Los Alamos
NATIONAL LABORATORY

Functional Characterization of Cellular Metabolism

AOP 1.3.1.100

Scott Twary

March 9, 2021

LA-UR-21-20841

Project Overview

Understand N sensing and signaling to uncouple N stress regulation of lipid accumulation for co-directed carbon allocations to biomass and lipids

Integrated line development strategy

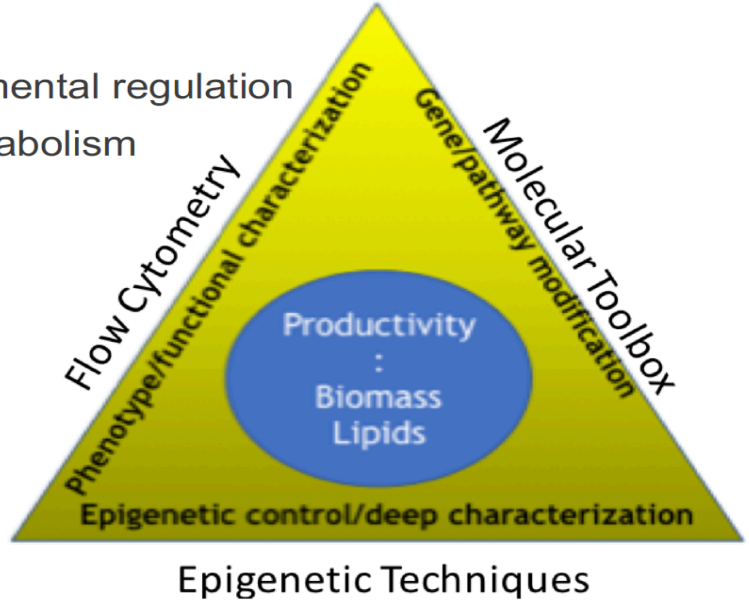
Flow Cytometry: rapid physiological characterization,

Non-GMO strain improvement

Epigenetics: modulation of environmental regulation

Genome Engineering: targeted metabolism

Capability development for identifying key gene targets, functional pathways, and regulatory mechanisms in algae applied to a novel strain improvement strategy to complement current 'omics approach.



1 – Management

- Scott Twary- PI Babetta Marrone- co-PI
  - **High Throughput Single Cell Analysis**
    - Babetta Marrone- Scientist
    - Claire Sanders- Technologist
  - **Epigenetics**
    - Christina Steadman- Scientist
  - **Biotechnology Strain Improvement**
    - Scott Twary- Scientist
    - Attelia Hollander- Technologist
    - Shounak Banerjee- Post Doctoral Associate

  Weekly team meetings, quarterly project reviews,
  monthly LANL and BETO algae team meetings
  Interface with Hovde: Engineering, Starkenburg: Blueprint and genomes, Dale: Multi-scale Characterization

2 – Approach

High Throughput Single Cell Analysis: Flow cytometry physiological assay development

- Developed assays for multiple algae species (*Picochlorum soloecismus, Nannochloropsis salina 1776, Tetraselmis striata LANL 1001)*
- Non-GMO strain improvement based on population sorting of these assay traits
- Expand characterization of primary and secondary physiological changes resulting from environmental and genetic changes

Milestones completed: Phenomic analysis completed applying five flow cytometry assays to epieffector modified *N. salina* and *T. striata* during growth cycle.

Demonstrate a linkage between the rational genetic engineering of *N. salina* and physiological performance using flow cytometry platform.

Epigenetics

- Antibody quantification of permissive and repressive histone modifications
- DNA methylation profiling and identification of epigenetically regulated genes
- Global epigenome modification through EpiEffectors

Milestone completed: Metabolism and productivity altered through global epigenome modification by EpiEffectors molecules in *N. salina* and *T. striata*.

Biotechnology Strain Improvement

- CRISPR/Cas genome engineering toolbox
- Targeted gene knock-outs involved in N sensing and signaling
- Overexpression of N assimilatory enzymes
- Altered N utilization responses resulting in greater lipid productivity

Milestones completed: Stable genetically modified lines of *T. striata* demonstrating reporter expression developed. Multi-gene stacking transformants in *N. salina* developed altering nitrogen sensing and signaling pathways.

3 – Impact

- Novel approaches investigated for strain improvement to meet BETO production goals and genetics and development objectives
  - Molecular engineering tools and strategies assist other AOP projects

- Comprehensive flow phenotyping leads to greater understanding of line modifications
  - Flow cytometry assays utilized by multiple BETO algae projects

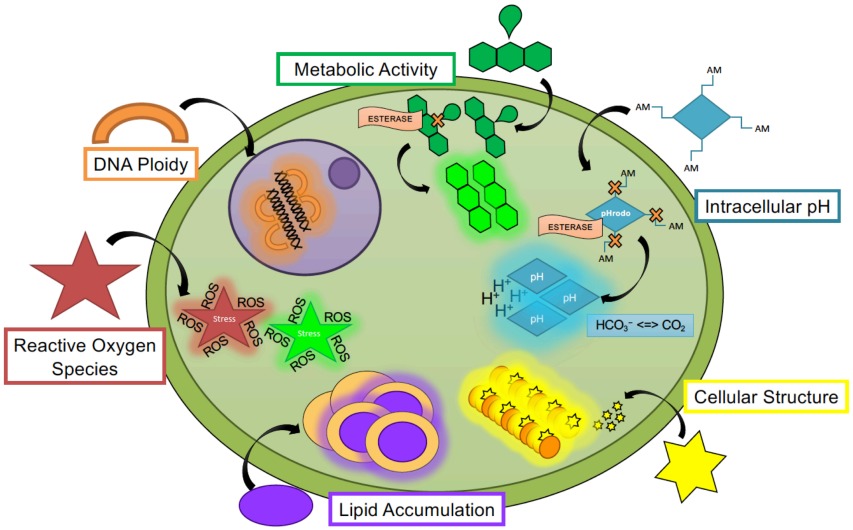
- Elucidating complex genetic and physiological mechanisms provides the basis for novel targeted strategies for both bioproduct and biofuel production

- 6 Publications produced from this work and 2 more in preparation

4 – Progress and Outcomes

- First characterization of epigenetic regulated genes in biofuel production species opens novel avenues for strain improvement, stress response regulation, and unique genetic regulation tools.
- Extensive transcriptome analysis for diurnal changes, nitrogen depletion stress, and epi-effector inhibition. Improved annotation models applied to new analysis pipelines for integrating multiple data sets
- Integrated flow sorted population improvement with genetic engineering
- Improved and optimized transformation efficiency for *Nannochloropsis* through cell cycle timing
- Molecular toolbox developed for *Tetraselmis*

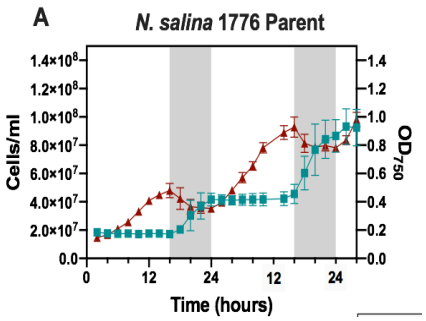
Flow Cytometry

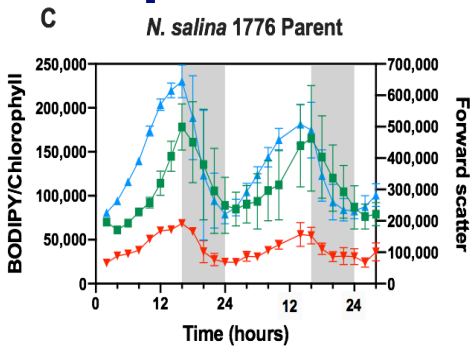


- Six assays optimized for multiple species
  - BODIPY – neutral lipid
  - Syto 9 and DyeCyle Orange – DNA content
  - FDA – esterase activity for metabolic activity
  - CM-H_2DCFDA – reactive oxygen species
  - pHrodo green AM – intracellular pH
  - Phalloidin – actin/cellular structure
- Applied to engineered lines and epigenetic experiments for in-depth characterization
- Flow sorting performed for potential non-GMO line improvement for three assays (ROS, DNA, esterase activity)

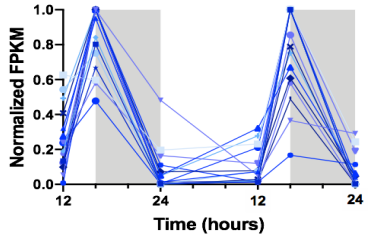
Steadman Tyler, C., Sanders, C., Erickson, R., Dale, T., Twary, S., Marrone, B. 2019. Functional and phenotypic flow cytometry characterization of *Picochlorum soloecismus,* Algal Research,43, doi.org/10.1016/j.algal.2019.101614.

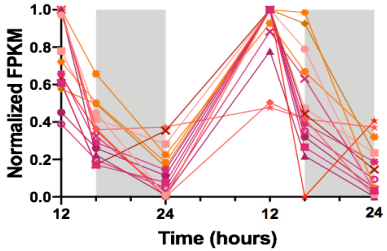
Cell Cycle Analysis Improves Transformation Efficiency



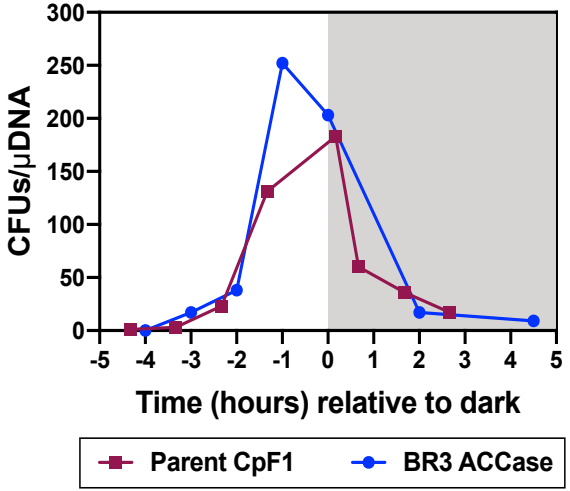


Flow cytometry characterization of synchronized cell growth





Gene expression analysis reveals synchronized expression of DNA repair and cell wall re-modeling genes.



Increased transformant colonies 1 hour before dark.

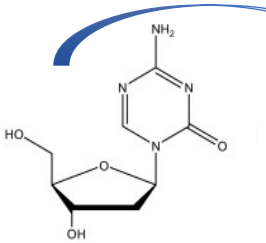
Claire K. Sanders, Shounak Banerjee, Migun Shakya, Blake Hovde, Attelia Hollander, Taraka Dale, Christina R. Steadman, Babetta L. Marrone, Scott N. Twary. 2021. **Cell cycle characterization of *Nannochloropsis salina* and relation to transformation efficiency**, Biomolecules, *In Review. (Special Issue on Algae Cell Cycles)*

Epigenetics

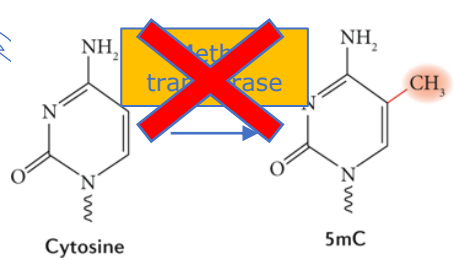
- Three species characterized for epigenetic changes during nitrogen stress
  - *Picochlorum soloecismus*
  - *Nannochloropsis salina 1776*
  - *Tetraselmis striata LANL 1001*
- Analysis pipeline developed for integrating whole genome bisulfite sequencing and transcriptomics to identify key regulated genes

EpiEffectors help determine functional importance of epigenetic modifications

EpiEffectors
Small molecules that alter function of chromatin modifying enzymes; usually "inhibitors"

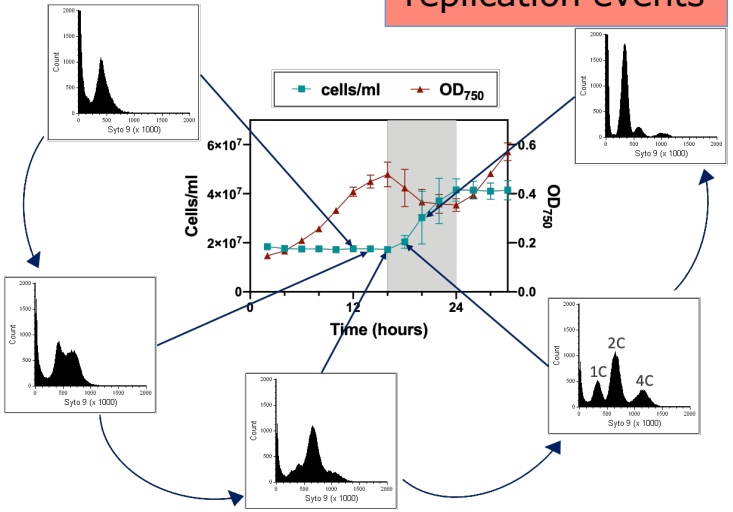


5-aza-2'-deoxycytidine (decitabine)



5AZA drug inhibits DNA methylation and causes loss of methylation over repeated DNA replication events

- **Histone deacetylation inhibitors**
  - SAHA Vorinostat
  - TSA Trichostatin
  - Tubacin



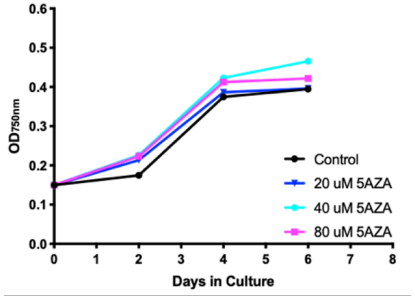
Identification of the initiation of DNA replication through Syto9 staining

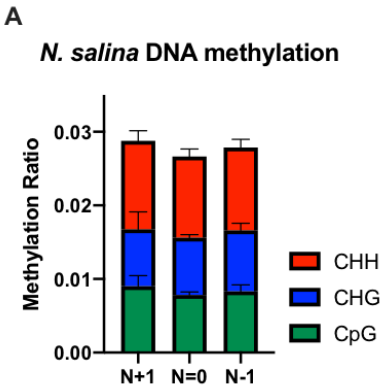
Christina R. Steadman,* Shounak Banerjee, Yuliya A. Kunde, Claire K. Sanders, Babetta L. Marrone, and Scott N. Twary. 2020. **Inhibition of DNA Methylation in *Picochlorum soloecismus* Alters Algae Productivity,** Front Genet. 2020; 11: 560444, doi: 10.3389/fgene.2020.560444

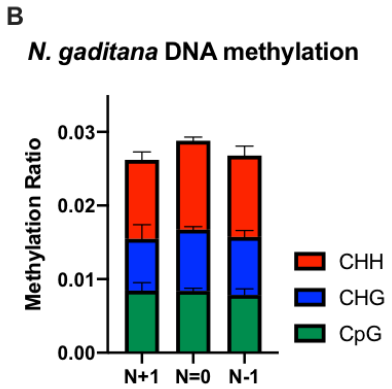
Nannochloropsis

Sample	Replicate Reading 1	Replicate Reading 2	Replicate Reading 3	Interpolated X (%5mC)
Negative Control (0)	0.0811	0.0802	0.0802	0
Negative Control (0)	0.0854	0.087	0.0761	0
NS1776 Control_1	0.0988	0.0974	0.0824	0
NS1776 Control_1	0.0903	0.1015	0.0874	0.00002
NS1776 Control_2	0.0886	0.0804	0.0817	0
NS1776 Control_2	0.0831	0.0887	0.0863	0
NS1776_Control DMSO	0.0845	0.0885	0.0839	0
NS1776_Control DMSO	0.0864	0.0875	0.0864	0
NS1776 20 uM 5AZA	0.0792	0.0831	0.0842	0
NS1776 20 uM 5AZA	0.0768	0.0781	0.082	0
NS1776 40 uM 5AZA	0.0834	0.0867	0.0918	0
NS1776 40 uM 5AZA	0.0845	0.0893	0.086	0
NS1776 80 uM 5AZA_1	0.0839	0.0928	0.0843	0
NS1776 80 uM 5AZA_1	0.0813	0.0847	0.0807	0
NS1776 80 uM 5AZA_2	0.0833	0.084	0.0818	0
NS1776 80 uM 5AZA_2	0.0829	0.086	0.0871	0

5-mc antibody based ELISA for DNA methylation analysis





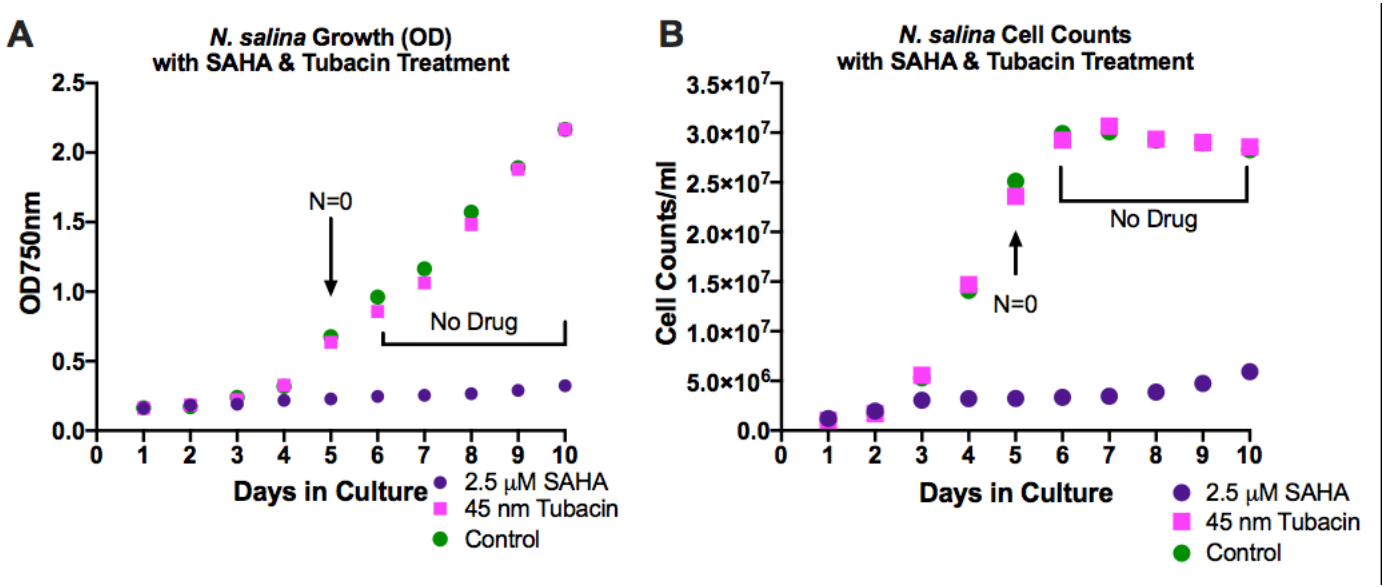


Whole genome bisulfite sequencing for genome methylation

- ELISA results below detection limits
- Genome methylation less than 3% of potential sites
- DNA methylation inhibitor has no effect

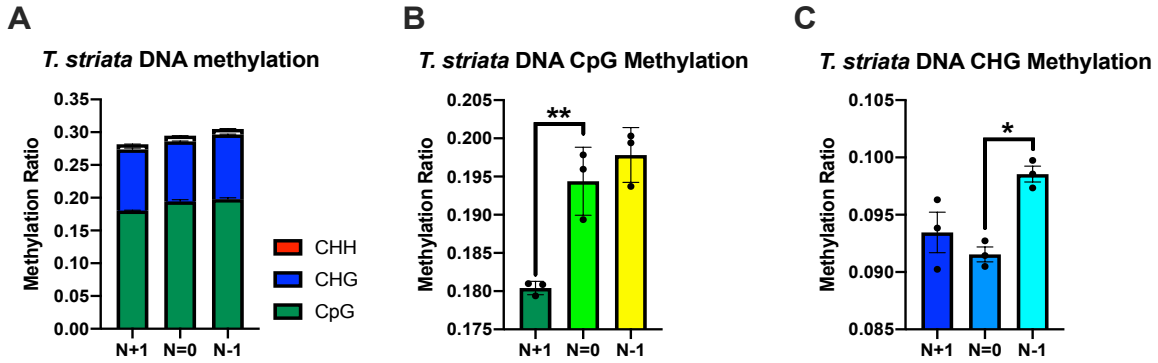
***Nannochloropsis* does not utilize DNA methylation for epigenetic regulation**

Nannochloropsis



Histone deacetylation inhibitor treatment results in altered growth. Bioinformatic analysis of *Nannochloropsis* genome reveals no methylation machinery but extensive histone modification enzymes.

Tetraselmis



Methylation site (cytosine)	Culture Condition	Methylation ratio replicates			Methyl ratio average	P-values	Methyl ratio difference	Change in methylation	Annotation
Tetraselmis_0037:60747	Replete	0.39	0.40	0.52	0.44	0.00			threonine-protein phosphatase 6 regulatory ankyrin repeat subunit A
	Starvation	0.75	0.82	0.91	0.83	0.02			
	Deplete	1.00	1.00	1.00	1.00	0.00	0.56	hypermethylated	
Tetraselmis_0095:3554	Replete	0.69	0.42	0.72	0.61	0.73			No annotation
No annotation	Starvation	0.71	0.70	0.20	0.54	0.16			
	Deplete	0.82	0.75	1.00	0.86	0.12	0.25	not significant	
Tetraselmis_0106:37078	Replete	0.27	0.42	0.86	0.52	0.37			No annotation
	Starvation	0.36	0.46	0.11	0.31	0.02			
	Deplete	0.71	0.80	0.68	0.73	0.30	0.21	hypermethylated	
Tetraselmis_0136:4550	Replete	0.57	0.52	0.58	0.56	0.81			Similar to CDCA7L: Cell division cycle-associated 7-like protein (Homo sapiens);
	Starvation	0.69	0.67	0.20	0.52	0.10			
	Deplete	1.00	1.00	0.74	0.91	0.02	0.35	hypermethylated	
Tetraselmis_0426:43776	Replete	0.15	0.50	0.56	0.40	0.07			No annotation
	Starvation	0.71	0.68	0.94	0.78	0.52			
	Deplete	0.59	0.80	0.73	0.71	0.10	0.30	hypermethylated	

30% of the genome sites are methylated and nitrogen stress response results in significant hypermethylation

Tetraselmis

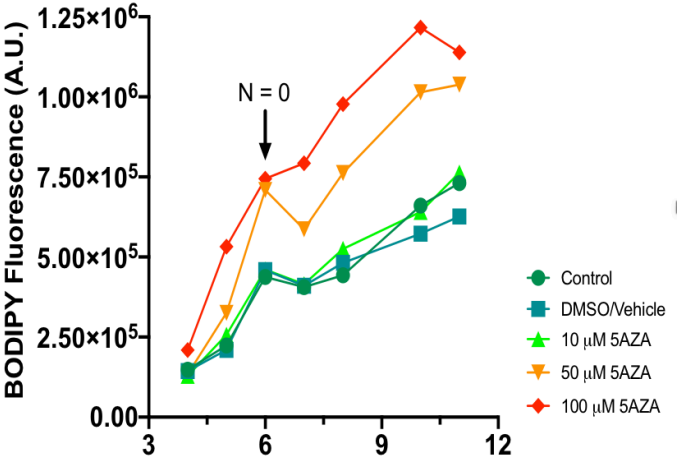
Flow Cytometry Analysis of DNA-methylation Inhibition

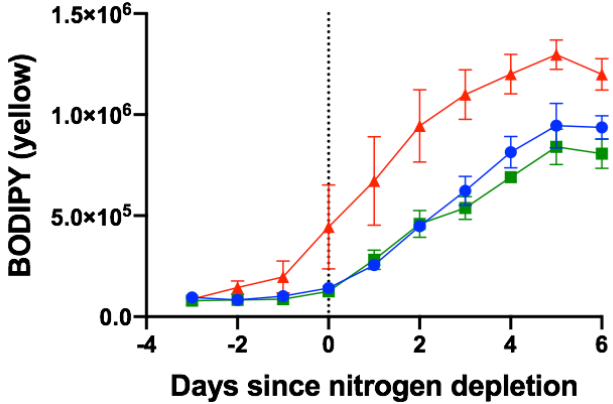
- 5-AZA treated cultures (100 uM)
  - 3 fold decrease in cell counts
  - 2 fold decrease in optical density
  - 2 fold decrease in dry weight accumulation
  - 1.5 fold **increase** in forward scatter (relative cell size)
  - 3 fold **increase** in chlorophyll fluorescence
  - 2 fold **increase** in lipid accumulation

In Process:

RNA-Seq

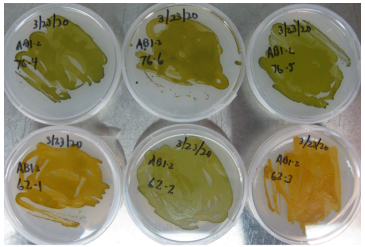
Whole genome bisulfite sequencing



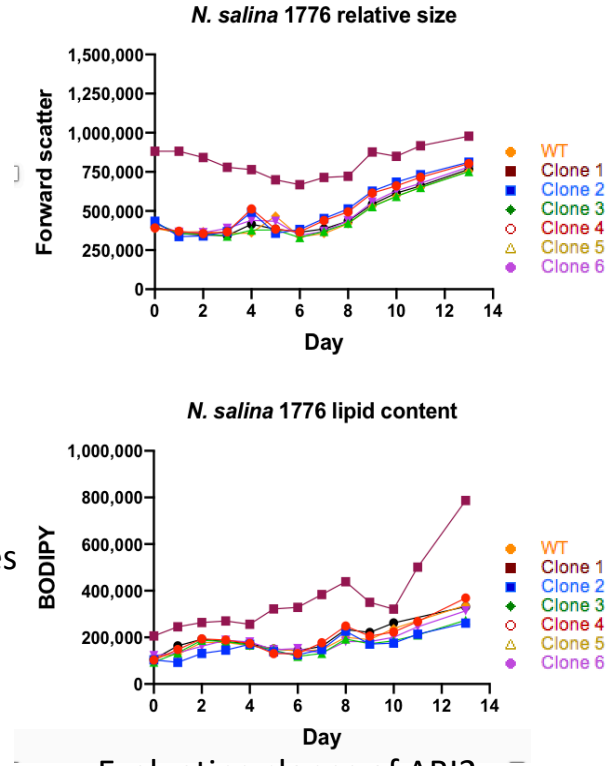


Molecular Toolbox

- Genetically stable overexpression lines created for multiple single genes into wild type or flow sorted improved lines for stacking functional advances in *Nannochloropsis*
  - ACCase
  - ABI2
  - NR, NiR
  - NO_3 transporter
- Cas 9 lines developed for *Nannochloropsis* and *Tetraselmis*
  - *Low efficiency of knock-outs and unstable lines*



ABI2 overexpression clones vary in phenotype



Evaluating clones of ABI2 overexpression results in arrested cell growth and greater lipid accumulation.

Summary

- Integrating flow cytometry, epigenetic characterization, and genome engineering
- Expanded analytical tools for physiological characterization
- Expanded molecular engineering tools for multiple species
- Expanded understanding of epigenetic regulation for multiple species
- Developed lines with greater lipid accumulation through both genetic engineering and epi-effector treatments

Quad Chart Overview

Timeline

- Project start date 10/1/2017
- Project end date 12/31/2020

	FY20	Active Project
DOE Funding	(10/01/2019 – 9/30/2020) *$650 K*	*$1950 K Total*

Project Partners* **N/A**

Barriers addressed

Aft C- Biomass Genetics and Development

Project Goal

Integrate epigenetic characterization, molecular genetic engineering and flow cytometry physiological characterization to advance strain improvement focused on nitrogen stress responses.

End of Project Milestone

Comparative epigenomic and phenomic characterization of genetically altered stacked transformant nitrogen responsive lines for comparison to wild type parent epigenomes and phenotypes for demonstrating an integrated protocol for strain improvement combining flow cytometry, genome engineering, and epigenome profiling.

Funding Mechanism

AOP 1.3.1.100

ADDITIONAL SLIDES

Responses to Previous Reviewers' Comments

"Focus future work on parts of project with greatest potential impact. Epigenetic work is most novel and molecular work is most challenging."

Multiple epigenetic regulators have been screened for evaluating efficacy and phenotypic responses across three species of algae. Differing regulatory mechanisms were identified and key responsive genes identified through combined analysis of transcriptome and genome sequencing analysis.

This work demonstrates the value of integrating multiple toolkit developments into one enhanced strain improvement method. Manipulation and evaluation of phenotype utilizing three different molecular approaches (phenotype, genotype, epigenome) increases the probability of generating a highly productive strain and generating a (more) holistic understanding of both physiological and epigenetic changes that occur from targeted genetic engineering strategies. This more comprehensive analysis allows greater elucidation of both primary and secondary responses, enriching the knowledge-base to support further advances in the field.

Publications

- Christina R. Steadman Tyler, Blake T. Hovde, Hajnalka E. Daligault, Xiang Li Zhang, Yuliya Kunde, Babetta L. Marrone, Scott N. Twary, Shawn R. Starkenburg. 2019. **High-Quality Draft Genome Sequence of the Green Alga *Tetraselmis striata* (Chlorophyta) Generated from PacBio Sequencing**. Microbiology Resource Announcements, Volume 8 Issue 43 e00780-19.
- J. A. Ohan, B. Hovde, X. Zhang, K. W. Davenport, O. Chertkov, C. Han, S. N. Twary, and S. R. Starkenburg. 2019. **Nuclear Genome Assembly of the Microalga *Nannochloropsis salina* CCMP1776**, Microbiol Resour Announc doi: 10.1128/MRA.00750-19
- Steadman Tyler, C., Sanders, C., Erickson, R., Dale, T., Twary, S., Marrone, B. 2019. **Functional and phenotypic flow cytometry characterization of *Picochlorum soloecismus*,** Algal Research,43, doi.org/10.1016/j.algal.2019.101614.
- Christina R. Steadman,* Shounak Banerjee, Yuliya A. Kunde, Claire K. Sanders, Babetta L. Marrone, and Scott N. Twary. 2020. **Inhibition of DNA Methylation in *Picochlorum soloecismus* Alters Algae Productivity,** Front Genet. 2020; 11: 560444, doi: 10.3389/fgene.2020.560444
- Claire K. Sanders, Shounak Banerjee, Migun Shakya, Blake Hovde, Attelia Hollander, Taraka Dale, Christina R. Steadman, Babetta L. Marrone, Scott N. Twary. 2021. **Cell cycle characterization of *Nannochloropsis salina* and relation to transformation efficiency**, Biomolecules, *In Review. (Special Issue on Algae Cell Cycles)*
- **Genomic characterization reveals significant divergence within Chlorella sorokiniana (Chlorellales, Trebouxiophyceae),** Blake T. Hovde, Erik R. Hanschen, Christina R. Steadman Tyler, Chien-Chi Loa,Yuliya Kundea, Karen Davenporta, Hajnalka Daligaulta, Joseph Msanne, Stephanie Canny, Seong-il Eyung, Jean-Jack M. Riethoven, Juergen Polle, Shawn R. Starkenburg. 2018. *Algal Research*, 35: 449-461.
- **Using Flow Cytometry and Multistage Machine Learning to Discover Label-Free Signatures of Algal Lipid Accumulation**, Mohammad Tanhaemami, Elaheh Alizadeh, Claire Sanders, Babetta L. Marrone, Brian Munsky. 2019. *Physical Biology 16(5): 055001.*

Presentations

- “Mapping the algal epigenomic landscape: new tools for manipulating algae*”,* C. Steadman. Pharmaceutical Sciences Seminar, University of New Mexico, Albuquerque, NM (2019).
- *“*Sequencing epigenomes to understand gene by environment interactions*”,* C. Steadman. Next Generation Sequencing Workshop, Los Alamos National Laboratory, Los Alamos, NM (2019).
- “Epigenetic manipulation of the DNA methylome in algae alters productivity”, C.R. Steadman*, S.N. Twary, B.L. Marrone, oral presentation *at the International Conference on Algal Biomass, Biofuels and Bioproducts, Boulder* CO, USA, June 2019.
- "Enhanced algae biomass and lipid production through mixotrophic cultivation with plants" A. Barry. Invited talk. US Microalgae, May 15-16, Fort Lauderdale, Florida.
- "Investigation of *Nannochloropsis* sp. cultivation with plant substrate addition: Ecology, productivity, lipid concentration, cellulase expression, and plant structure analysis" Amanda N. Barry, Jenna Schambach, Anna Finck, Christopher Hunt, Peter Kitin, Erik Hanschen, Shawn Starkenburg, Brian Vogler. Poster. *International Conference on Algal Biomass, Biofuels and Bioproducts*, June 17-19, Boulder, CO.
- “Rapid genetic engineering of *Nannochloropsis* sp. to synergize biomass and lipid productivities”, S. Banerjee*, C.K. Sanders, A.D. Hollander, C.R. Gonzalez Esquer, B.T. Hovde, B.L. Marrone, S.N. Twary, poster presented at the *International Conference on Algal Biomass, Biofuels and Bioproducts,* Boulder CO*, USA*, June 2019

Presentations

- “Monitoring Cell Cycle and Lipid Accumulation in Microalgae” Claire K. Sanders, Babetta L. Marrone, Scott N. Twary. 34th Congress of the International Society for Advancement of Cytometry; June 22-26, 2019; Vancouver, BC, Canada
- “*Epigenetics in action: from mechanisms to biological impacts* “ C R Steadman, New York University, Langone Medical School, Environmental Medicine Seminar Series, New York City, NY (2019)
- “Mining the genomic landscape of microalgae for epigenetic treasures”. Steadman, C.R. Gordon Research Conference on Dynamics of Epigenetic Regulation: Mechanisms and Beyond. July 20-21, 2019, Holderness NH
- “Understanding epigenetic regulation of harmful algae blooms to enhance cultivation of algae production strains” Steadman, C.R. 13th Annual Algae Biomass Summit by Algae Biomass Organization September 16-19, 2019, Orlando FL
- “*Gene by environment considerations for bio-restoration* “ BETO BioRestore Workshop, Chicago, IL (2019)
- ”Combining multiple line improvement strategies for enhancing algal productivity” S. Banerjee, C. Sanders, B. Marrone, A. Hollander, J. Schambach, S. Twary, *International Conference on Algal Biomass, Biofuels and Bioproducts, 2020.*
- “Flow cytometry as a tool for biomanufacturing” Claire K. Sanders, Niju Narayanan, Ramesh Jha, Carol K. Carr, C. Raul Gonzalez-Esquer, Scott N. Twary, Taraka Dale. Cytometrists of Western States; February 21, 2020; Scottsdale, AZ.