SANDIA BIOSCIENCE

SAND2016-7763PE

# Engineering 'Green' Algae: Reducing Metabolic Waste for High Biomass Productivity

BSAP Review

8.17.2016

Anne Ruffing, Todd Lane, Jordan McEwen, Pam Lane, and Lucas Strickland

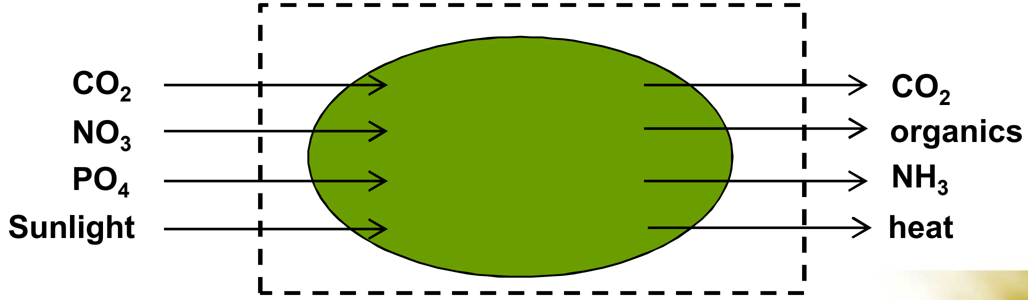
Funding: LDRD – Biosciences (FY16-FY18)

# Problem Statement: Algal Biofuels = Biomass Productivity

- Biomass productivity limits economic feasibility of algal biofuels
- BETO calls targeting algal biomass productivity
  - 2014 TABB – Targeted Algal Biofuels and Bioproducts
  - 2013/2016 ABY – Advancements in Algal Biomass Yield
- BETO goals from Multi-Year Program Plan (MYPP) March 2016
  - By 2018, demonstrate algal productivity of 2,500 gal/acre/yr
  - By 2020, demonstrate algal productivity of 3,700 gal/acre/yr
  - By 2025, demonstrate algal productivity greater than 5,000 gal/acre/yr

$$\frac{dBiomass}{dt} = \sum \dot{m}_{in} + \sum \dot{m}_{generation} - \sum \dot{m}_{out} - \sum \dot{m}_{consumption}$$

- **Light harvesting**
- **Photosynthetic efficiency**
- **Carbon Fixation**



# Hypothesis:
## Eliminating Metabolic Waste in Natural Algal Metabolism Will Improve Biomass Productivity

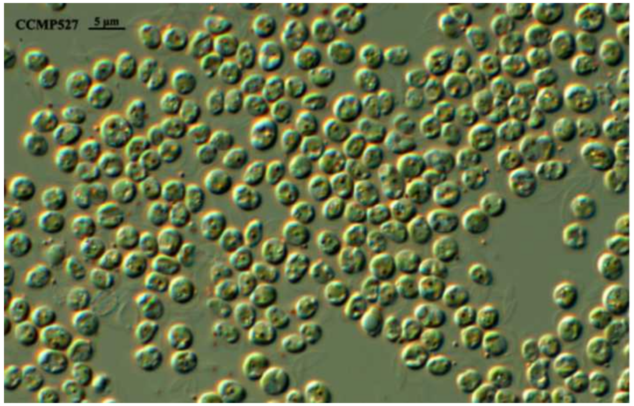
- Dark Respiration
  - Loss of $CO_2$ via glycolysis and TCA cycle
  - Generates $H_2O_2$
  - Accounts for 20 - 60% loss of fixed carbon **daily**[1]
- Photorespiration
  - RuBisCO may utilize $O_2$ as substrate
  - Results in net loss of 1 to 3 $CO_2$ and 1 $NH_3$
  - Accounts for 15 - 55% rate of $CO_2$ fixation[2-3]

**Net improvement in biomass productivity = 35-115%**

1. Geider R. (1992) Respiration: Taxation without representation. 333-360.
2. Goyal A. (2002) Physiologia Plantarum 116:264-270.
3. Moroney JV et al. (1986) Plant Physiol. 82: 821-6.

# *Nannochloropsis gaditana*: An 'Industrial' Alga

- *Nannochloropsis* species are hardy algae, shown to thrive under a diverse range of environmental conditions
- *Nannochloropsis* species are reported to have high lipid content (~50%) and fast growth (20 $g/m^2/day$)[4]




- *Nannochloropsis* species are under investigation for commercial application (ATP3, Sapphire, Cellana, NAABB)
- Draft genome sequence and publically available[5]
- Little information about dark respiration or photorespiration in this species.

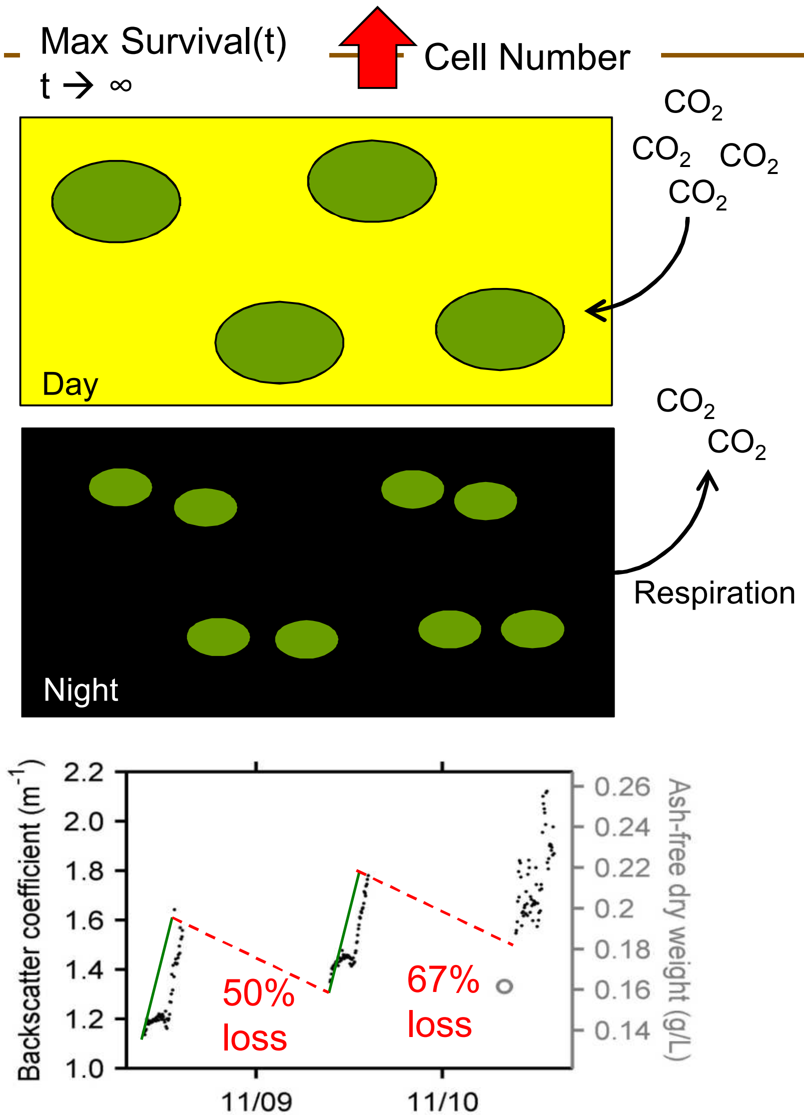
While this effort will focus on *N. gaditana*, the dark respiration and photorespiration targets and genetic tools developed in this work are likely applicable to other 'industrial' algal strains.

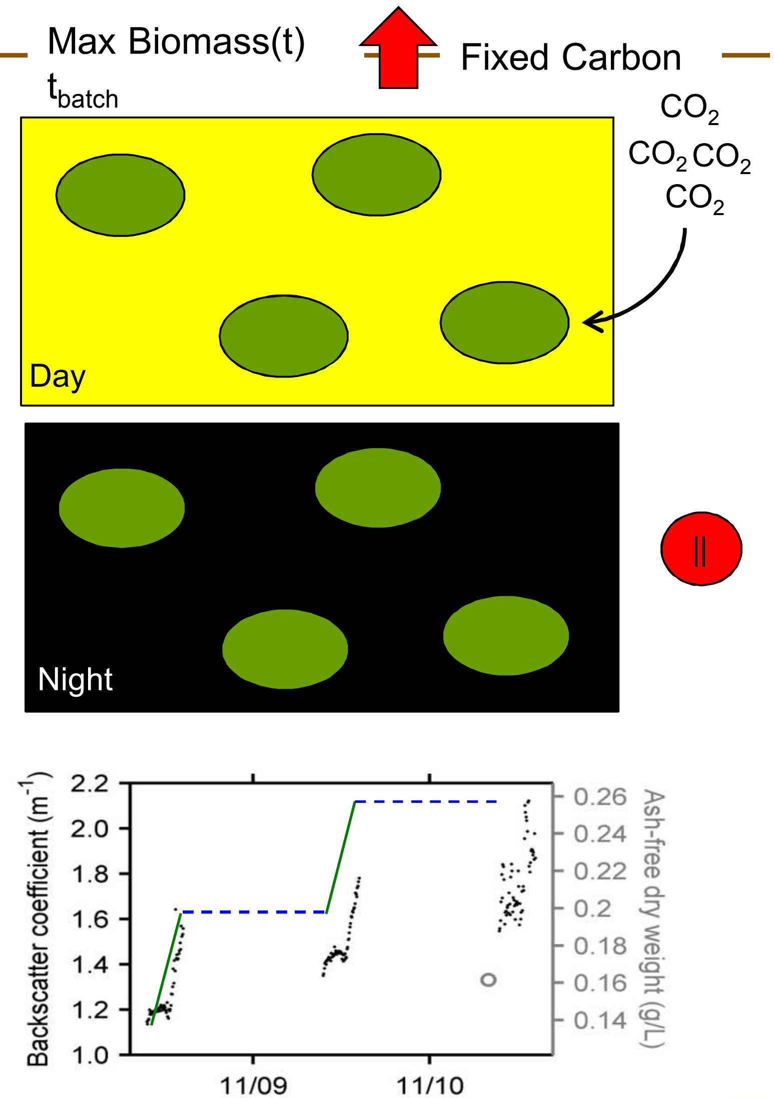
4. Jinkerson et al. (2013) Bioengineered. 4(1):37-43.
5. Radakovits et al. (2012) Nature Comm.

# Project Goals

- Develop CRISPR/Cas9 genome editing tools for *Nannochloropsis gaditana*
- Investigate dark respiration using chemical inhibitors
- Investigate glycolate production and excretion in *N. gaditana* (photorespiration)
- Target dark respiration in *N. gaditana* using CRISPR/Cas9 tools
- Target photorespiration in *N. gaditana* using CRISPR/Cas9 tools
- Demonstrate scale-up potential of modified *N. gaditana*

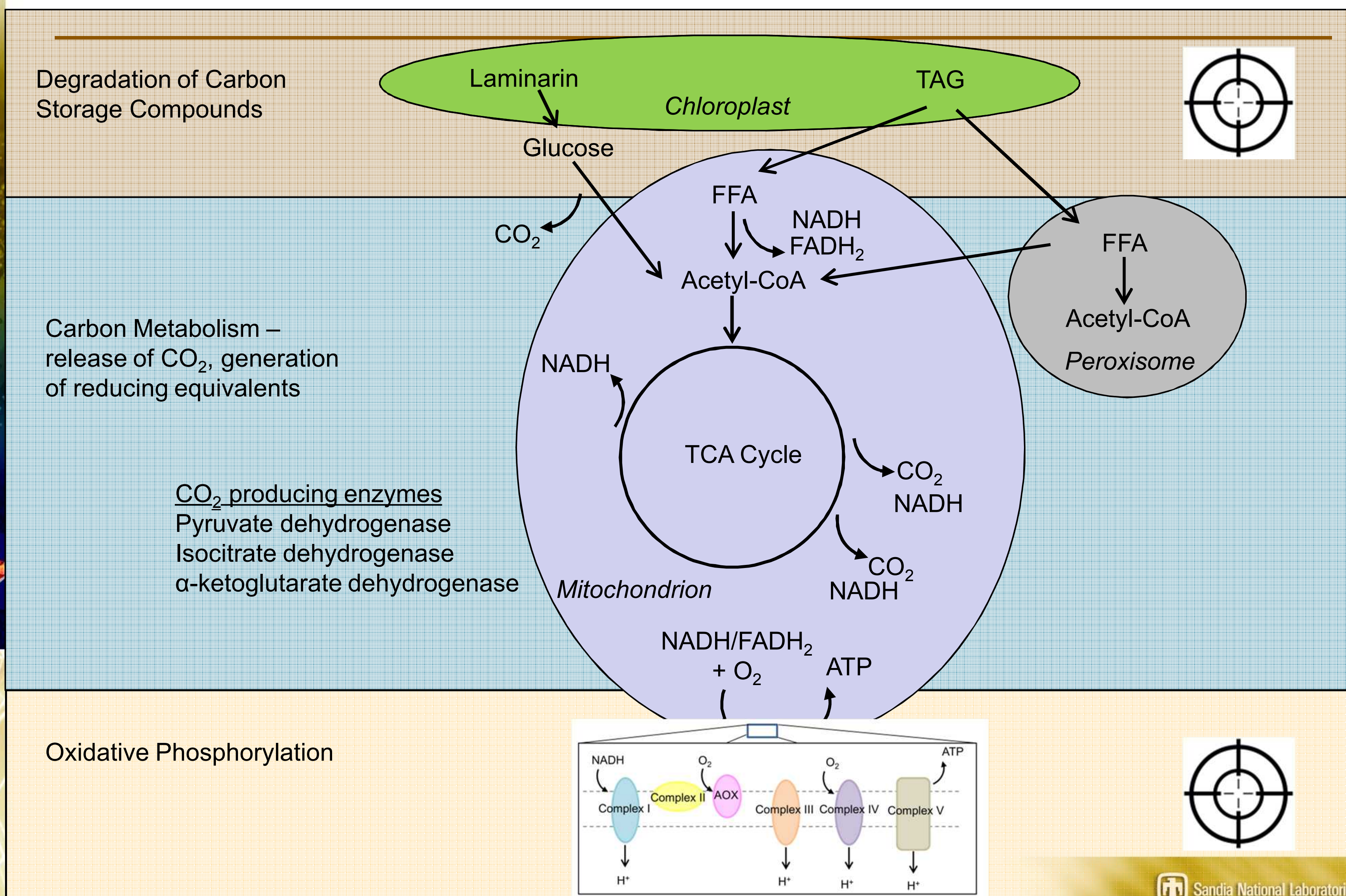
# Dark Respiration: Survival vs. Biomass



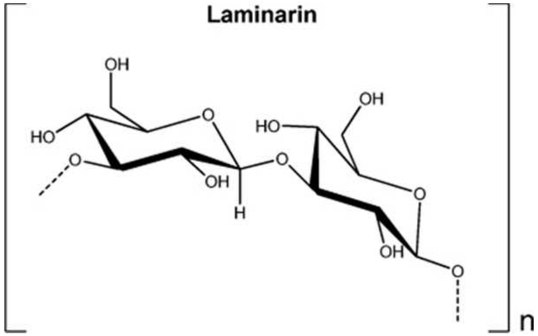




6. ATP3 data for Nannochloropsis salina in mini-raceway ponds at ASU – Tom Reichardt

# Technical Approach: Target Dark Respiration



# Respiration Targets: Laminarin and TAG Degradation

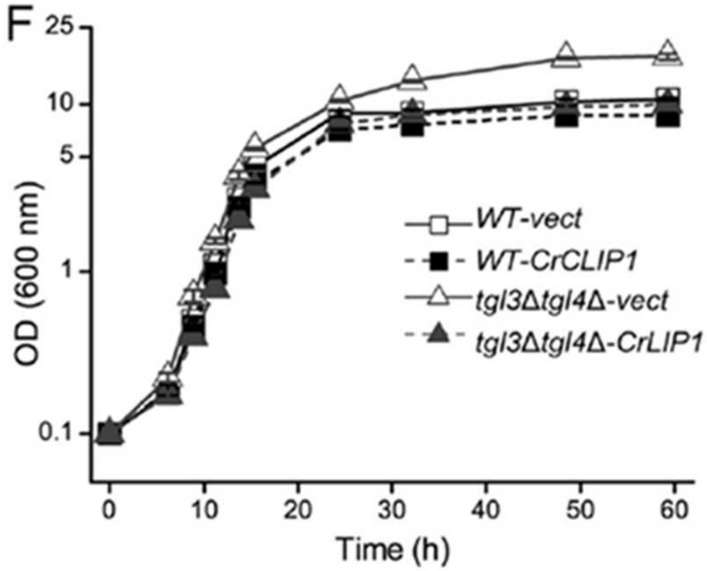



Laminarin

- Laminarin – β1,3-glucan with β1,6 branches
- Degraded by laminarinase
- A single laminarinase is present in *N. gaditana* (Nga02655)

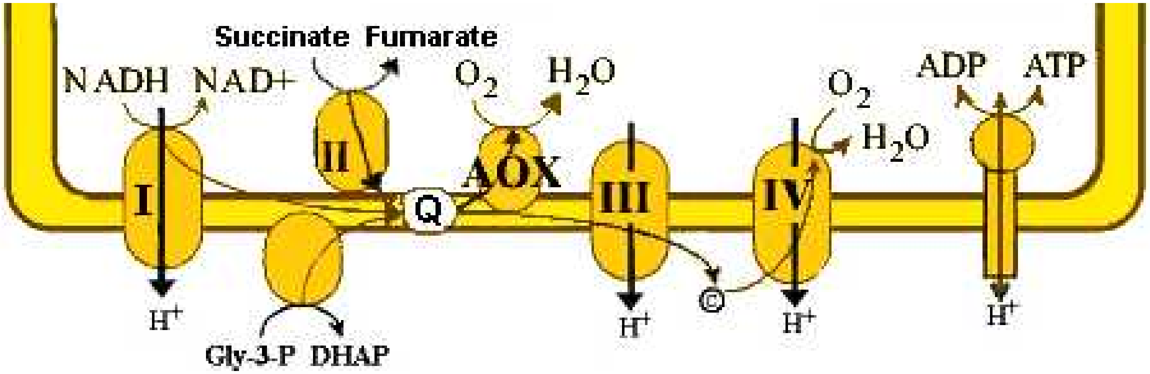
TAG

- TAG – triacylglyceride – glycerol backbone with fatty acid side chains
- Degraded by lipases
- ~ 20-30% increase in OD with *Saccharomyces cerevisiae* Δ*tgl3*Δ*tgl4* mutant (lacking TAG lipases)
- Higher cell diameters for *S. cerevisiae* Δ*tgl3*Δ*tgl4* mutant




7. Li X, et al. (2012) Eukaryotic Cell 11(12): 1451-1462.

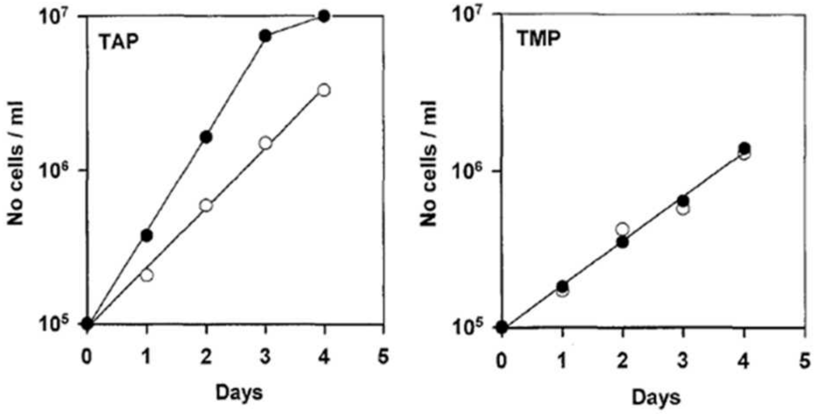
# Respiration Targets: Oxidative Phosphorylation




Complex I: NADH dehydrogenase
Complex II: succinate dehydrogenase
AOX: alternative oxidase
Complex III: cytochrome bc1 complex
Complex IV: cytochrome C oxidase
Complex V: ATP synthase

- Algae have 2 pathways for oxidative phosphorylation: cytochrome C and alternative oxidase pathways
- *Chlamydomonas reinhardtii* mutants of complexes III and IV show no change in photoautotrophic growth under continuous light

**Mixotrophic**



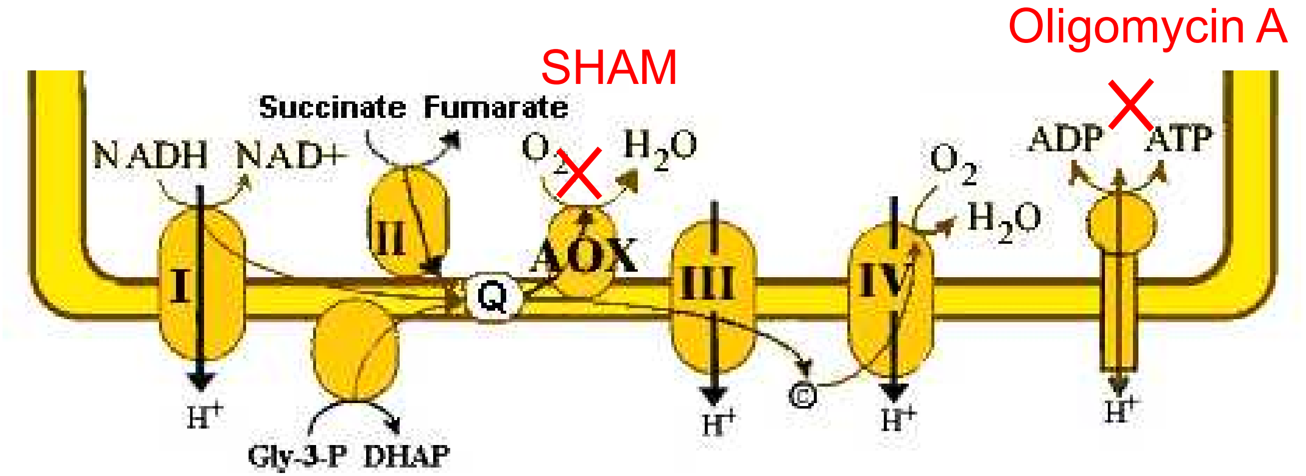

**Photoautotrophic**
(continuous light)

●Wild type
○Complex IV mutant

8. Collin et al. (1995) Mol. Gen. Genet. 249: 179-184.

# Physiological Studies

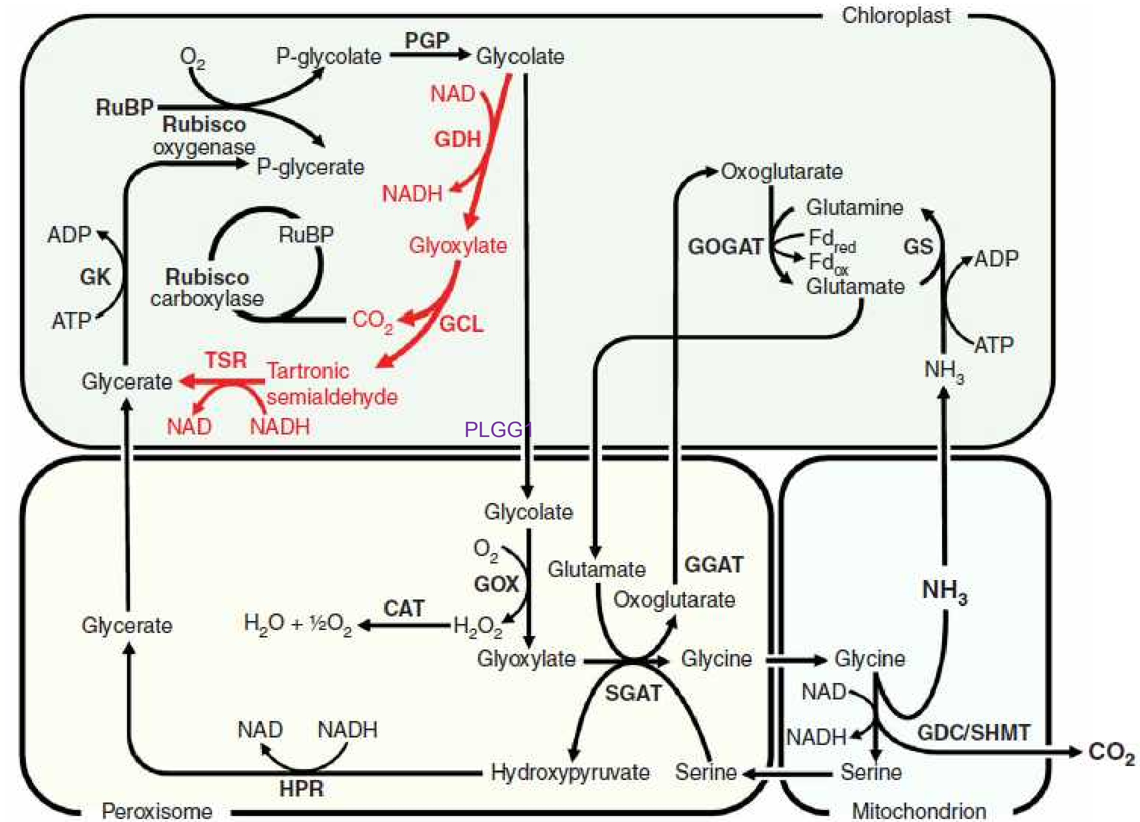
- Quantitative diurnal measurement of laminarin and TAG accumulation/degradation in *N. gaditana*
- Determine the effect of dark respiration inhibitors on overall biomass productivity in *N. gaditana*
  - Oligomycin A
  - Salicylhydroxamic acid (SHAM)
- Measure glycolate excretion in *N. gaditana* under high and low $CO_2$ conditions

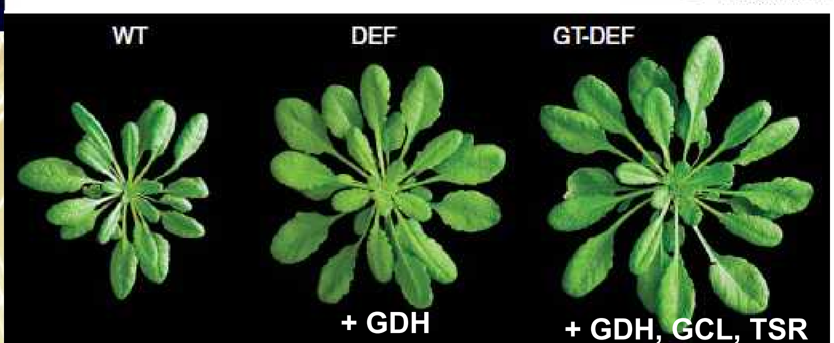


# Photorespiration in Plants

Bacterial photorespiratory bypass pathway

- Does not require re-fixation of $NH_3$ (ATP consuming)
- Does not generate $H_2O_2$
- Releases $CO_2$ in chloroplast, near RuBisCO
- Reduced number of transport reactions



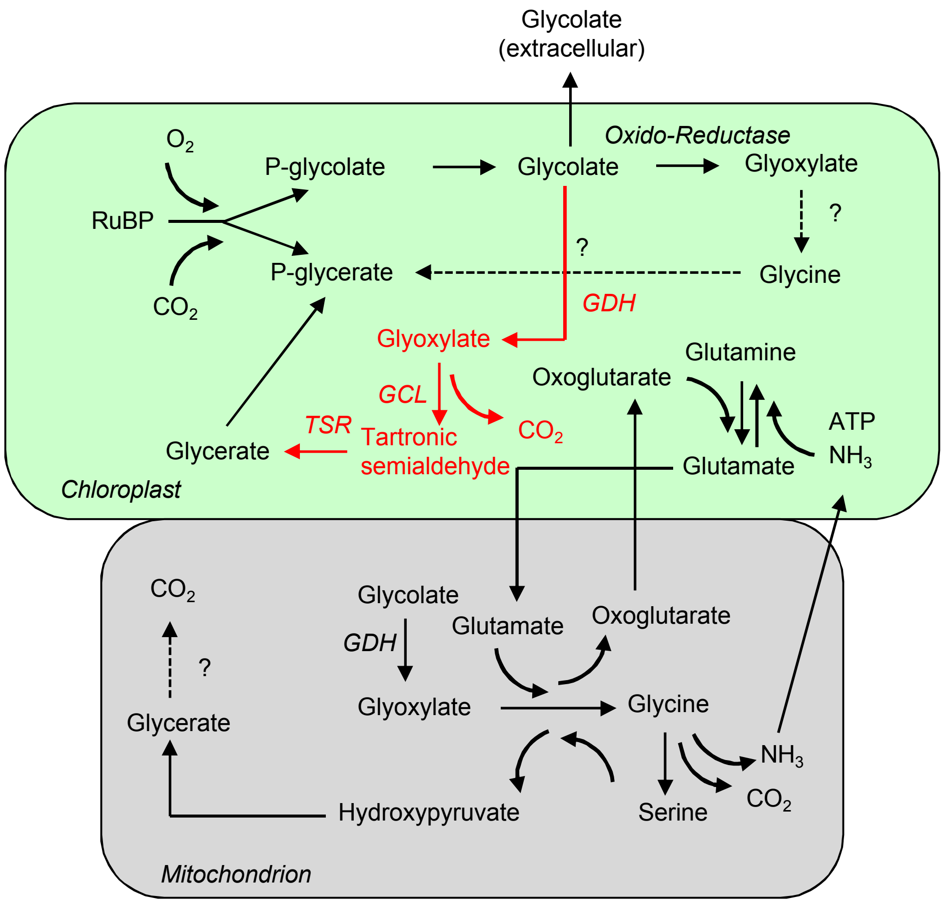




- Expression of bacterial photorespiratory bypass pathway in *Arabidopsis thaliana* enhanced biomass productivity

9. Kebeish et al. (2007) Nature Biotech.25(5):593-599.

# Photorespiration Targets in Algae

- Algae do not have glycolate oxidase
  - No $H_2O_2$ generation
- Evidence suggests that glycolate metabolism is present in both chloroplasts and mitochontria[2]
- Glycolate is excreted by algae (net loss of 3 $CO_2$)
- Glycolate can be fully metabolized rather than recycled (net loss of 3 $CO_2$)
- $NH_3$ loss, ATP consumed to re-fix




2. Goyal A. (2002) Physiologica Plantarum 116: 264-270.

# List of Targets for *N. gaditana*

| Pathway | Target Description | Gene Target |
|---|---|---|
| Laminarin degradation | Laminarinase | Nga02655 |
| TAG degradation | TAG lipase CrLIP1 (*Chlamydomonas reinhardtii*) | Nga01367 |
| | TGL3/TGL4/TGL5 (yeast)<br>SDP1 (*Arabidopsis thaliana*) | Nga03028 |
| Dark respiration | Cytochrome c oxidase COX1 | Nga50030, Nga50029 |
| | Alternative oxidase AOX1 | Nga03289 |
| Photorespiration | Glycolate dehydrogenase | *glcD* (*E. coli*) |
| | Glycolate carboxyligase | glcE (*E. coli*) |
| | Tartronic semialdehyde reductase | *glcF* (*E. coli*) |

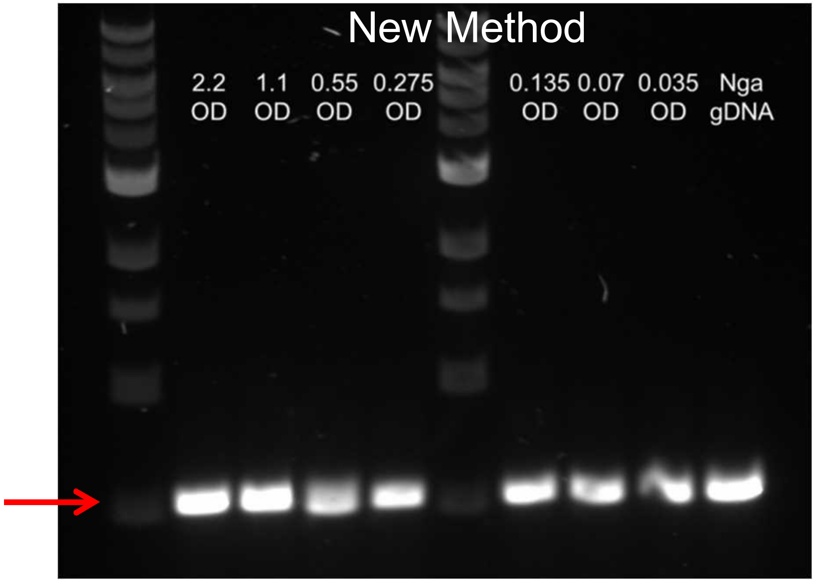
# RESULTS

# Protocol Optimizations for *N. gaditana*

- Enriched F/2 determined to be best growth medium for *N. gaditana* (out of 5 media)
- Zeocin resistance, conferred by *ble*, chosen as selection marker
- Baseline measurements of oxygen evolution (i.e. rate of photosynthesis) and oxygen consumption (i.e. rate of dark respiration) for *N. gaditana*
- Established method for gDNA isolation (for RFLP analysis)
- Established method for quantifying lipid content of *N. gaditana* (to assess lipase mutants)
- Established method for measuring glycolate (product of photorespiration)

# Development of PCR Screening Method for *N. gaditana*

- PCR screening of *N. gaditana* – Literature reports gDNA isolation of each candidate for subsequent screening[11]
- Traditional colony PCR used for bacterial screening is unsuccessful (tough algal cell wall)
- Modified method developed for colony PCR:
  - Heat 1 hour at 95˚C to break down cell wall
  - Traditional PCR amplification
  - Demonstrated screening of β-tubulin promoter




11. Kilian et al. 2011 PNAS 108(52): 21265-21269.

# Bioinformatics to Identify Target sgRNA Sequences

Program: CRISPRseek - R based software package available from Bioconductor

Selection criteria for sgRNAs for gene knockout:

- Select for sgRNAs with zero predicted off-targets (max mismatch = 4)
- Select sgRNAs with highest gRNA efficacy score
- Use two highly rated sgRNA targets with > 100 bp cut along with integration cassette (1 kb homology arms and *ble* resistance) for selection

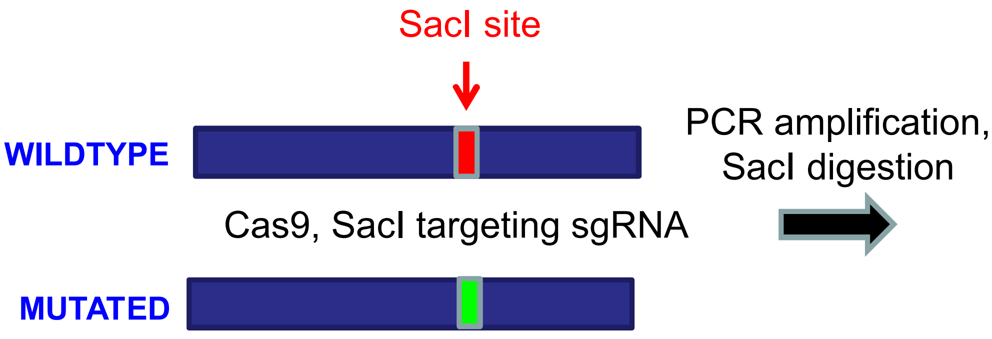
**Nga01367 - lipase**

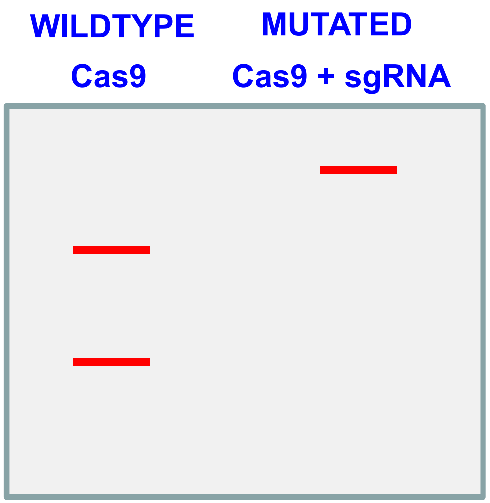
| names | gRNA efficacy | gRNAsPlusPAM | top5OfftargetTotalScore |
|---|---|---|---|
| Nga01367_genome_gR187f | 0.808532 | GGGCGGGTGGGTAAGCCGCGCGG | NA |
| Nga01367_genome_gR248r | 0.821709 | GTATCGGCTGAAGAGCTCCAAGG | NA |
| Nga01367_genome_gR72f | 0.882135 | TTAAAGACCGGATCGTCTGGCGG | NA |
| Nga01367_genome_gR1502r | 0.896311 | GATCGAGTAACGGTTCGAGGCGG | NA |

```
>Nga01367_genome
ATGGTCCCGGCCCGGGTGTGCGGCGCCTGCAAGGGCCAGCTGGAGGACG
AGGACCTTAAAGACCGGATCGTCTGGCGGATGGTGCGGGTCGAGGCCTTTCTGAAGGACCGTTTGATCCCTTACTTTGCC
CCGGGGGACCATACGCAGCTGGACAGGGCCTTGCGGACGGTGGGCGGGTGGGTAAGCCGCGCGGCGCGCCGGGCCCCGCC
CCTGCGCAGCACAACGGCCTTGGCTGGCGAGGCCTTGGAGCTCTTCAGCCGATACGGGTACGCGGGCGTAGCGGGGTCT
TGCTTCGGCACGAGCACGTGGAGGCGGTCGAGCTGCTCAAGGAGGTGTCGGGGGTGGACGCCGCCTGGCCCGTGACGGGA
AGCCAGCTGAGCGCGGCGATGTACTACCTGCTGGCACGGGGAAGGGGGGAGCGAGGAGCGGCTCCCGATGCCGAGCAGGA
GGCGCACCGAGGATGCCCACCGGCCTCGGACAGCTTGATGCAGGACCTGTTGGACGTGGCGCCCTTGGCCTTGCATTTCG
CCTACTGCGACAACCTGGTGGAGATGCAACTCAAGGCACAGCAGCAAGGGTGGCGGTTGGTGTTCGCCTACGCGCCCCCC
GCCGCCCAGGCTGGGCAGCCGGCCTTCGTCTTGCTCTGCCACTTGACCGAAAAGGAGGCTTGCCTGGTGGTGCGTGGTCC
CGACCGGGCGCAGGATGTTCTCGTCGACATCCGGGGCCTGCCCATGCCCTTTCCCCTCGCGGGGGAGGGGGCCGGGAGCG
GGGAGAAGGGCAGTCAGGACAAGGAGAGCGGGTGGGCGAACGTCTCGACGGAGTGGATGGCTTCCTGTGGAGCGGCCGAG
GCGGGCCATTGGCTCTTCAGCGAGGTCTACCCCCATCTCCATCGGCTGGCCAAGGAGGGCTACTCCTTGACGCTGGCGGG
GCACTCGGTGGGCGGGGCGGTGGCGGCCTTGCTGGGGGTGCTGATGCGAGAGGAAGGGATGACGGAGGGGTTGCGGTGCT
ACACATTTGGTAGCCCTGCCTGCGTGAACCAGAAGCTGGCTCAAGTGTGCGAGGCCTTCGTGACCACGGTGGTGCTGCAC
GACGACGTCATTCCGCGGGTGACACCCACGGGCGTGCGGGGCCTCTTGAAAGACCTGCTCTCGGAGCGGGAGAGGGCAGA
GCAGCATTGGCAGGACGACGTGGAGGCGATCATCGTCAGGAGCAAAGGCCATTGGGCGCCACGGTGGAGGCGGGTCTTGT
CGCGGGGGGGGCGGAGAAGGGGGCGGCCCCCGGGAGCCGGTCACTGTCCTTCATTCGGAGCGAGGACGAGGGGGAGAGGG
GGGAAGGGGAGGGGGAAGGGTGGACTGGCGAGGACGAGGGAAGGTGAGGGAAAAGAGGGAGGGGACGGGGAGGGCGCGGG
GGAGGGGTGGGACAGCTGCGACGACGACCTCGGGAACTACTGGGGCGTGGGCTGCACGCGTGACGTGGTGTCCGTGGGCT
CCGGCGCCGCCTCGAACCGTTACTCGATCCGGCACGAGGACGGCACCACCACCACGGTGAACCTGGCCCTCGCCCGGCGC
CGGCTCTTGGATTCGGGCGGGGACGCGGCGGCTGACCGAGGGAGCTCGGTCTCGAAAAATGTCACGGGCGCCTGCCCCGC
GCCTTGCGGGCTTGGCGAGGCGGGCGTCAACTCCGCGCTCACGTCATCGGGGACTTCTGTTCCGAGCCTGGGGGCTGCCT
CGTCTCCCGGGGCTGAGAGTGGGGAGGCTGGAAGGAGAGAAGGGGGGGAGGAATTAGGGGGGGGAGGGGCGGGGGAAGGC
CGGCTGGAAGGATTCTCGTCGACTCTCACGCCCCTAGGCTTGGGGGATGGAGACGACACGGACGATTGGGGGGAGGATGG
GGGCGAGGGGGCAGAGAGGGCGGGGGAGGAAGCGCAGGCGTGGATGGGGGACCGCACGGGGTCGCTGCAGGAGGGGGAGA
GCGAGGGAGAGGAAGGGGAGGAGCTGCAGGGCAGGTGGCTCGGATCGAGGGATGCCCCCCCAGCATCTTCGGACGGCATG
GGCGCCGAGGAAGAAGGAGGCGCGGGCCTGGAGCAAAGCTTGGCGCTCTGGAATTTGTTTGGGAGTGAGGGGTCGGAGGC
GGCGGCGGCGGCGCCCGGGAGAGAGCCGGACTCCCGGGCAGTGCTCGAGGTAGAGGGCGTGCCCGTGGACACGAAGCAAG
TCTCCGTCAGCACCACCGCGCCCACCGCAGCTGCCGACTCCACCTGCTCCTTCTCCTCCTCCACCTCCTCCTTGTCCTCG
TCGAGCCCATCGCCGCCGGCGCCTGTGACCGCCAGCACGGGCCAGCTCTCTCGGCAATCTTTAGCTTCAAGTTGGCGGGG
CGCCTTGACCATCCCCCGCTGGGAAGGAGGGAGGAAGGGAGGGAGGAAGCAAAAGTGAGGAAAAGGAGGAAGGAAACGAA
GAAGAAGAAGAAGAAGAGGAGGAGGAGGAGGAGGAAGAGGAGGAAGAGGACTGCGGGATACTGGTGGAGGACGTGAGCCT
CCCAGAGCTATACCCGCCGGGCCGTCTGGTTCATATCTACAGCTACCGCGGCGTCTATAAGGCCTGCATGCCCCCCCGAA
GCTTCCCGGGCCTGCGCCGCATCCCCCTGCAGGGGAATTTGCTGAAGGACCACGCCCCCACGGCCTACTTCTCCGCTCTC
TGCGAAGTGATCGACGTCCGCCGAGCCCCGCAGCCGCCGCCCGCGTGGAGGGGTTCAAGGAGAAGGAGGCCTGCGTCTG
CTGTGCGGTGGATTTGACCTGGCAGCGGGCCACGGCCAGCGAGGCGCATCGGGACCGGGAGAAGCACAATTGTCGCTGCT
GCGGTGGACTGGTTTGTCAGGATTGCTCCCGGCATCGTCGGGCCTTGCCCTCCATCGGGCTCTCTGCGCCTGCTCGGGTC
TGCGACCGGTGTTTTTTCGGGGGAAAGCAGTCGGCAGTGGAGGCGACGGAGCGTGGATGA
```

# Bioinformatics to Identify Target sgRNA sequences - RFLP

- Restriction Fragment Length Polymorphism (RFLP)




- CRISPRseek sgRNA search criteria for restriction enzyme sites:
  - Look at 2 different restriction enzyme sites directly upstream of PAM site
    - SacI: GAGCT/C
    - SmaI: CCC/GGG
  - Look at 3 different locations within the genome for each target
    - Selected 3 largest contigs in *N. gaditana* CCMP 526 genome and applied search criteria (zero off-target, high gRNA efficacy score)

# Plasmid Construction for Cas9 Tools in *N. gaditana*

| Plasmid | Construct | Application |
|---|---|---|
| pCMV-Cas9-GFP | $P_{CMV}$-Cas9-2A-GFP | Mammalian promoter for Cas9 expression (Sigma-Aldrich) |
| pJTM001 | $P_{TUB}$-Cas9-2A-GFP | *N. gaditana* β-tubulin promoter for Cas9 expression |
| pJTM002 | $P_{VCP}$-Cas9-2A-GFP | *N. gaditana* violaxanthin/chlorophyll *a*-binding protein promoter for Cas9 expression |
| pJTM006 | $P_{TUB}$-Cas9-2A-GFP, $P_{TUB}$ – ble (Chlamy) | Includes *ble* gene optimized for expression in *Chlamydomonas* for genome integration |
| pJTM007 | $P_{VCP}$-Cas9-2A-GFP, $P_{TUB}$ – ble (Chlamy) | |
| pJTM010 | $P_{TUB}$-Cas9-GFP | Includes a Cas9-GFP gene fusion for visualization of the cellular localization of the Cas9 protein |
| pJTM011 | $P_{VCP}$-Cas9-GFP | |
| pSacI1-CMV | $P_{U6ng}$-SacI1-gRNA, $P_{CMV}$-Cas9-2A-GFP | Plasmids containing putative U6 promoter from *N. gaditana*, target sequence for SacI-1, guide RNA scaffold, and Cas9 expressed from one of three different promoters (CMV, TUB, or VCP) |
| pSacI1-TUB | $P_{U6ng}$-SacI1-gRNA, $P_{TUB}$-Cas9-2A-GFP | |
| pSacI1-VCP | $P_{U6ng}$-SacI1-gRNA, $P_{VCP}$-Cas9-2A-GFP | |

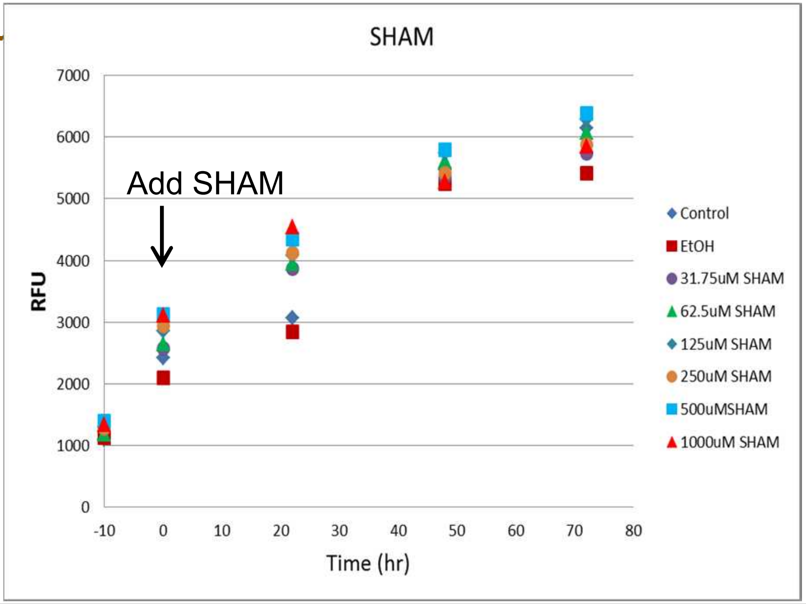
# Transformation of *N. gaditana*

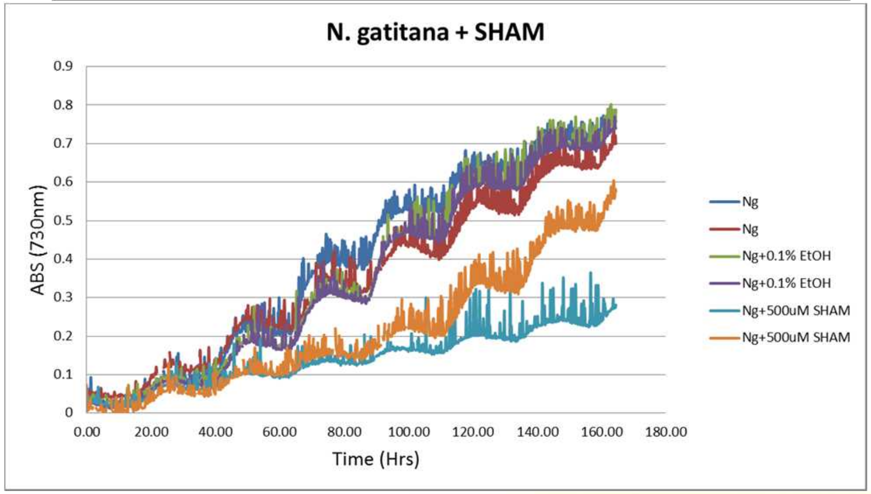
- High voltage electroporation (11,000 V/cm)
- Low transformation efficiency with random integration of zeocin resistance (*ble*)
  - 0 to 68 colonies with $5 \times 10^8$ cells and 3 μg of linear DNA
- Transformation Vector
  - *N. gaditana* β-tubulin promoter used for *ble*
  - Removed introns and codon optimized *ble*
- Optimization of electroporation conditions
  - Recent transformation results = >1,000 colonies

**Note: Cas9-mediated mutation is still possible with low transformation efficiency but will require genome integration of Cas9 and marker insertion/screening**

# Preliminary Investigation of Dark Respiration Inhibitors

- At 100µE 20˚C SHAM in EtOH showed no effect on grow across a range of concentrations
- Dark respiration in highest after high light growth phase and at high temperature
- At 1000µE (37% full sun) and 25˚C 500µM SHAM inhibited growth
- SHAM may also be inhibiting glycolate metabolism[2]
- Dark respiration losses of *N. gaditana* at 1000µE and 25˚C amount to an average daily loss of 10-35%
- Next steps:
  - Quantify dark respiration and photorespiration rates as a function of illumination and temperature
  - Test lower SHAM concentrations





2. Goyal A. (2002) Physiologia Plantarum. 116: 264-270.

# Path Forward

Tool Development

- Complete optimization of transformation process for *N. gaditana*
- Genome integration of Cas9
- Demonstrate Cas9-mediated nuclear modification

Physiological studies

- Investigate dark respiration inhibitors under more realistic environmental conditions
- Measure glycolate production (photorespiration) under high and low $CO_2$ conditions

FY17

- Construct and characterize laminarinase and lipase mutants
- Construct and characterize dark respiration mutants
- Demonstrate 1,000 L scale growth of *N. gaditana* in indoor mini-raceway ponds

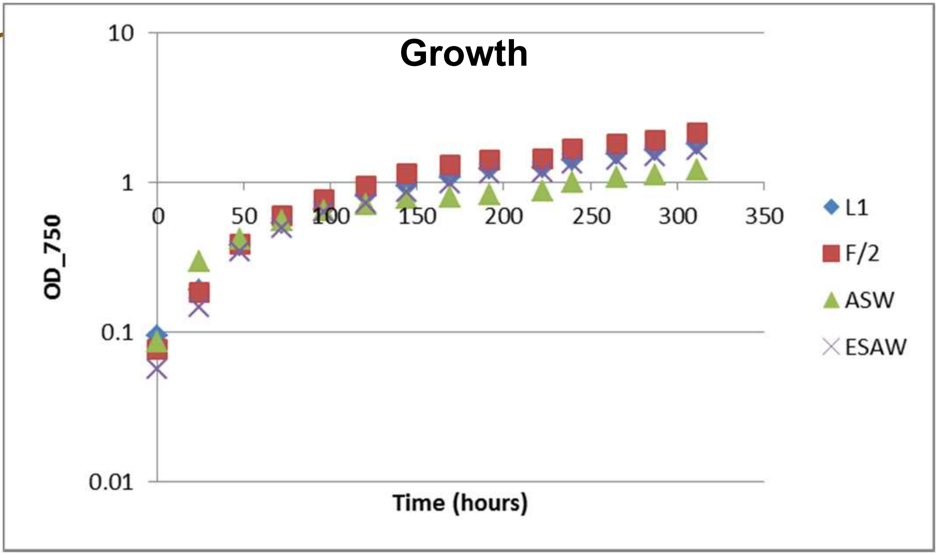
# Future Funding Opportunities

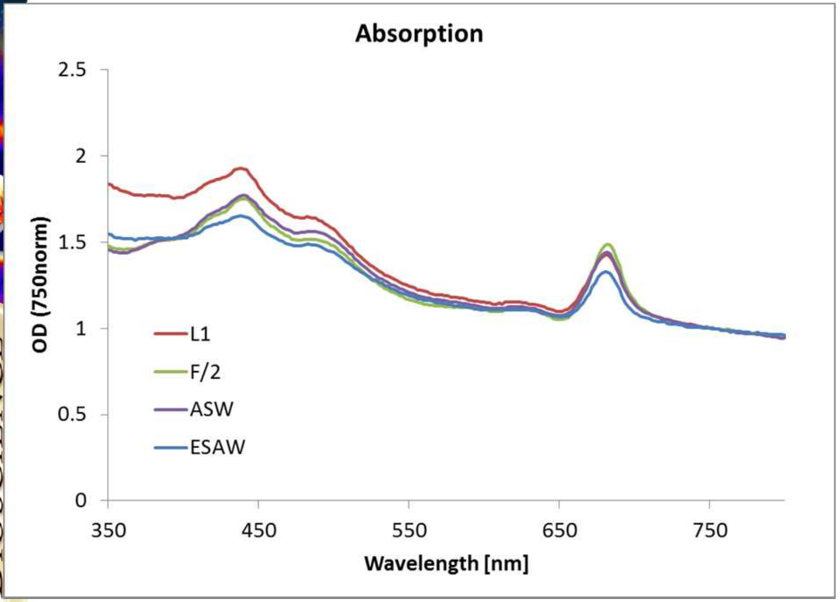
- JGI DNA Synthesis proposal submitted in July for optimization of CRISPR-Cas9 technology in *N. gaditana*
- Anticipated BETO FOA on Algae Toolbox Development, Fall 2016

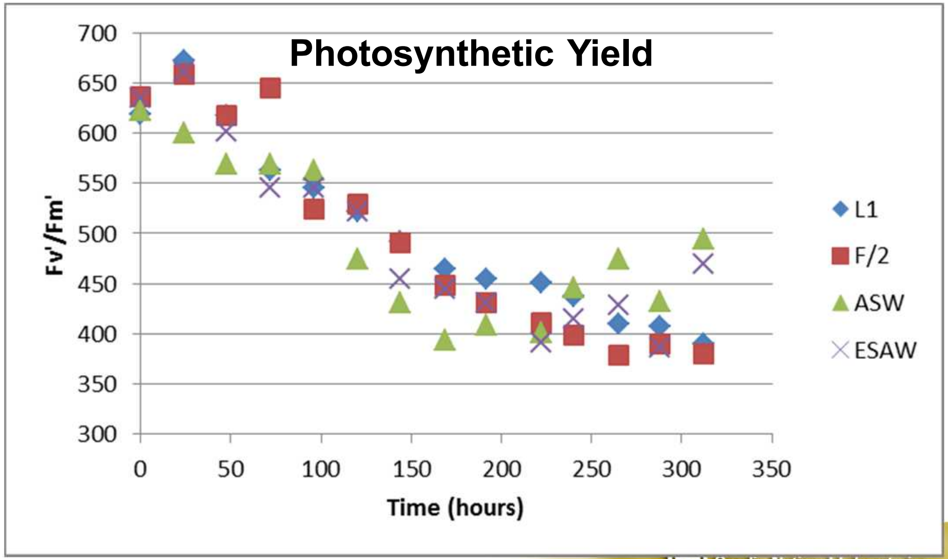
# ADDITIONAL SLIDES

# Medium Optimization for *N. gaditana*

- 4 media:
  - L1
  - ASW
  - F/2
  - ESAW
- F/2 has highest final OD and no change in photosynthetic pigments



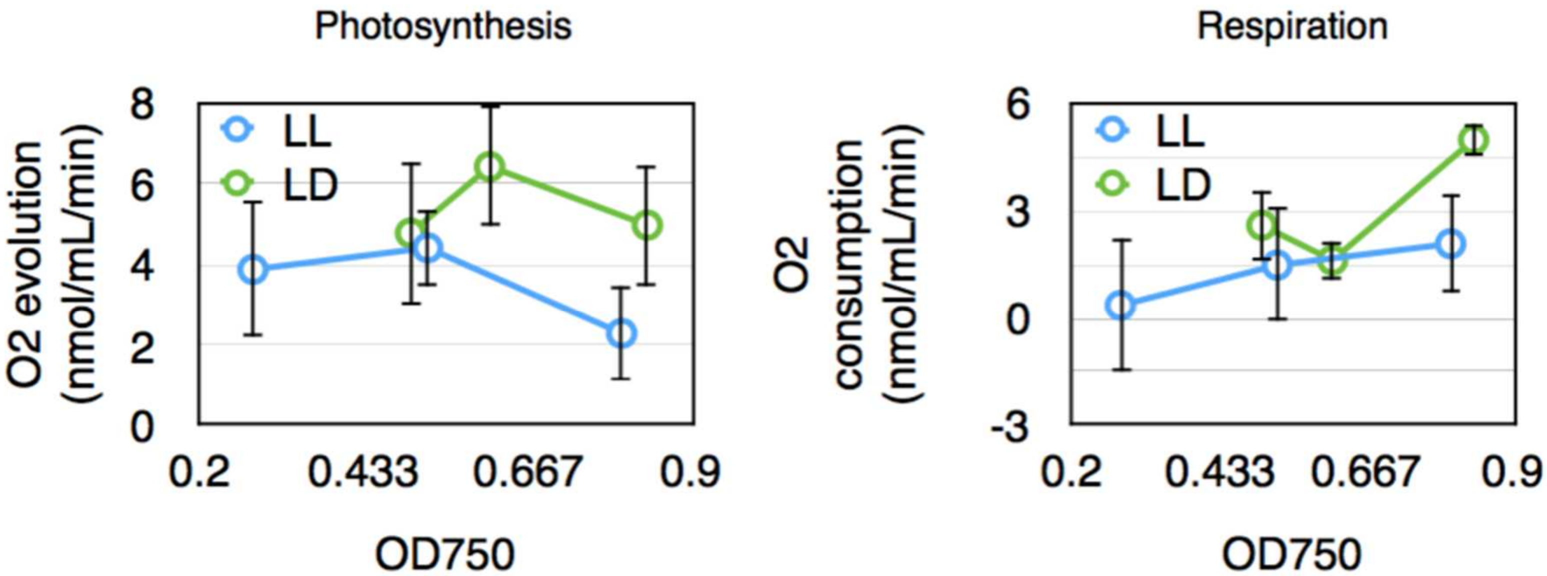



# Zeocin Resistance of *N. gaditana*

No zeocin | 2 μg/mL zeocin | 5 μg/mL zeocin

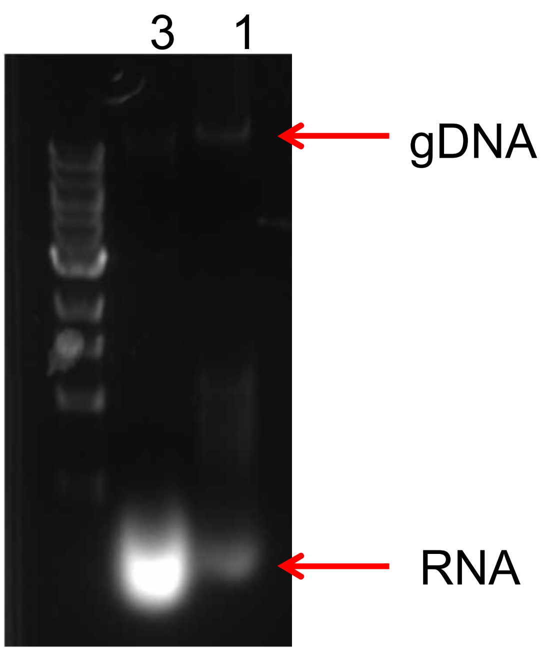
# Measuring Oxygen Evolution and Consumption in *N. gaditana*

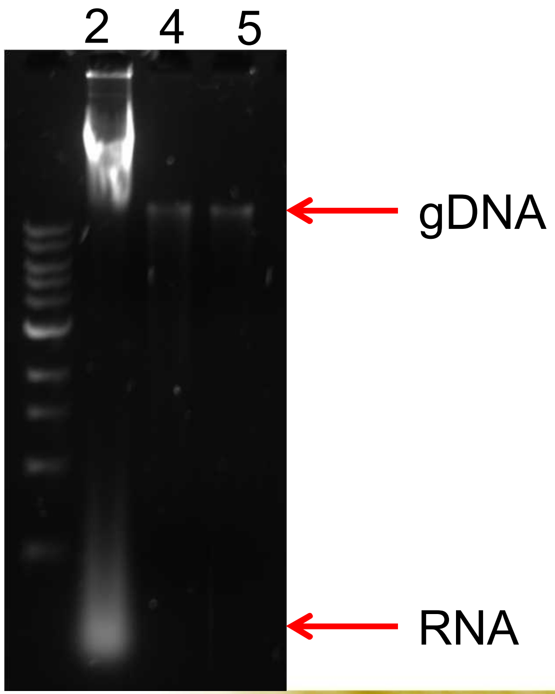


# Genomic DNA Isolation of *N. gaditana* for RFLP

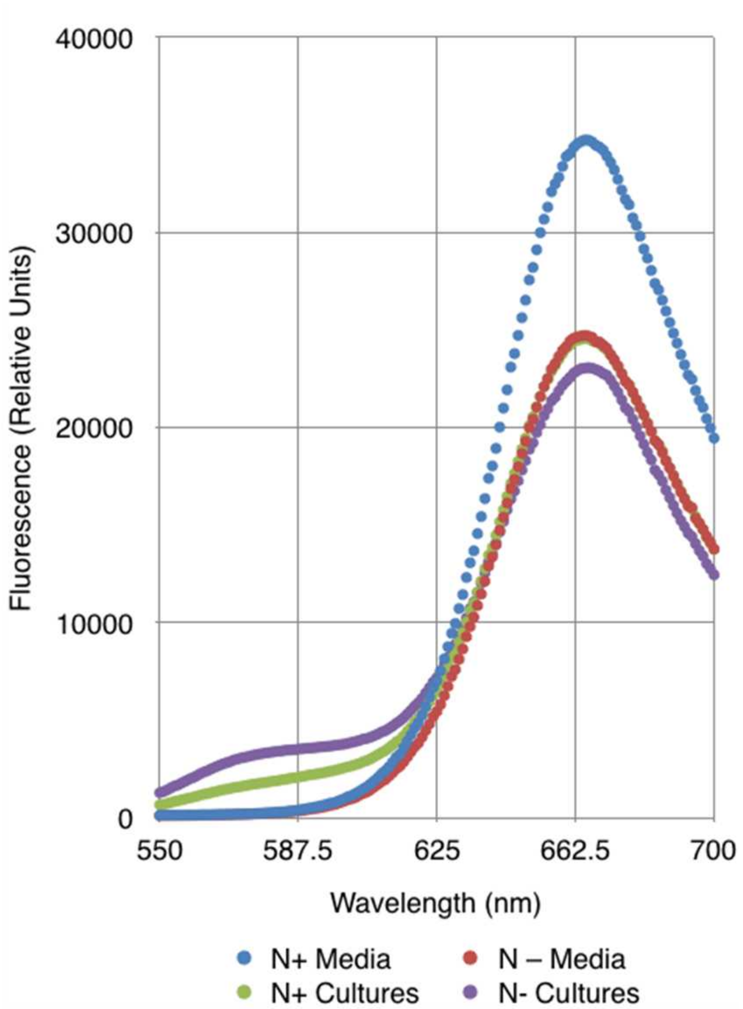
1. Chemical isolation (phenol-chloroform-isoamyl alcohol), no RNase
2. Chemical isolation with 2x volumes of solvents, with RNase
3. Qiagen Plant DNeasy isolation with 0.1 µm bead lysing, no RNase
4. Sigma bacterial isolation kit (lysozyme and proteinase K lysis), with RNase
5. Sigma bacterial isolation kit (lysozyme, proteinase K, and 0.1 µm bead lysing), with RNase

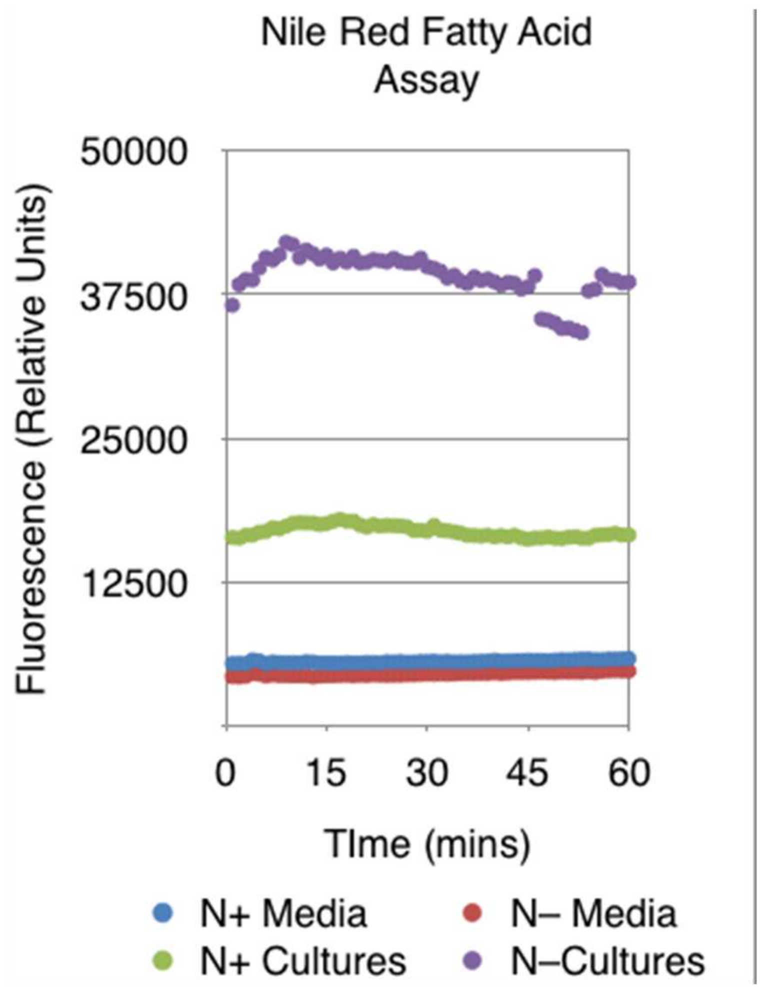
| # | ug DNA Isolated |
|---|---|
| 1 | 43.2 |
| 2 | 61.3 |
| 3 | 126.7 |
| 4 | 10.2 |
| 5 | 11.9 |



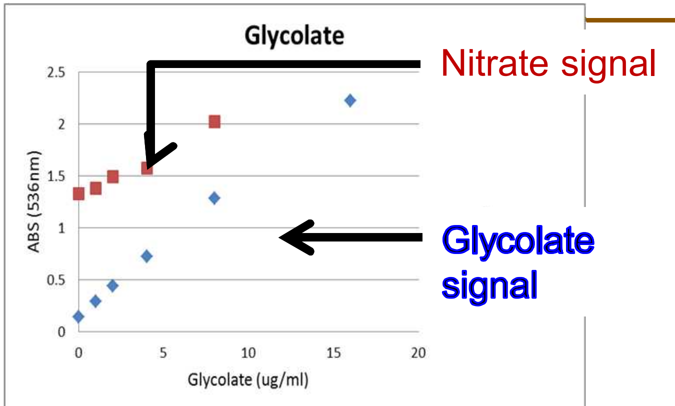



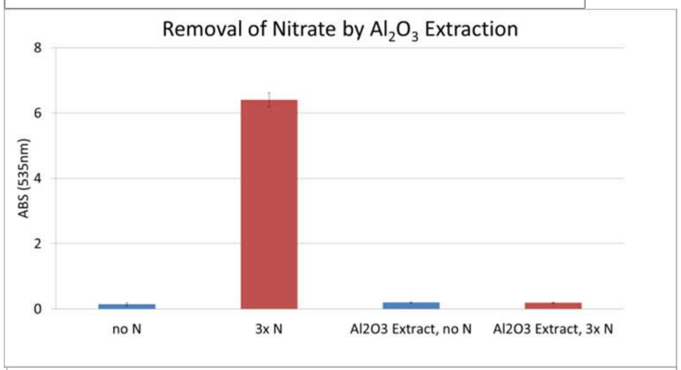
# Lipid Quantification in *N. gaditana*

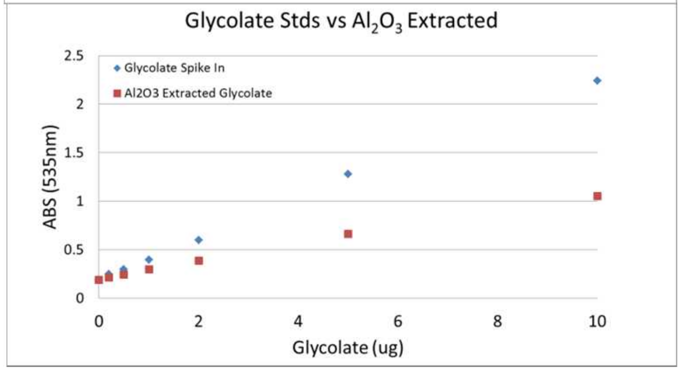


# Photorespiration (Glycolate) Quantification in *N. gaditana*




- Chromogenic assay (Caulkin's)
  - *Caulkin's rgt: 18.5mg 2,7 dihydroxynaphthalene in 100ml conc $H_2SO_4$
  - Heat to 100 ° C for 20 min
- Nitrate is a positive interferent
- Three methods for removing or correcting nitrate signal
  1. Run nitrate assays on all samples and nitrate standard curve and subtract nitrate signal
  2. Extracte glycolate with ethyl acetate-leave nitrate behind—Only 5% efficient in recovering glycolate (data not shown)
  3. Extract glycolate with alumina ~50% efficient (compare to 65% in literature) very consistent fraction across range of concentration
     - Effectively removes nitrate
     - Possible to concentrate glycolate from dilute samples
     - Elution in $H_2SO_4$ is compatible with Caulkin's assay