Functional Characterization of Cellular Metabolism

U.S. Department of Energy (DOE)
Bioenergy Technologies Office (BETO)
2019 Project Peer Review

Advanced Algal Systems

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Los Alamos NATIONAL LABORATORY EST.1943

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Goal Statement

Goal

Design an integrated strain improvement platform utilizing environmental, epigenetic, and genetic factors for targeted advances with rapid, comprehensive phenotyping leading to greater understanding of these modifications.

Outcomes & Relevance

Define the genetic pathway regulating nitrogen (N) sensing and signaling to produce an improved algae line with faster production due to rapid N assimilation and potentially uncouple N stress induction of lipid accumulation.

Novel capability development

- **Integrated strain improvement strategy**
- Expanded suite of flow cytometry physiological assays
- CRISPR/Cas genome engineering toolbox for *Nannochloropsis salina*
- Epigenetic profiling and specific gene responses to environmental stress
  - EpiEffector modification and regulation of genome

Quad Chart Overview

Timeline: Ongoing Project

- Current merit review period:
- October 1, 2017-September 30, 2020
- 33% complete

	Total Costs Pre FY17**	FY 17 Costs	FY 18 Costs	Total Planned Funding (FY 19-Project End Date)
DOE Funded	2,200 K	650 K	1,100 K	1,334 K
Project Cost Share*				N/A

Barriers addressed

Aft-C. Biomass Genetics and Development:

Creation of novel integrated strain improvement methods and new tool applications.

Objective

To integrate flow cytometry, epigenome regulation, and genome engineering for elucidation of N stress sensing and signaling and application towards novel line improvement strategies

End of Project Goal

An improved algae line with increased productivity over 30% of baseline.

1- 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

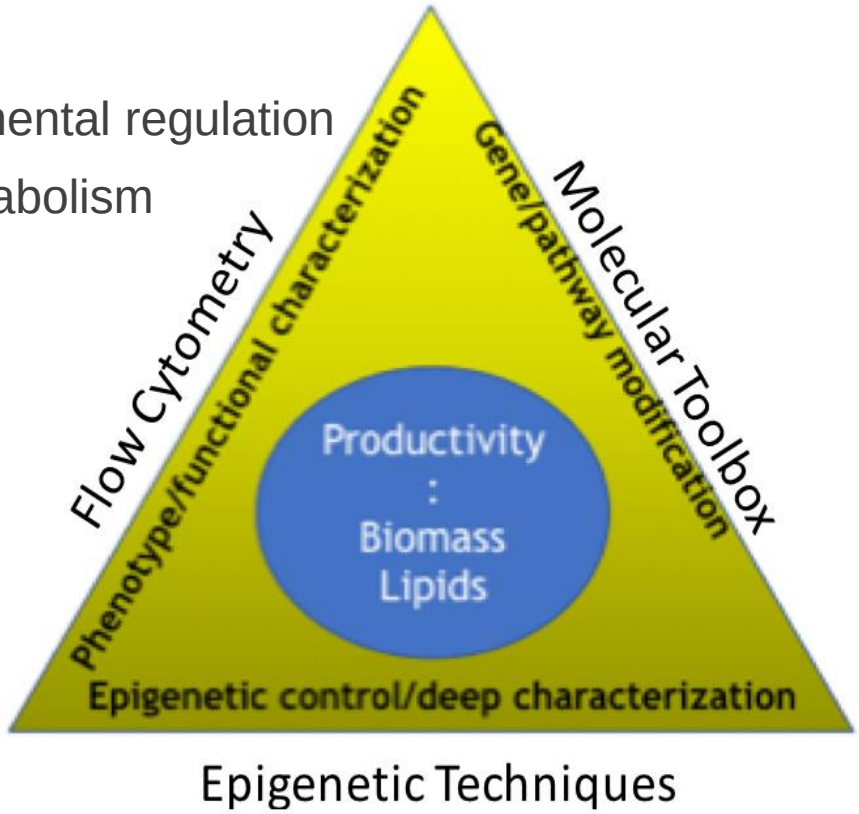
Flow Cytometry: rapid physiological characterization,

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.



2 – Approach (Management)

- Scott Twary- PI Babetta Marrone- co-PI
  - **High Throughput Single Cell Analysis**
    - Babetta Marrone- Scientist
    - Claire Sanders- Technologist
  - **Epigenetics**
    - Christina Steadman- Post Doctoral Fellow
  - **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 PACE, Hovde: Engineering, Starkenburg: Blueprint and genomes, Dale: Multi-scale Characterization

2 – Approach (Technical): Three Key Components

High Throughput Single Cell Analysis: Flow cytometry physiological assay development

- Developed assays for multiple algae species (*Picochlorum soloecismus, Nannochloropsis salina, Tetraselmis striata)*
- 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

Epigenetics

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

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

High Throughput Single Cell Analysis

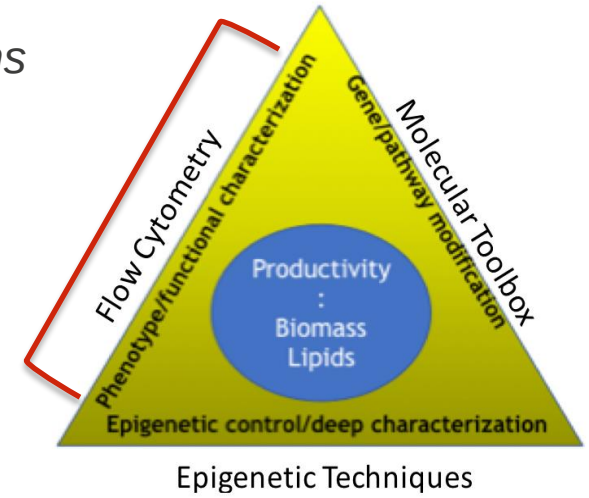
Flow cytometry physiological assay development

- **Milestones (complete)**
  - Flow cytometry assays of pH, cell viability, cell structure, Reactive Oxygen Species, lipids, and DNA ploidy of *P. soloecismus* and *N. salina*
  - Comparative phenotypic profiles of *N. salina* selected lines utilizing physiological flow cytometry assays

- **Milestones (in progress)**
  - Improved lines of *N. salina* created through flow cytometry cell metabolism assay population sorting

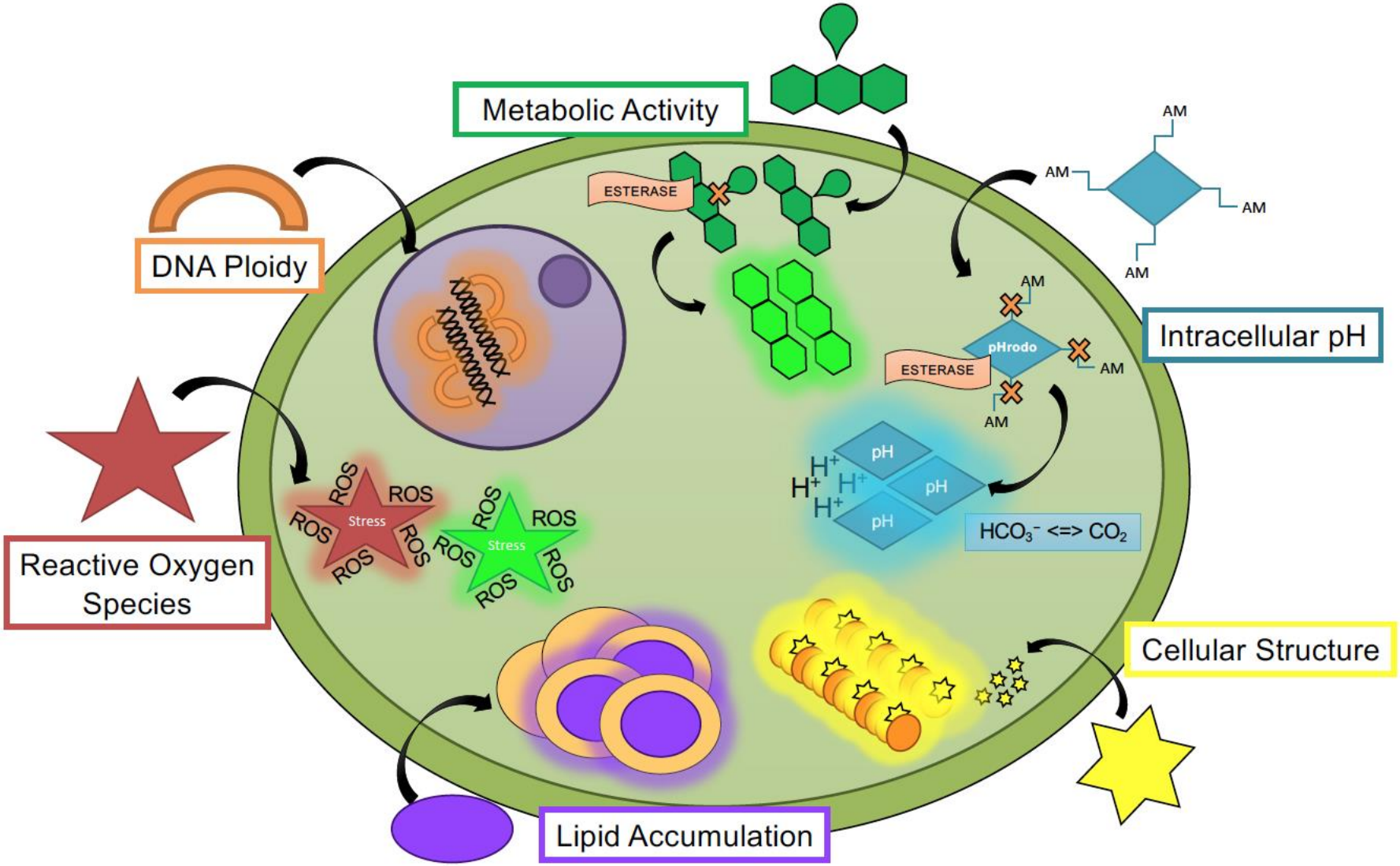
    – Previous work created a FACS line from N. salina with greater lipid accumulation during replete N conditions named BR3, validated under outdoor growth. Comparative transcriptome completed for differential analysis to 1776 parent.

- **Milestones (future)**
  - Expand assay application to new algae species
  - Integrated strain improvement process



High Throughput Single Cell Analysis

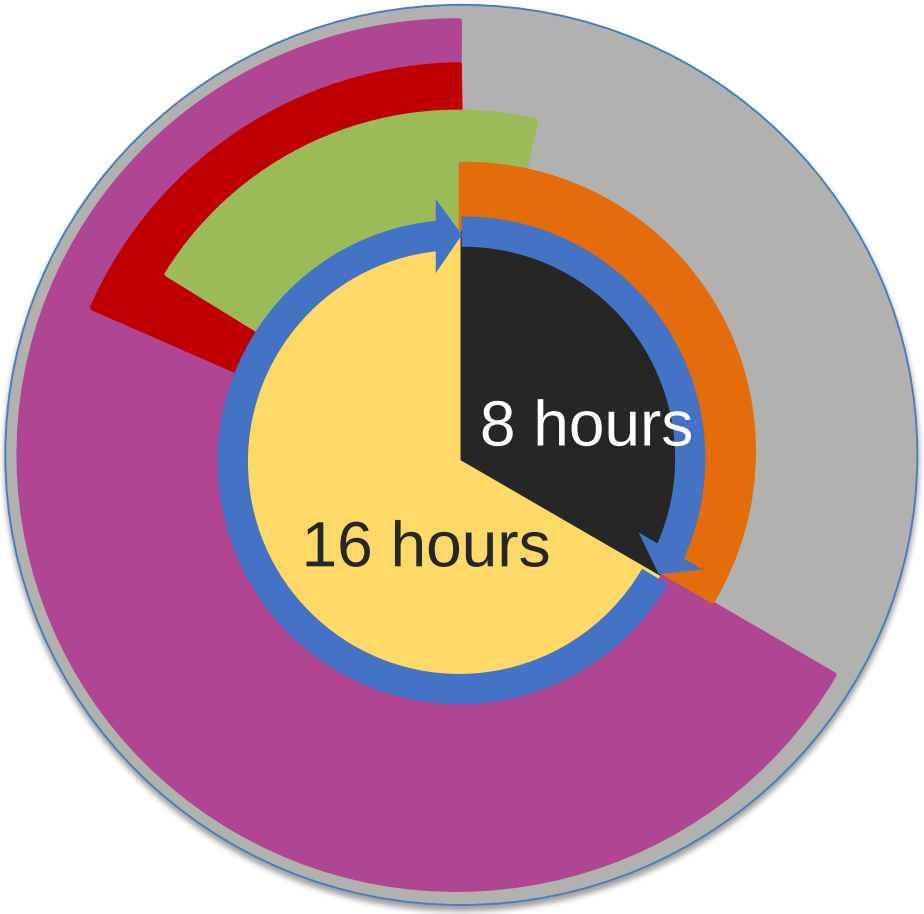
Six assays developed and applied to multiple species and selection lines.
Application to non-GMO strain improvements.



Flow Cytometry Characterization: DNA Replication and Cell Division

Light Synchronized Cell Cycles Vary by Algae Species

Colored bars highlight cell division times for each species



Nannochloropsis salina

- Cell division initiated and occurs throughout the dark cycle

Picochlorum soloecismus

- Cell division initiated near the end of the light cycle

Chlorella sorokiniana

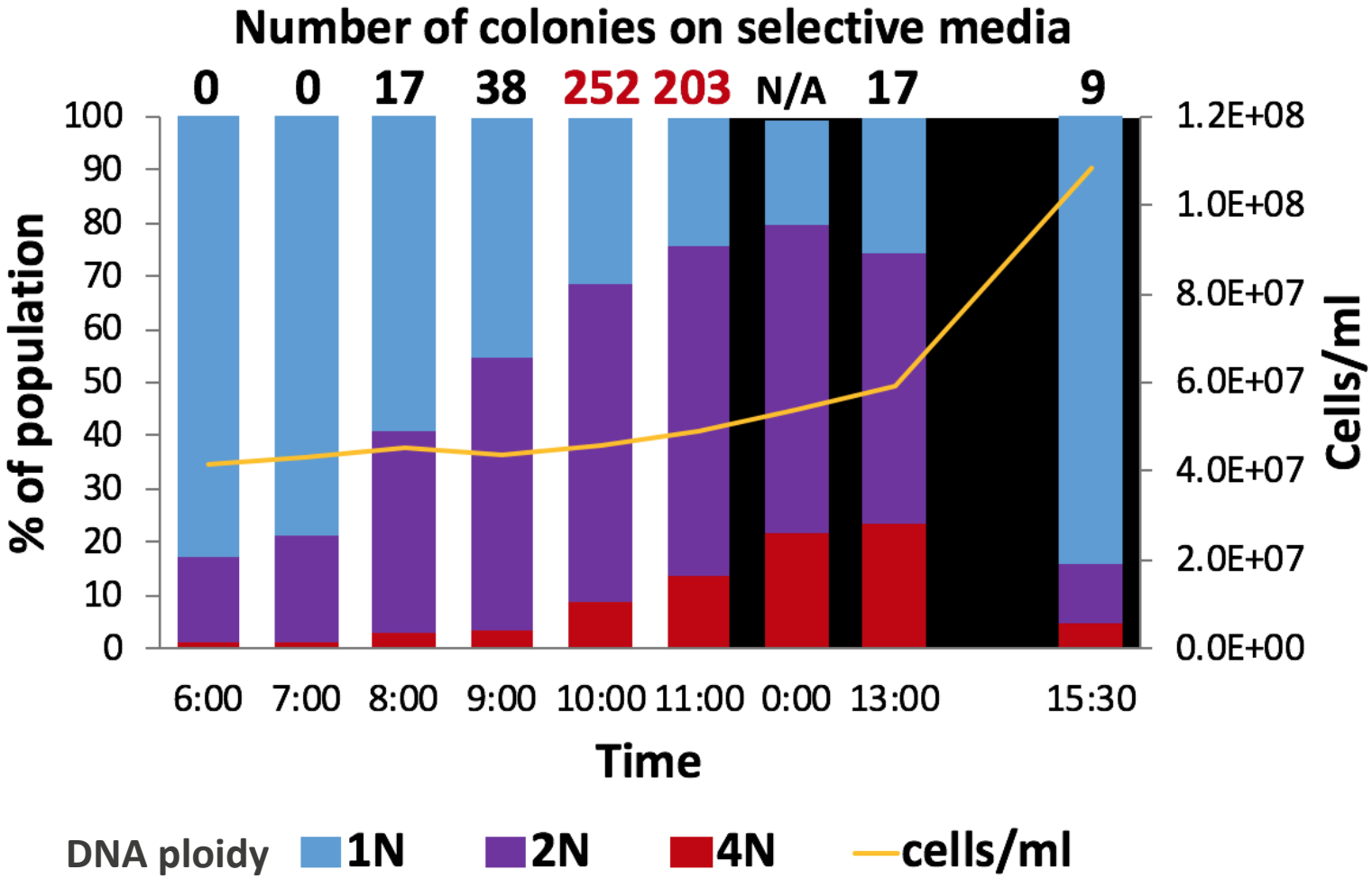
- Cell division initiated near the end of the light cycle (strain variability noted)

Tetraselmis striata

- Cell division occurs throughout the light cycle only

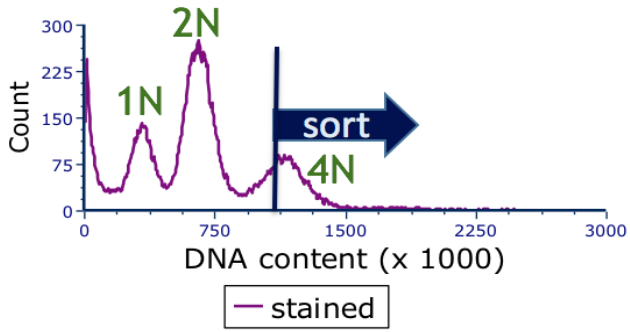
DNA replication typically begins 3 to 4 hours before cell division

Flow Cytometry Characterization
Nannochloropsis Transformation Efficiency During Cell Cycle



- Two distinct phases of DNA replication and cell division noted
- Genetic transformation efficiency highest during transition between phases

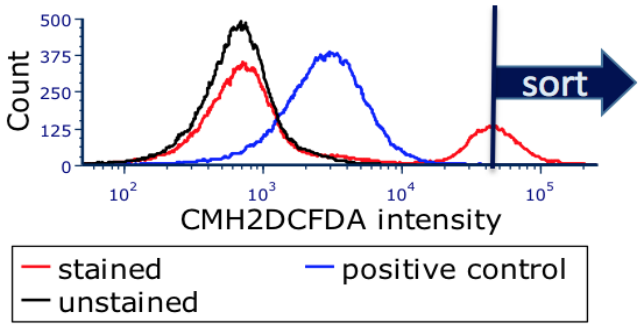
Flow Cytometry Characterization
Non-GMO FACS Population Improvement Based on Function



DNA content

Hoechst 33342
High DNA content

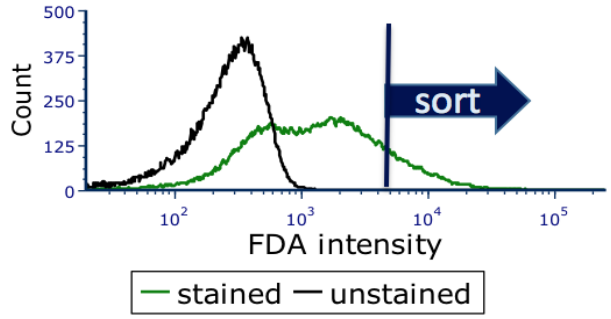
Two generations completed



High metabolic activity

CM-H_2DCFDA
Reactive Oxygen Species

One generation completed



Fluorescein diacetate (FDA)
Esterase activity

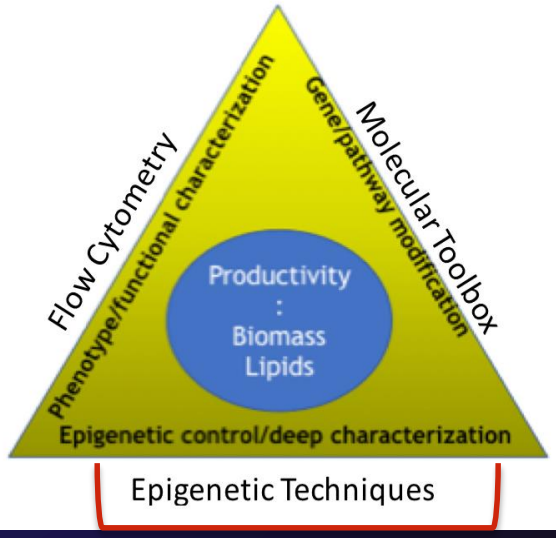
One generation completed

Epigenetics

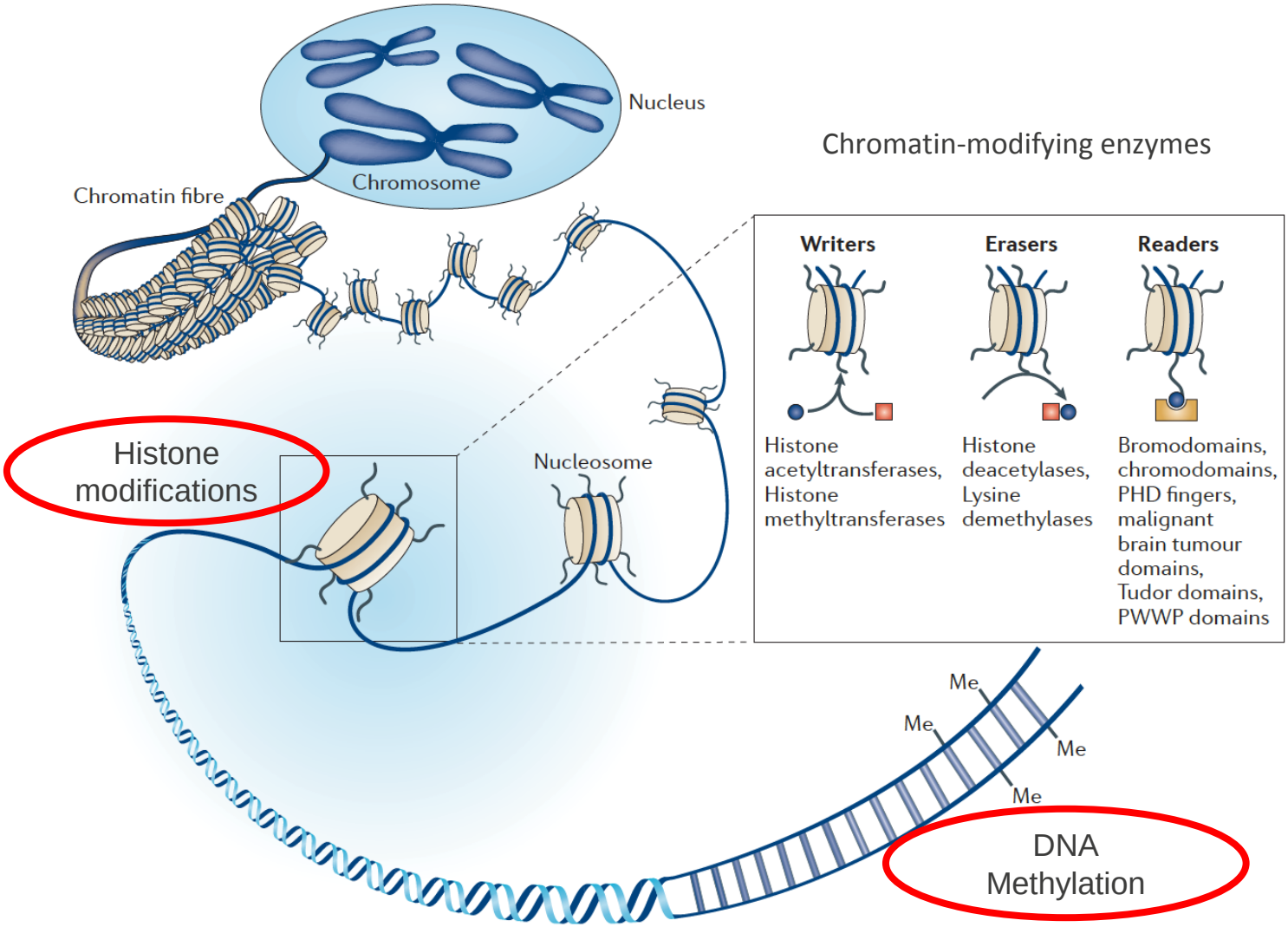
- **Milestones (complete)**
  - Histone modifications verified by immunoblotting
  - Protocol developed for DNA methylation epigenetic assays

- **Milestones (in progress)**
  - DNA methylome sequencing to identify regulated genes
  - EpiEffector genomic and phenotypic outcomes
    - » Completed full genome sequence and annotation of *Tetraselmis striata* for detailed analysis
      Genome announcement *in preparation*

- **Milestones (future)**
  - Genetic analysis for comparative gene regulation between species
  - Integrated strain improvement process



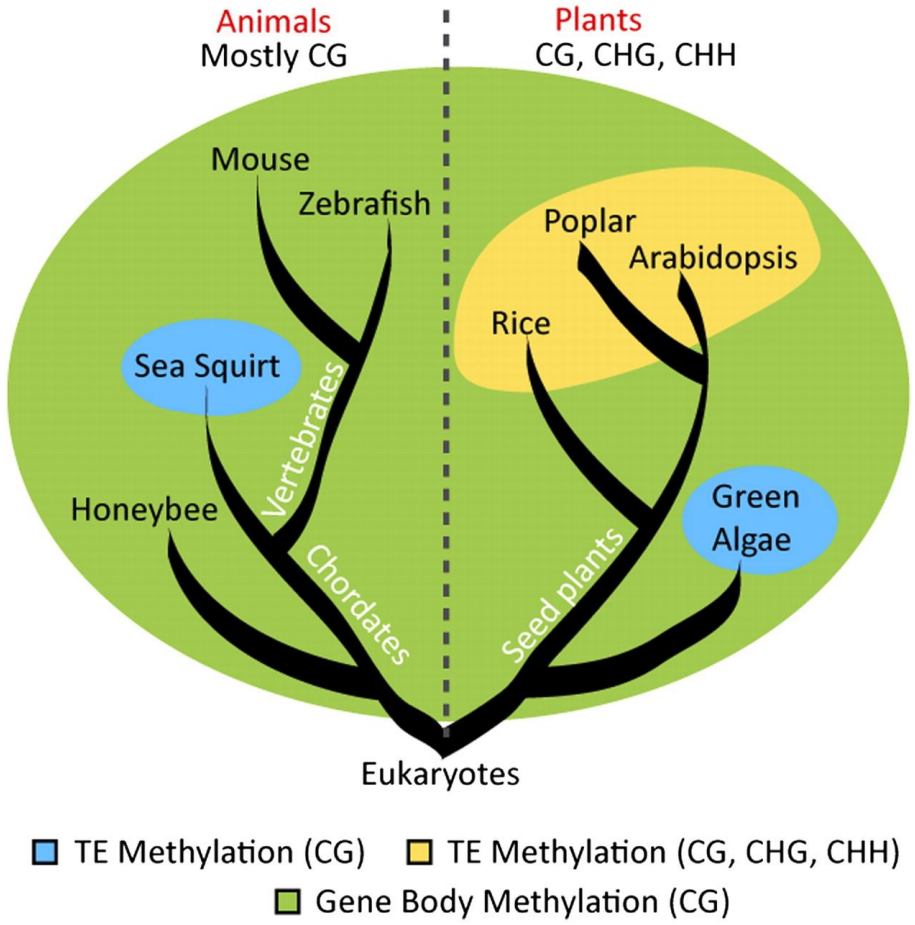
Epigenetics
Determine epigenetic responses and specific epigenome changes



Epigenetics
Genomic profiling using ELISA reveals reduced DNA methylation (DNAme) in microalgae

Eukaryote	Genomic DNAme
*Picochlorum soloecismus**	1.3%
*Nannochloropsis salina**	1.1%
Volvox carteri	1.1%
C. reindhardtii	0.75%
Human brain	4.8%
Arabidopsis (plant)	6.7%

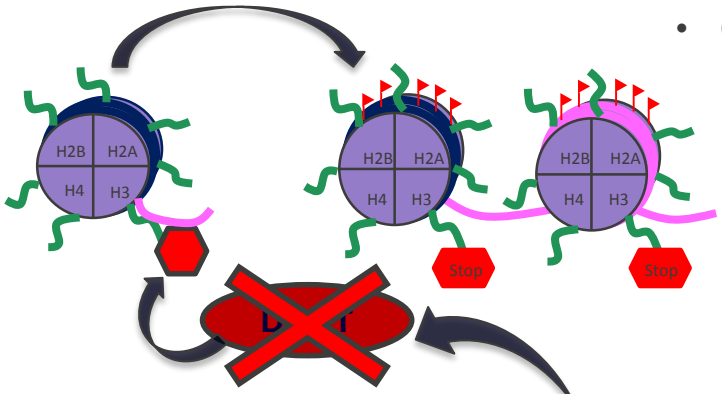
- Developed antibody-based (5mC) ELISA assay for global DNA methylation assessment in algae
- DNA methylation (DNAme) in plants evolved to silence transposable elements
- More complex genomes have greater DNAme on CpGs
- Algae methylate DNA more like plants than animals but have less TE

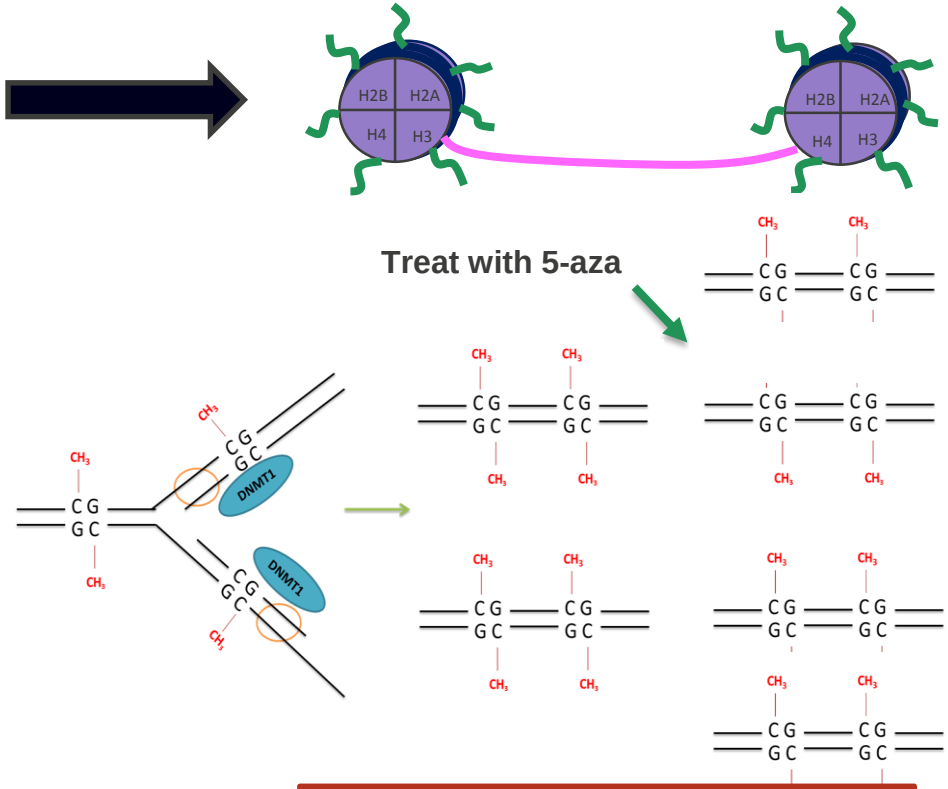


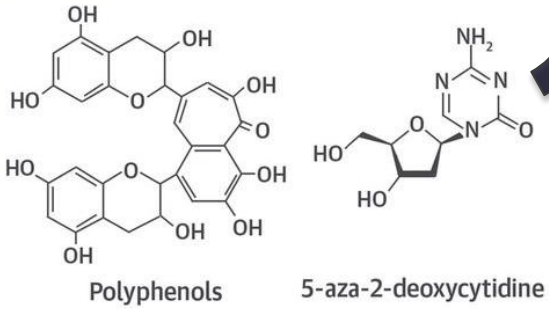
Lee et al 2010 PNAS

Epigenetics
EpiEffectors induce changes in the epigenome

- Treat with 5-aza to inhibit DNMT; loss of methyl groups from DNA
- **Open chromatin** results in more genes being read/expressed







EpiEffectors
Small molecules that alter function of chromatin modifying enzymes (i.e. 5AZA inhibits DNMT)

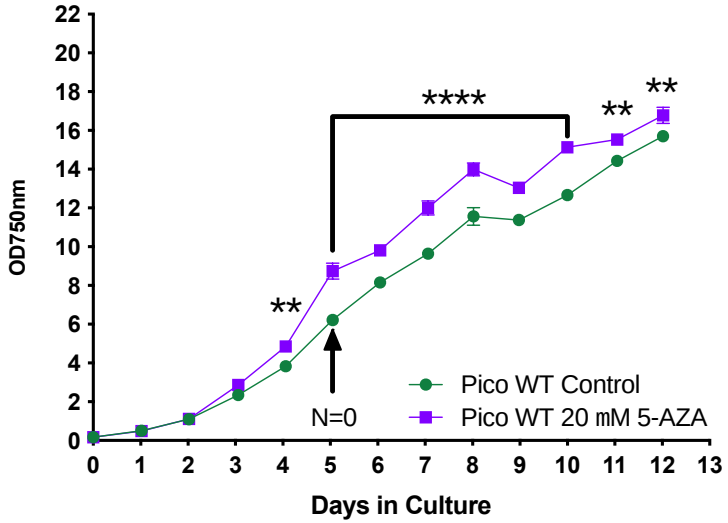
5-aza treatment reduces Picochlorum DNA methylation from 1.3% to 0.8%

Epigenetics

Treatment with methylation inhibitor alters genomic DNA methylation, enhances lipid accumulation but does not slow growth

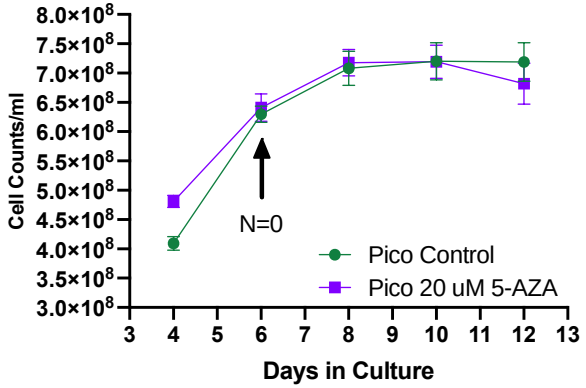
- EpiEffector that inhibits methylation during DNA replication
  - Applied every 24 hours prior to S-phase
- Epigenome sequencing analysis in progress detailing methylation responses
- Similar response across algae species (*Tetraselmis*, *Picochlorum* sorted strain, *Nannochloropsis* in progress)

Growth of *P. soloecismus*



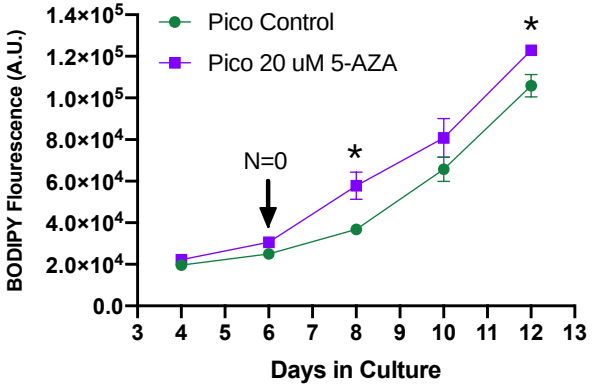
Potentially, loss of DNA methylation results in altered regulation of N stress response

Cell Counts



Cell growth is not affected by 5-aza treatment

Lipid Accumulation



5-aza treatment increases lipid accumulation

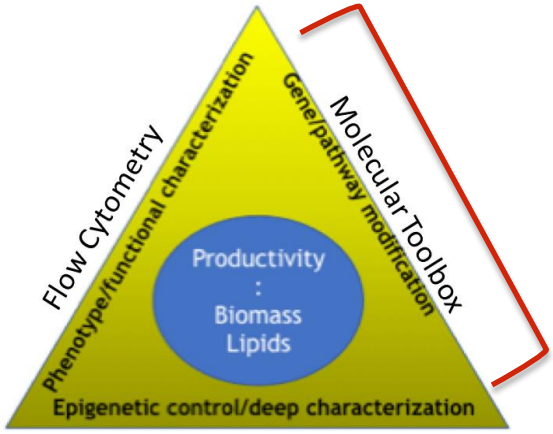
3 – Technical Accomplishments/ Progress/Results

Biotechnology Strain Improvement

- **Milestones (complete)**
  - engineered Cas9 and CpF1 (RNA-guided DNA endonuclease enzymes) expressing lines in *N. salina*

- **Milestones (in progress)**
  - Knock-out lines ABI2, CIPK23 (phosphorylation regulating proteins), Nitrate regulated transcription factor (NIT2), Nitrate transporter (NRT)
  - Overexpression lines cytosolic and chloroplast localized glutamine synthetase (GS), Asparagine synthetase (AS)
    - Stacked GS and AS overexpression into **Cas3** line and **BR3** line
    - Stacked acetyl-co-A carboxylase overexpression into **BR3** line
      - » 50% increase in BODIPY staining

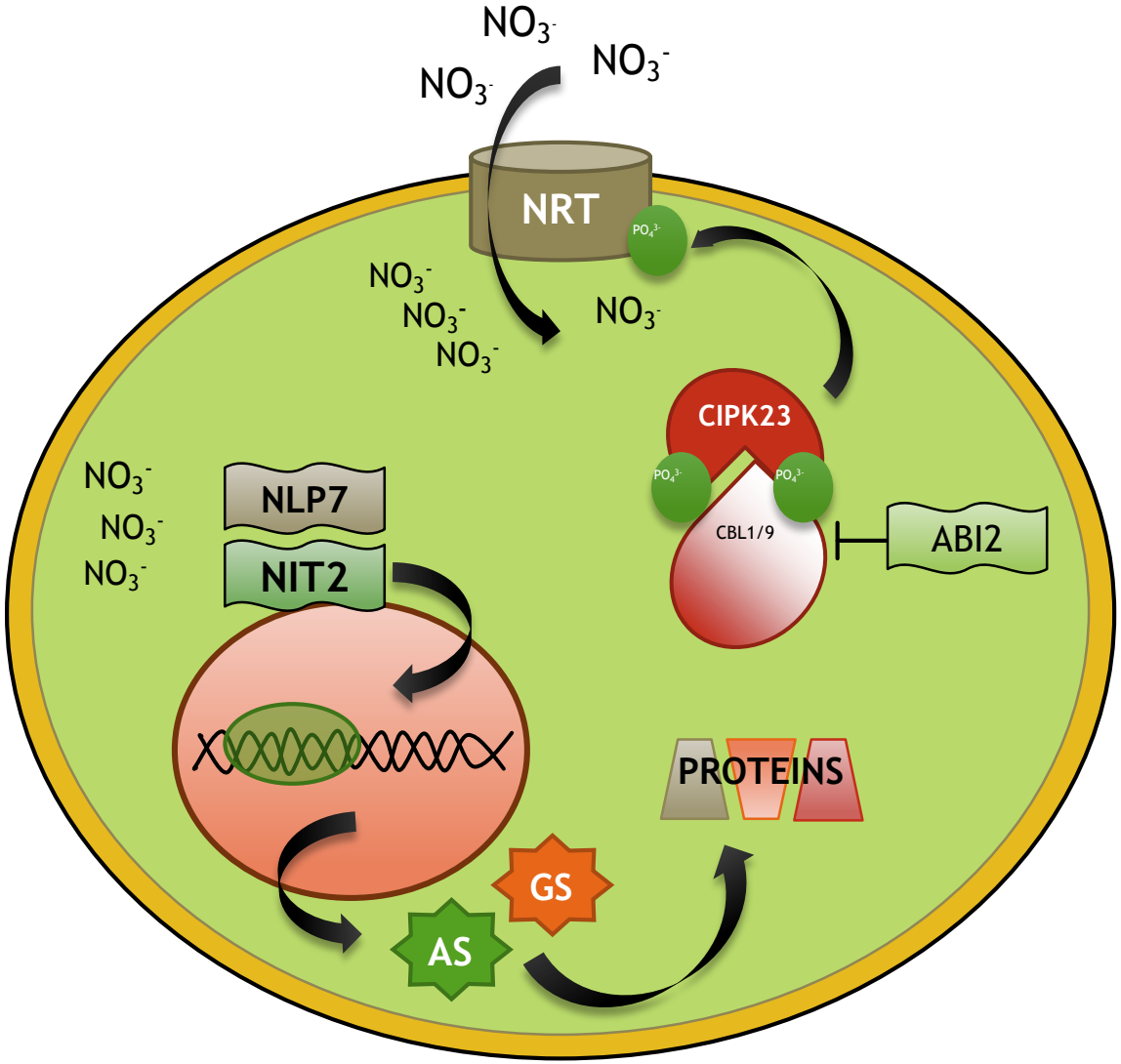
- **Milestones (future)**
  - Transcriptome analysis of N sensing responsive lines for novel gene targets and pathway elucidation
  - Integrated strain improvement process
    - First work completed integrating flow cytometry selection and targeted genome engineering overexpression improving line performance



Epigenetic Techniques

NO_3 Sensing and Signaling

Gene homologs identified through plant genome analysis

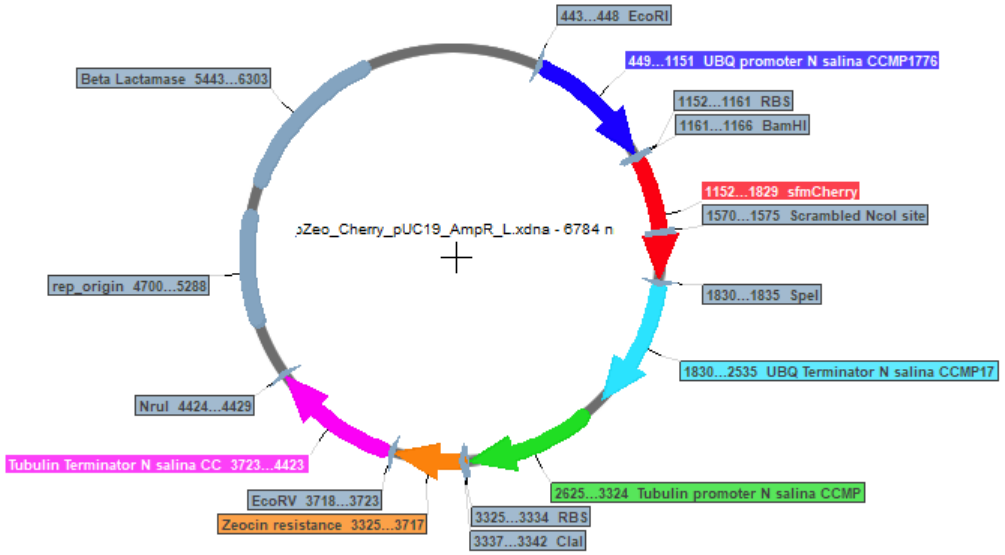


Genome Engineering

Guides targeting multiple genetic sites in the *N. salina* genome designed and validated in vitro

CRISPR Targeted Genes

- Neutral landing sites, Nitrate reductase, O5'PD
- CIPK23
- ABI2
- Nitrate transporter
- NIT2

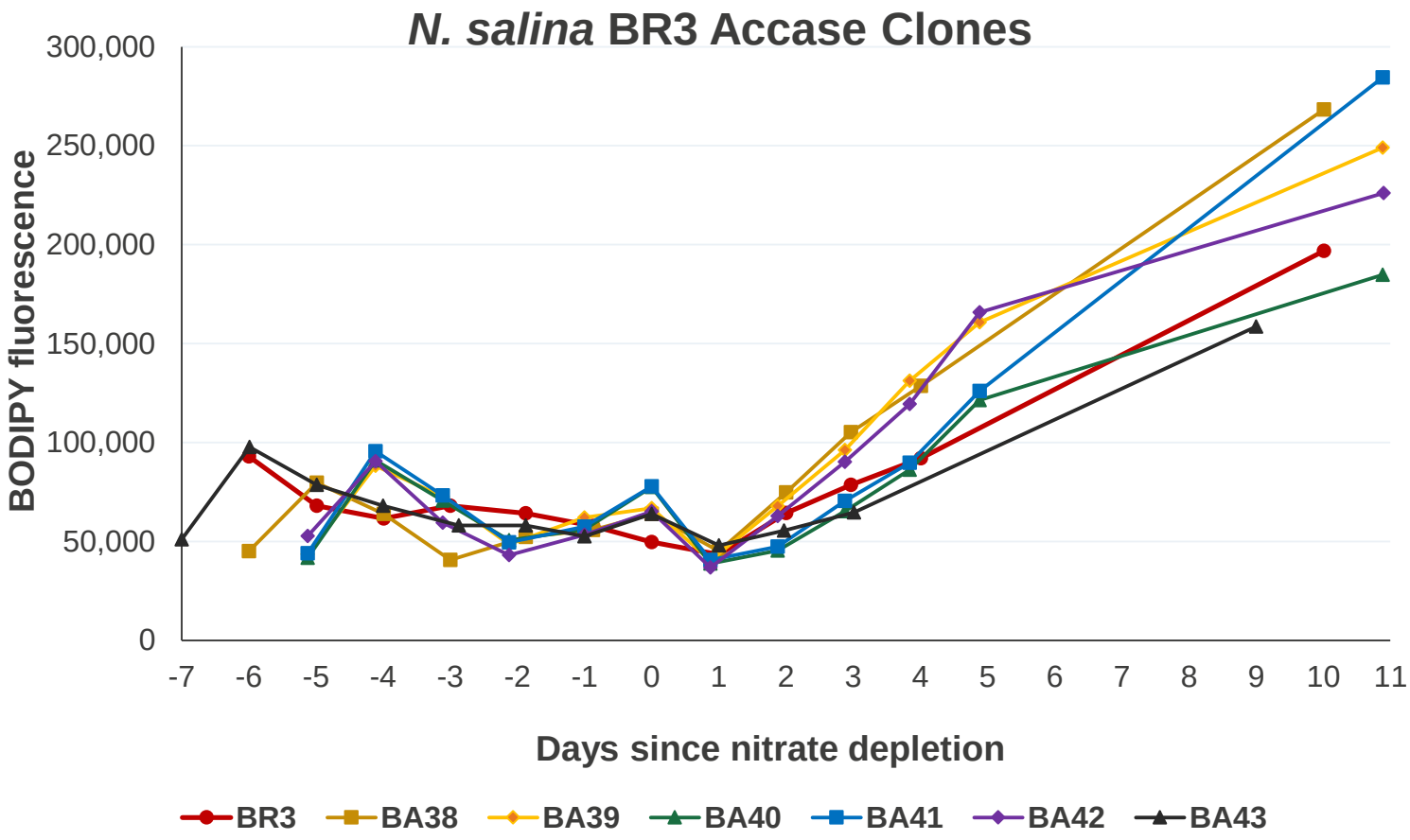


Transformant Selection

- Nitrate reductase
  - Chlorate metabolized to toxic chlorite by active enzyme
  - NR knock-out allows for selection with chlorate/ ammonia
- Orotidine 5-phosphate decarboxylase
  - 5-Fluorotic acid metabolized to toxic product inhibiting uracil metabolism
  - O5PD knock-out allows for selection with 5-FOA/ uracil
- Antibiotic resistance selection cassette (4 options)
- Fluorescent reporters: **rapid expression analysis and flow sorting of transformants**
  - GFP, mCherry
  - B-gal fluorescent metabolite

Preliminary characterization of ACCase overexpression in *N. salina* 1776 BR3 line.

A 50% increase in BODIPY over the parent is observed



Integrating flow sorting improved population with directed genome engineering

4 – Relevance

Delivering improved production strains and novel capabilities for focused strain improvements

Goal

- Design an integrated strain improvement platform utilizing environmental, epigenetic, and genetic factors for targeted advances with rapid, comprehensive phenotyping leading to greater understanding of these modifications.
- **Directly meets BETO mission goals of developing technologies for algae strain improvement to meet biomass yield and productivity goals.**
- Sustainability requires production of high value products and high rates of biomass production through regulated carbon allocation.
- Novel strain improvement technologies will advance and expand domestication of an algae crop for industrial applications and meet BETO milestones.
- Elucidating complex physiological mechanisms provides the basis for novel targeted strategies for both bioproduct and biofuel production.

5 – Future Work to complete milestones

Flow Cytometry
- Application of flow cytometry assays to *Tetraselmis striata*
- Continued flow sorting population improvement

Epigenetics
- Define epigenetic genome methylation profiles for multiple species
- Identify key epigenetically regulated N stress responsive genes
- Define EpiEffector effects on cell physiology

Genetic Engineering
- Characterize targeted gene knock-out transformants and identify N sensing and signaling responsive elements
- Determine N assimilation responses for assimilation enzyme
- Over-expression transformants

******Integrate capabilities into strain improvement method***
- *Go/No-Go Decisions*
  - Multiple individual approaches for line improvement will be performed in parallel with integrated complementary selection to create methods for stable improved productivity.

Summary

1. Overview	Capability development for identifying key gene targets, functional pathways, and novel regulatory mechanisms
2. Approach	Integrating flow cytometry, epigenetic characterization, and genome engineering
3. Progress	Unique population improvement parameters, novel EpiEffector modification responses, expanded CRISPR toolbox.
4. Relevance	Strain improvement methods for Improved productivity, environmental resilience, and increased sustainability to meet BETO milestones.
5. Future Work	Select improved cell populations based on physiological flow cytometry assays. Determine how epigenome manipulation affects physiological and phenotypic outcomes. Expand the molecular toolbox for targeted knock-outs to identify novel responsive genes. **Integrate new capabilities into a strain improvement strategy.**

- **Additional Slides**

Responses to Previous Reviewers' Comments

- "**Overall, continued improvement in the creation of genetic information on potential cultivation species is important.**"
  - We have expanded the exploration of genetic regulation in algae through novel developments in epigenome profiling, transcriptome comparative analysis of improved populations (BR3), and genome sequencing and annotation of new strains (*Tetraselmis striata*).

- "**Future cytometry work is perhaps of less value, particularly if it is envisioned as a real-time diagnostic tool.**"
  - We have focused the application of flow cytometry away from diagnostics towards supplemental physiological characterization and novel trait improvement tools. Physiological assays allow for greater characterization of both primary (expected targets) and secondary metabolic changes. Assays can be combined in one analysis allowing for rapid cell measurements. These assays can also be used to select functional individual cells from within a population for non-standard line improvements. For example, we have created the stable *N. salina* line BR3 selected for lipid accumulation during N replete conditions. This line has been utilized for comparative genetic analysis, genome engineering, and epigenome responses to stress. Its phenotype has been validated in outdoor trials.

Publications and Presentations

- **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.
- **Functional and phenotypic flow cytometry characterization of *Picochlorum soloecismus* DOE101 isolates**, Christina R. Steadman Tyler*, Claire K. Sanders, Reece S. Erikson, Taraka T. Dale, Scott N. Twary, Babetta L. Marrone. 2019. submitted, *Algal Research.*
- **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. submitted, *Physical Biology.*
- **High quality complete genome of the algae *Tetraselmis striata (Chlorophyta)* generated from PacBio sequencing**, Christina R. Steadman Tyler, Blake T Hovde, Hajnalka E Daligault, Yuliya Kunde, Babetta Marrone, Scott N. Twary, Shawn R Starkenburg, *In preparation.*

Presentations continued:

- 2019. 34th Congress of the International Society for Advancement of Cytometry. **Monitoring Cell Cycle and Lipid Accumulation in Microalgae.** Claire K. Sanders, Babetta L. Marrone, Scott N. Twary
- 2019. The 9th International Conference on Algal Biomass, Biofuels and Bioproducts. **Epigenetic manipulation of the DNA methylome in algae alters productivity**. Christina R. Steadman, Scott N. Twary, Babetta L. Marrone.
- 2019. University of Miami Biochemistry and Molecular Biology Research Day. Miami. FL. **Cell Cycle Regulation in Microalgae.** Claire K. Sanders, Babetta L. Marrone, Taraka Dale, Scott N. Twary.
- 2018 The 8th International Conference on Algal Biomass, Biofuels and Bioproducts. **Growth parameters of synchronized algal cultures and relation to genetic transformation efficiency**. Claire Sanders, Babetta Marrone, Taraka Dale, Cesar Raul Gonzalez-Esquer, Attelia Hollander, Scott Twary.
- 2017 The 7th International Conference on Algal Biomass, Biofuels, and Bioproducts. **Phenotypic and functional characterization of microalgae species using novel molecular flow cytometry analysis**. Christina R. Tyler, Claire K. Sanders, Scott N. Twary, Babetta L. Marrone.

Presentations continued:

- Twary: Fourth year as Chair of organizing committee Biofuels and Biomanufacturing sessions (three sessions) at TechConnect World 2016-2019.
  - 2018 TechConnect World Innovation Conference. Invited Speaker. **Managing Carbon Use Efficiency in Algae Ponds for Sustainable Production**. Scott Twary.

- **ATP^3 (Algae Testbed Public-Private Partnership) Workshop: Large-Scale Algal Cultivation, Harvesting and Downstream Processing.**

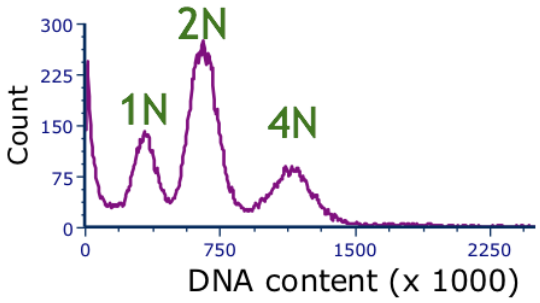
  Claire Sanders: Lecture and Lab sessions

  Flow Sorting for Desirable Traits task within the Algal Flow Cytometry Laboratory Module and lecture on Algal Flow Cytometry. May 2017, Los Alamos, NM.

Cell Cycle Analysis

We sought to look at DNA replication and cell division to see if we could identify a time point more amenable to transformation.

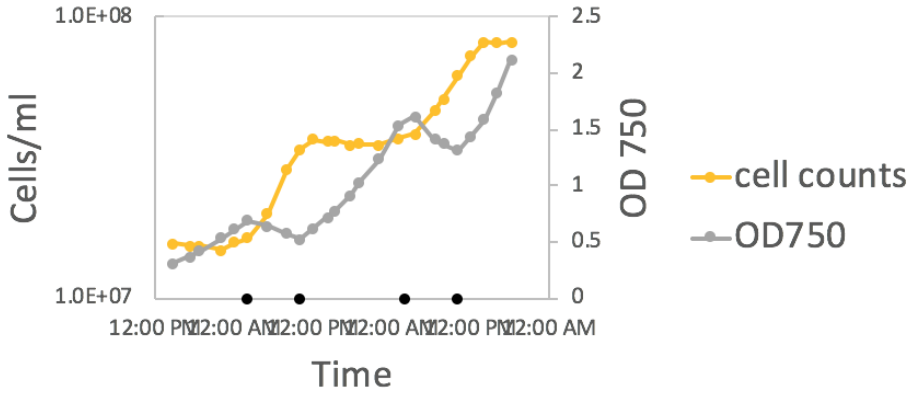
- *N. salina* is thought to be
  - haploid, 1N
  - replicate to diploid, 2N
  - divide back to 1N
  - This results in 2x cell number after each division



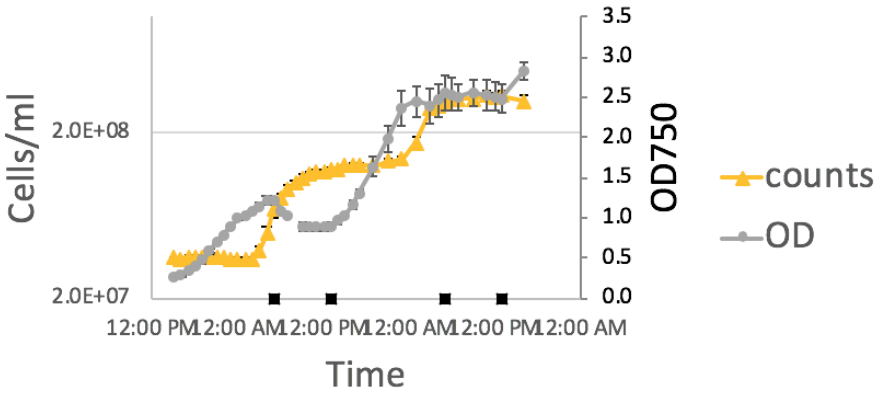
- Three stoichiometric DNA binding dyes were tested
  - DyeCycle Orange
    - Ideal for multiplexing with other assays due to spectral emission
    - Toxic to cells
  - Syto 9
    - Stains all nucleic acids
    - Toxic to cells
  - Hoechst 33342 – This has been used for sorting
    - Non-toxic
    - UV excitation required, making instrumentation more limiting
- We found a sub-population of cells that replicated to 4N prior to cell division
  - This population was greater in BR3 than the parent
    - This is probably the reason for increased growth rate
    - This plan: sort on this 4N population

Synchronized cell culture growth in relation to light:dark cycle

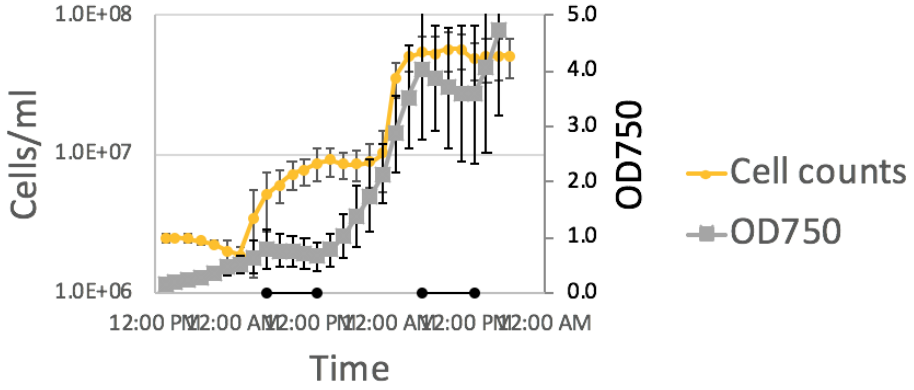
Nannochloropsis salina



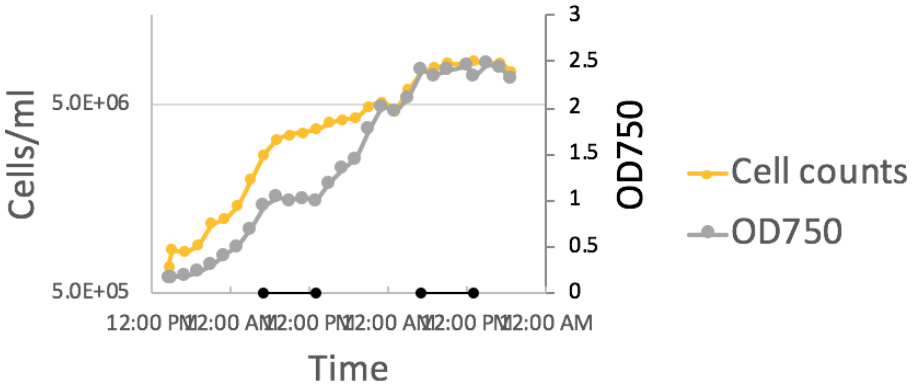
Picochlorum soloecismus



Chlorella sorokiniana



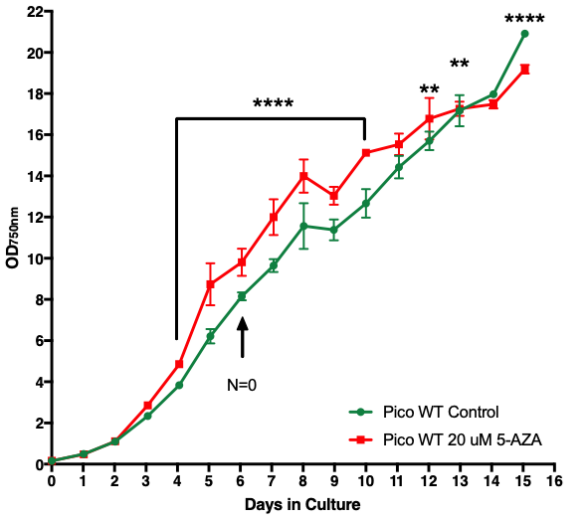
Tetraselmis striata



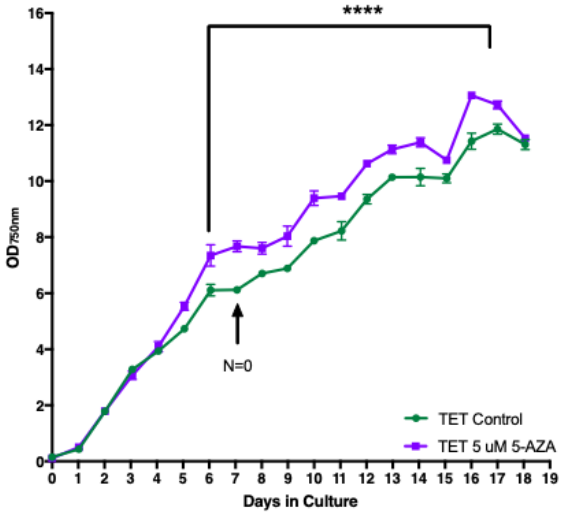
5-Azacytidine Treatment (EpiEffector)

- EpiEffector that inhibits methylation during DNA replication
  - Applied every 24 hours prior to S-phase

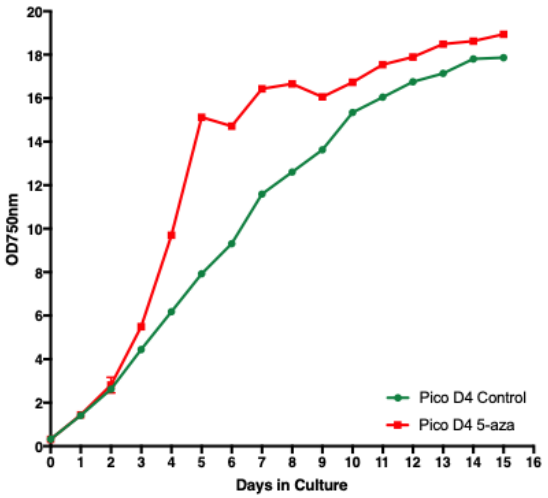
Picochlorum



Tetraselmis



Picochlorum D4



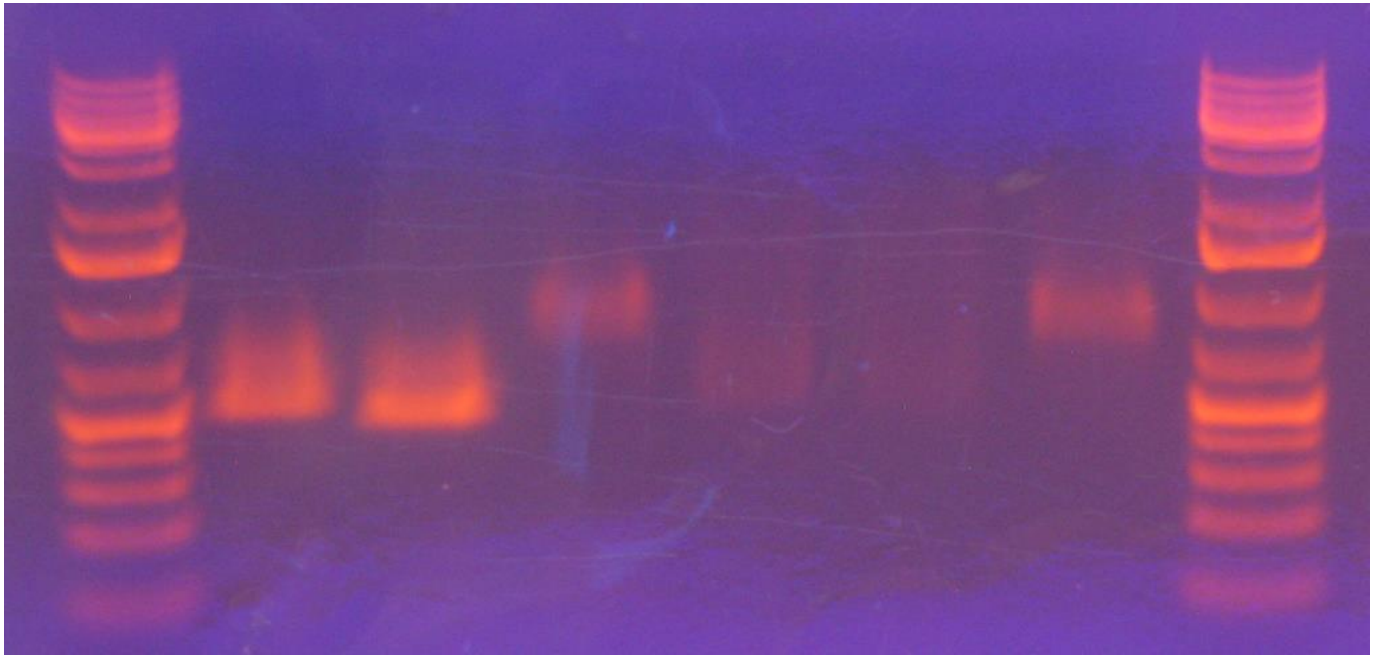
- Similar response across algae species (*Nannochloropsis* in progress)
- Preliminary analysis suggests cell growth is not affected but lipid accumulation increases
- Epigenome sequencing analysis in progress detailing methylation responses

qPCR of Cas9 expressing *N. salina* and *N. gaditana* transformants validating gene insertion and expression

3 primer sets spanned across the large Cas9 nucleotide sequence

N. salina cDNA

N. gaditana cDNA



Overexpression of N Assimilation Gene (AS) in *N. salina* 1776 results in greater rates on nitrate uptake and faster early growth.