bicarbonate medium.*
- *Quantify the pH, O_2 and temperature tolerances of evolved* Nannochloropsis *sp.*
- *Quantify the pH, O_2 and temperature tolerances of evolved* Cyanobacterium stanieri *strain HL69.*
- *Quantify biomass production metrics at GAI facility*
- *Evolve strains/consortia that can produce 30 g/m^2/d biomass is solar simulating photobioreactors (Go/No-go decision point).*

Summary

- **Overview**

Directed evolution is a powerful tool to improve biotechnology phenotypes.

- **Approach**

By using laboratory culturing facilities that better correlate with outdoor environmental conditions, selective pressures can be imposed that improve strain performance.

- **Technical Accomplishments/Progress/Results**

GAI229 has been transferred to both Mines and PNNL for strain characterization and phenotyping; Cyanobacterium stanieri *strain HL69 has very promising growth metrics in GAI medium; Directed evolution studies are underway.*

- **Relevance**

Improved photoautotrophic biomass yields are essential to lower feedstock costs. GAI229 is already very promising and directed evolution in being probed to further improve yields.

- **Future work**

Directed evolution is ongoing and additional strains are being tested for growth in GAI media.

Publications, Patents, Presentations, Awards, and Commercialization

Presentations

- Posewitz M.C., *Characterization of Algal Phototrophs for Potential Biotechnology Applications.* January 2019 Western Photosynthesis Conference. Friday Harbor, WA.

Research Initiated August, 2018.