DOE Bioenergy Technologies Office (BETO) 2023 Project Peer Review

Algal Productivity Enhancements by Rapid Screening and Selection of Improved Biomass and Lipid Producing Phototrophs (APEX) EE0008904

Advanced Algal Systems – April 3rd 2023

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DE-FOA-0002029: FY19 BIOENERGY TECHNOLOGIES OFFICE MULTI-TOPIC FUNDING OPPORTUNITY ANNOUNCEMENT
Period of Performance: 10/2020 – 9/2024

Collaboration with Pacific Northwest National Laboratory, Queensland University of Technology and Global Algae Innovations

Project Overview

- **FY19 BIOENERGY TECHNOLOGIES OFFICE MULTI-TOPIC FUNDING OPPORTUNITY ANNOUNCEMENT - Cultivation Intensification Processes for Algae**
  - **Strain/trait of interest characterization and adaptation of novel and/or existing strains to novel cultivation conditions in an indoor/outdoor/indoor iterative experimental framework**
  - **Successful applications will accomplish the objective by showing closer correlation between promising laboratory results and "mass culture" campaigns resulting in "high-performance" cultivation outcomes**
  - **50% improvement in harvest yield (g/m^2/d AFDW) and robustness paired with a 20% improvement in quality**

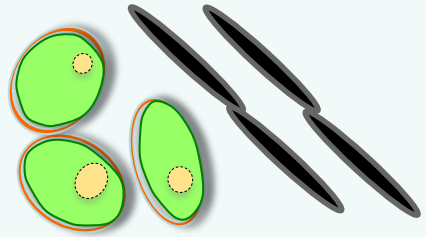
- *Our project aims to attain high-biomass AND high-lipid productivity. Specifically, lipid yields >31% with biomass productivities >23 g/m^2/day are targeted.*
- *Mutagenesis used to generate a mutant library of GAI high-productivity strain (e.g. Nitzschia sp.) and Nannochloropsis mutants. Atmospheric and room temperature plasma (ARTP) mutagenesis used to generate insertions and deletions.*
- *Algal breeding being pursued using Nitzschia sp. to generate genetic diversity for the isolation of high-lipid AND high biomass strains. High-risk/reward.*
- *Demonstrate scalability of high-lipid AND high-productivity strains from the laboratory to outdoor algal farm.*

1 – Approach

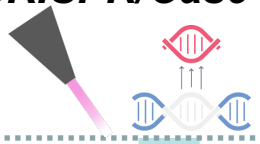
- ***Strains of GAI Nitzschia and species of Nannochloropsis are mutagenized using atmospheric and room temperature plasma (ARTP) mutagenesis. This approach generates high levels of insertions and deletions that are more stable to reversion relative to single base mutations.***
- ***High-lipid strains selected using flow cytometry and cell sorting. This pool is filtered for high growth in custom-built environmental bioreactors for the best growing cultivars from high-lipid sorts. This process iterated depending on results.***
- ***Bioprospecting for high-productivity AND high-biomass strains used as risk mitigation.***
- ***The largest challenges include the ability generate/isolate strains of interest and to maintain mutants without reversion.***
- ***The Go/No-Go metrics included the ability to reach 23 g/m^2/d and 31% lipid from laboratory bioreactors that scale that ultimately scale to the GAI farm facility.***
- ***Risk mitigation involves multiple unrelated approaches to targeted goals (Nitzschia, Nannochloropsis, bioprospecting) and genetic diversity (mutagenesis and breeding).***
- ***The primary technical metric is algal productivity (g/m^2/d) and quality (gallons of gasoline equivalent).***

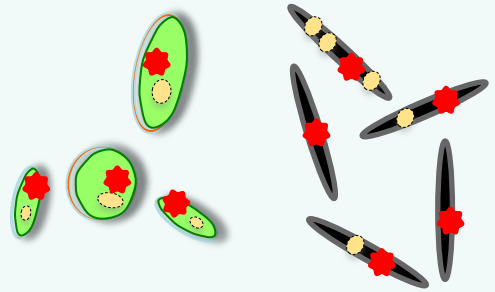
Approach Schematic

Nitzschia inconspicua,
Nannochloropsis gaditana



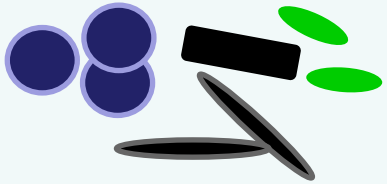
Plasma mutagenesis
CRISPR/Cas9 editing





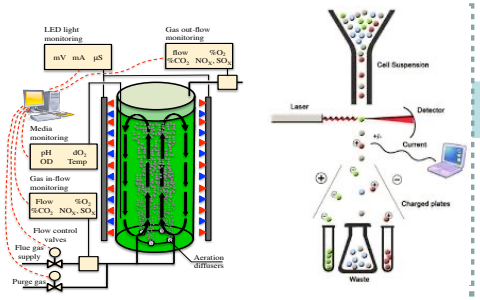
Mutagenized populations and strains

High-productivity, high-oil strains from bioprospecting

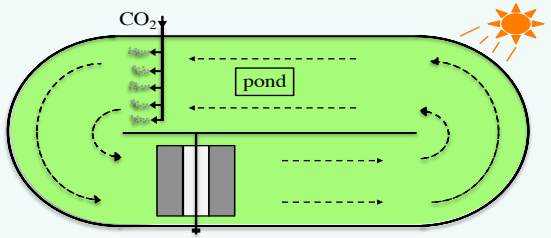


Strain selection & breeding

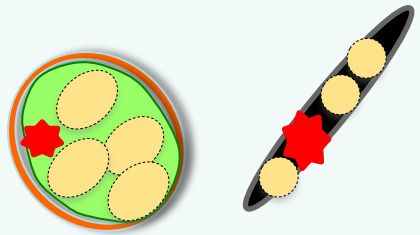
Bioreactor/FACS selection



Testing & demonstration in production ponds



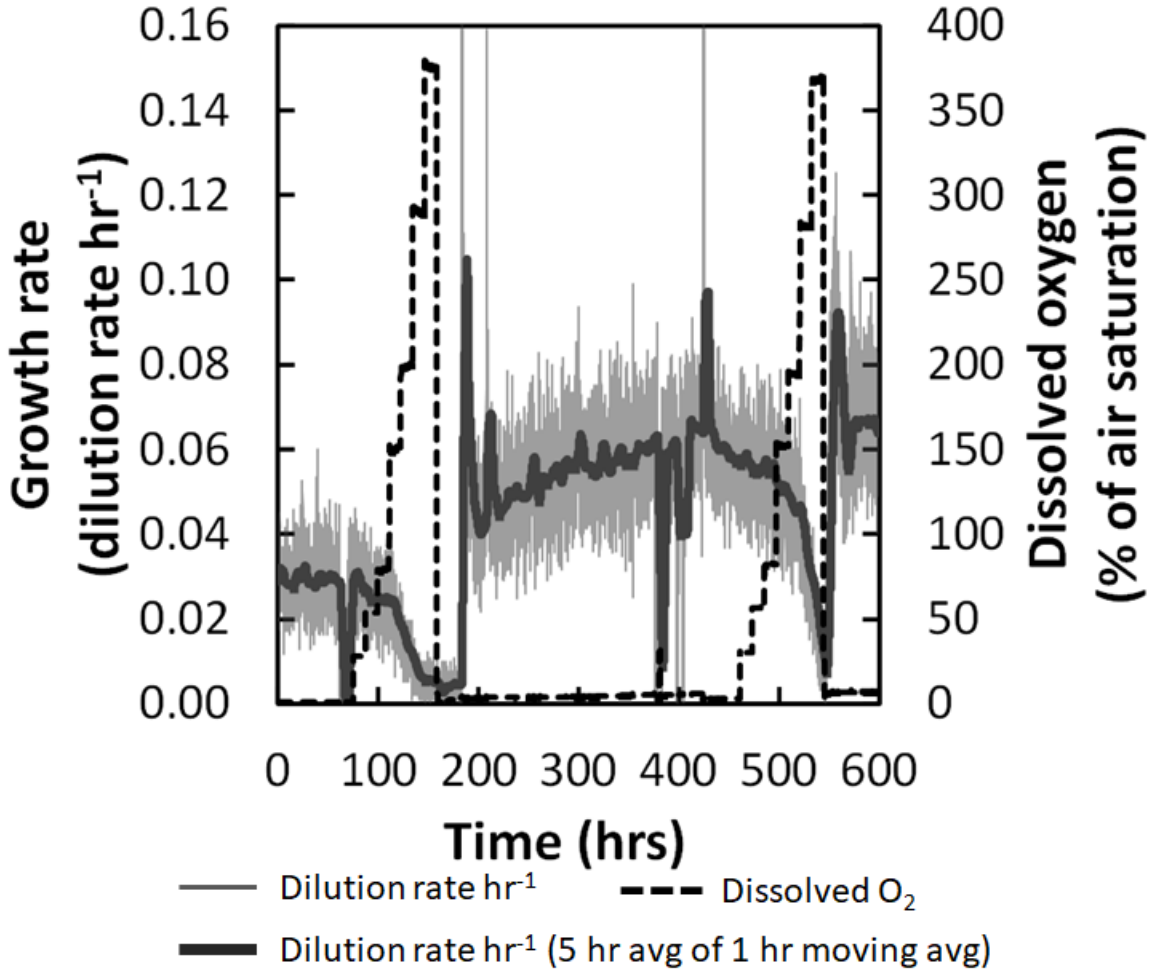
Growth optimization, testing & scale-up in intermediate size lab-based miniaturized ponds

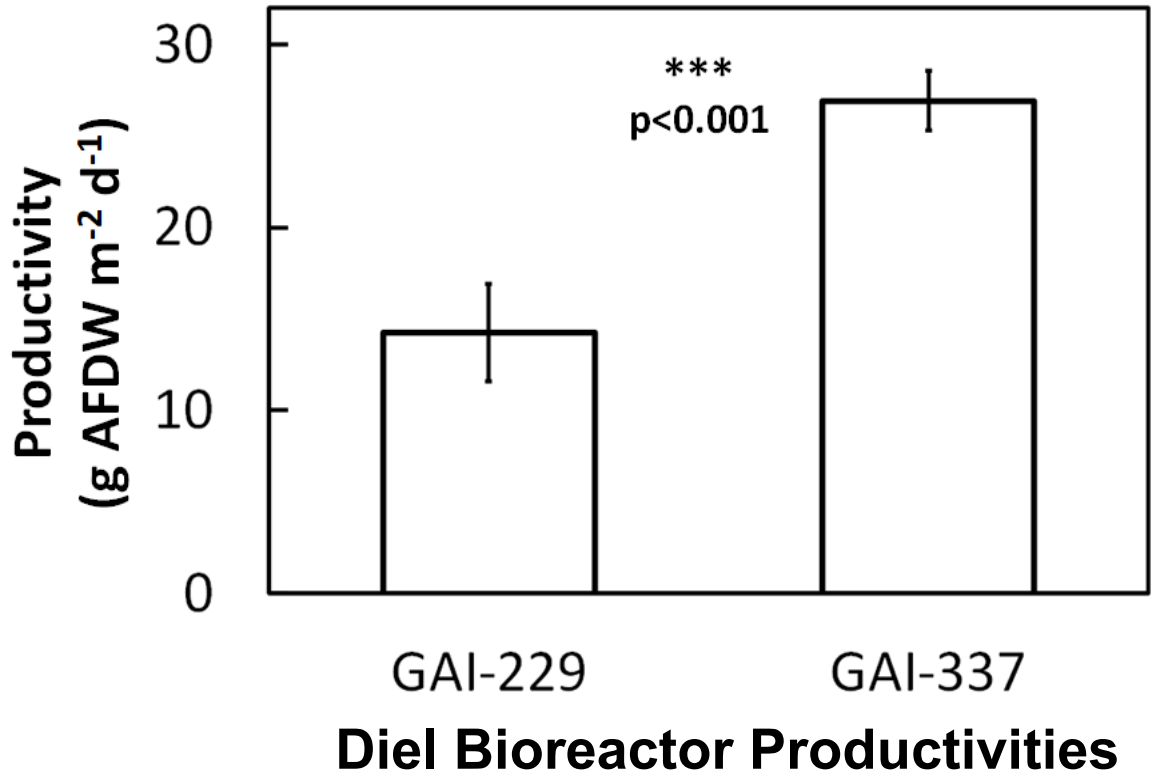


Selected high-productivity/high-oil strains

2 – Progress and Outcomes

Directed Evolution for O_2 Tolerance

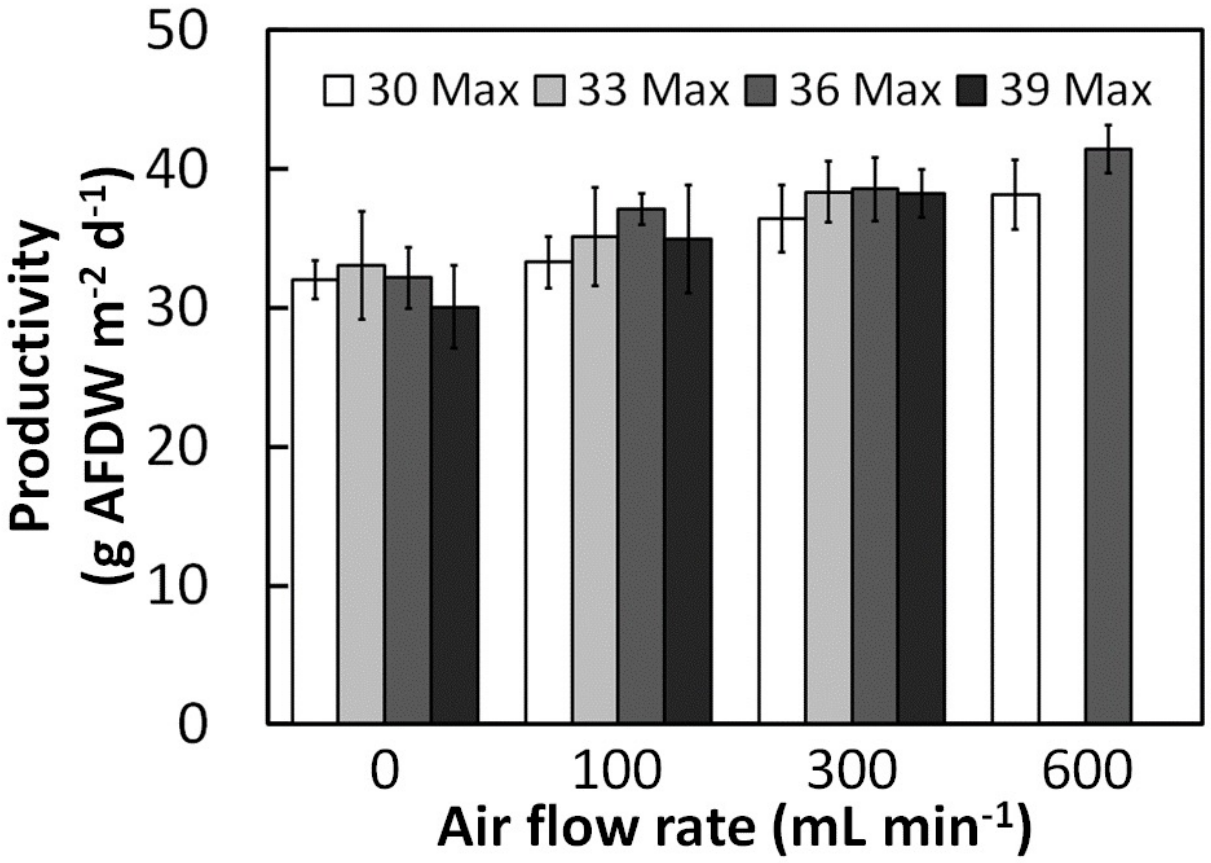


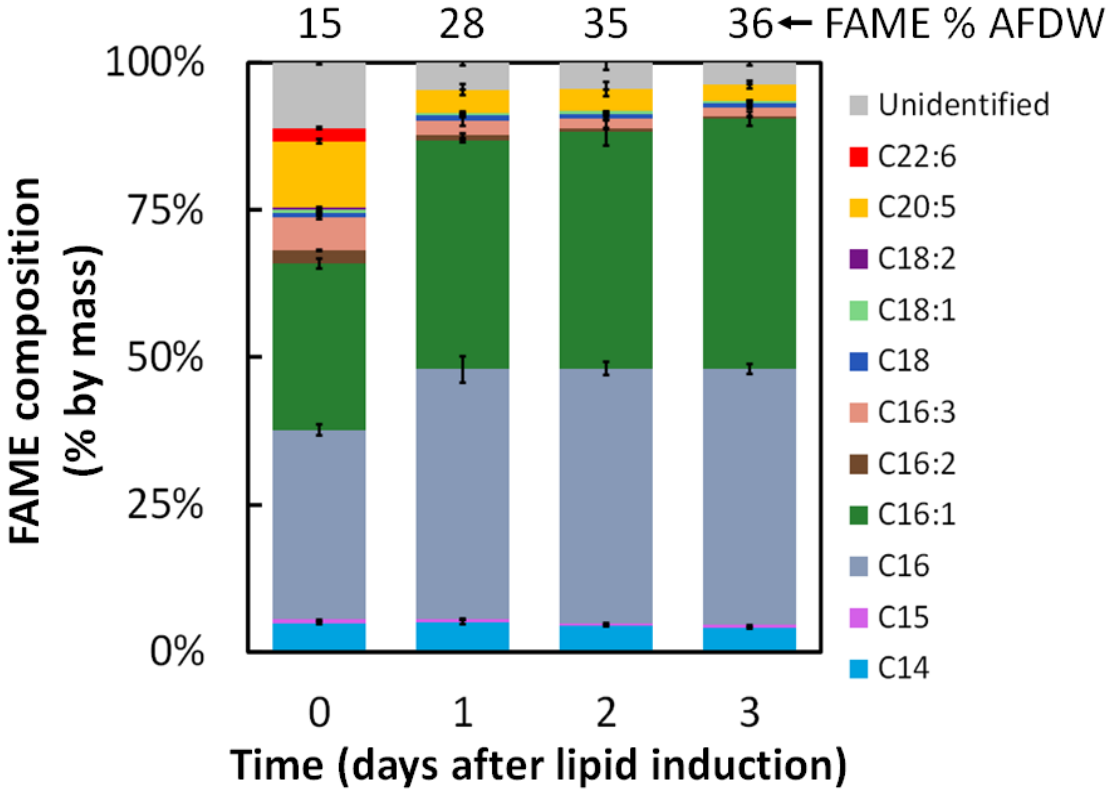


Diel Bioreactor Productivities

Initial Mutagenesis Focused on *Nitzschia inconspicua* (GAI-229) That Was Adapted to High (pond relevant) O_2 Levels (PEAK Project). This Directed Evolution Campaign Generated an O_2-adapted Consortium (GAI-337) With Higher Bioreactor Areal Productivity That Was Mutagenized Using Plasma Mutagenesis and Screened for High Lipid Strains. PATENT PENDING: METHODS FOR PRODUCING AND CULTIVATING HIGH PRODUCTIVITY ALGAE STRAINS

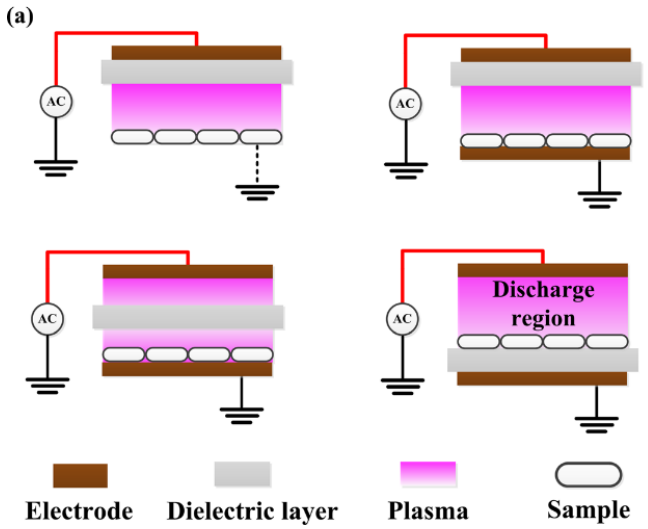
2 – Progress and Outcomes

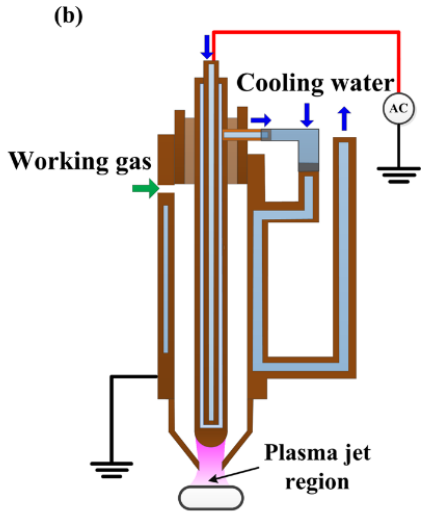


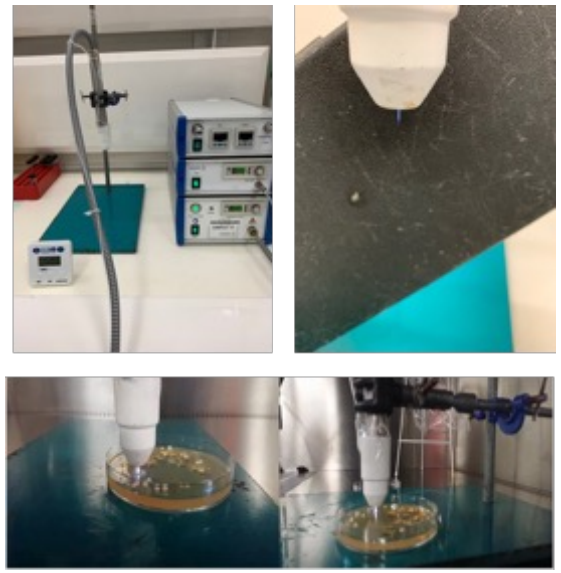


GAI-337 Growth Regimes Were Improved to Achieve Bioreactor Yields of 30-40 g/m²/d Depending on Diel Scripts and Mixing. Lipids Were Quantified and Assessed in Replete Media and Nutrient Limited Media. *Nitzschia inconspicua* Makes Both DHA and EPA Fatty Acids.

2 – Progress and Outcomes
Plasma Mutagenesis





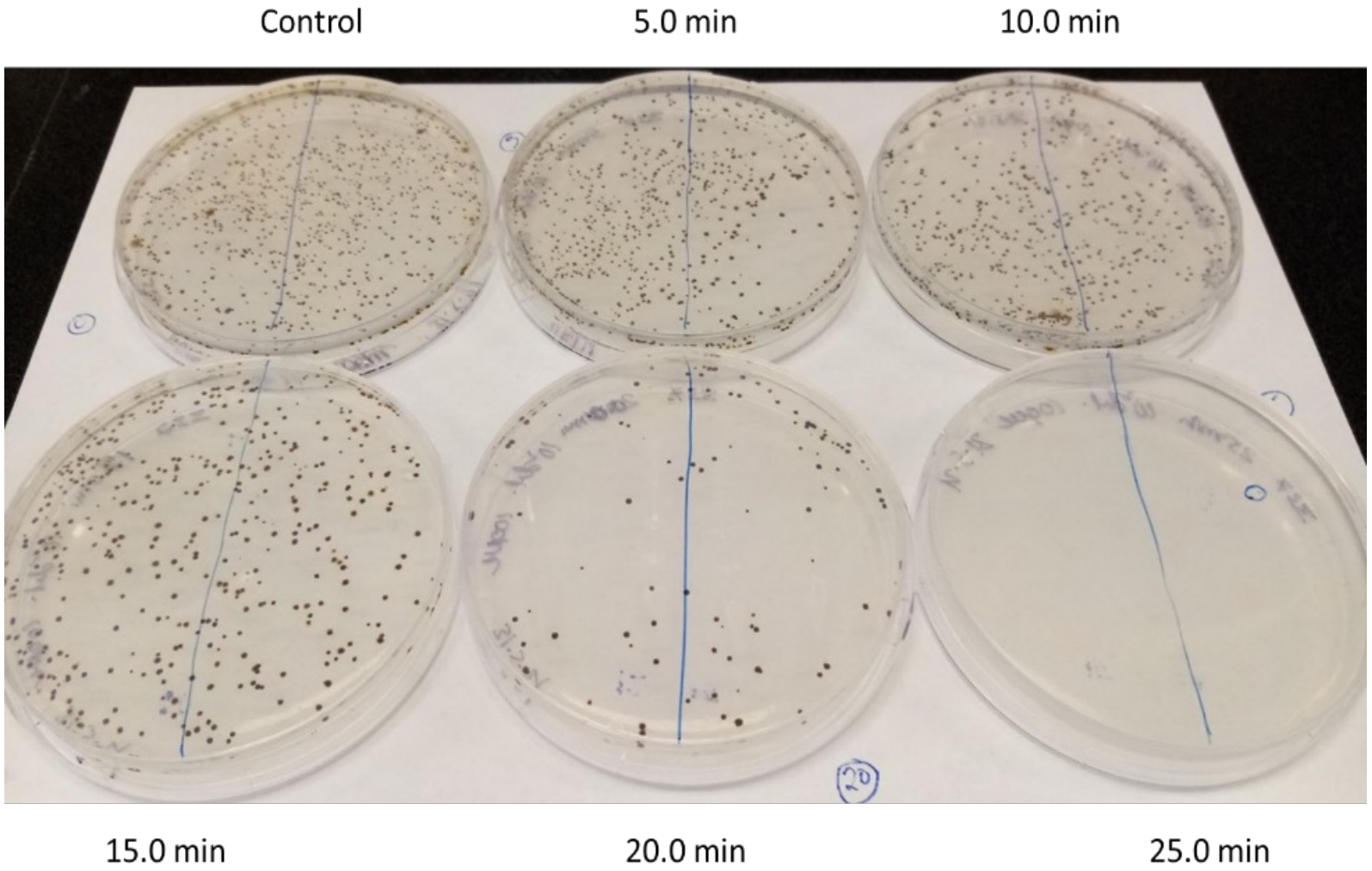


Plasma pen

Multi-pin plasma plate

- **Atmospheric and Room Temperature Plasma (ARTP) mutagenesis uses the radio-frequency glow discharge plasma jets to generate mutations**
- **Distinct from traditional mutagens because of low and controllable gas temperatures, abundant chemically reactive species (UV radiation, charged particles, neutral reactive species, electromagnetic frequency, heat), rapid mutation, high operation flexibility**
- **Three ARTP machines available: with indirect action pattern (plasma pen), with direct action (multipin plate), and bubble pen**

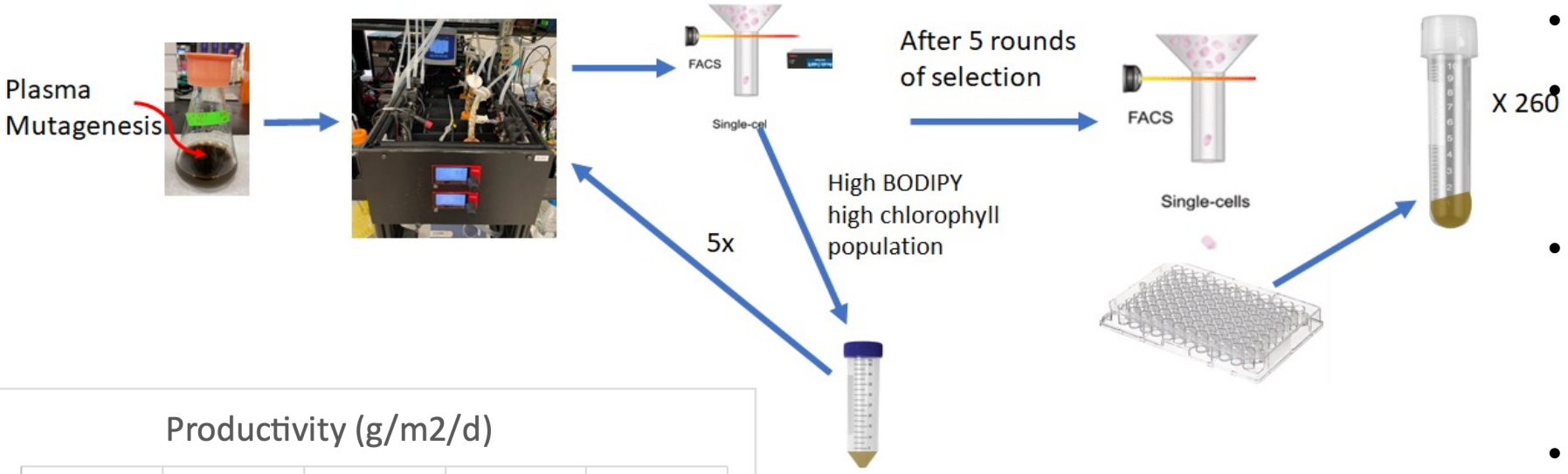
2 – Progress and Outcomes
Plasma Mutagenesis



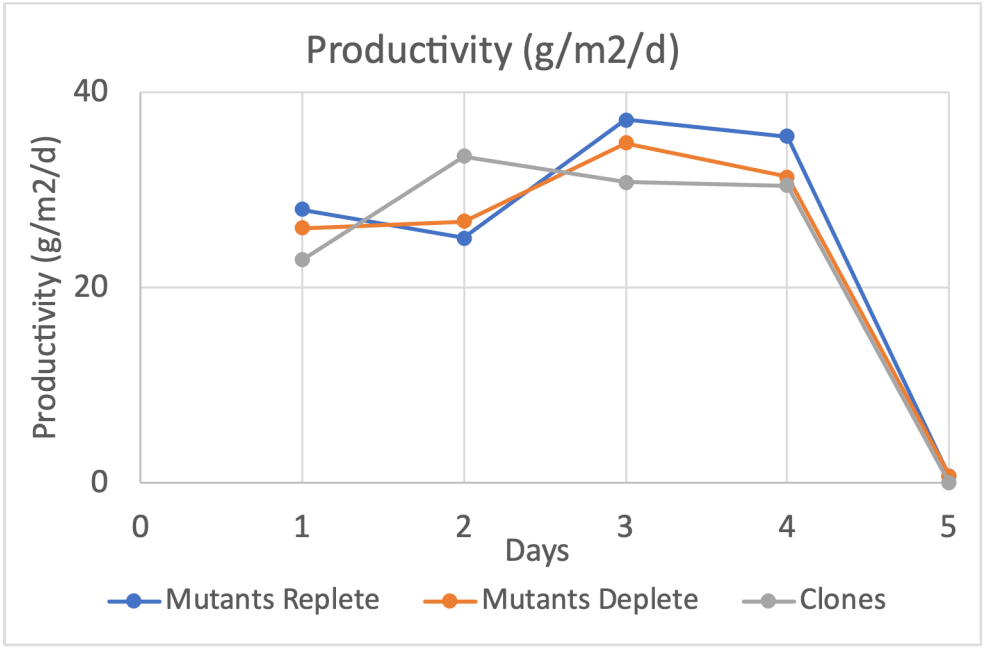
Representative Plates from Plasma "Kill Curve"
Different Treatment Times Lead to 10%-90% Viability

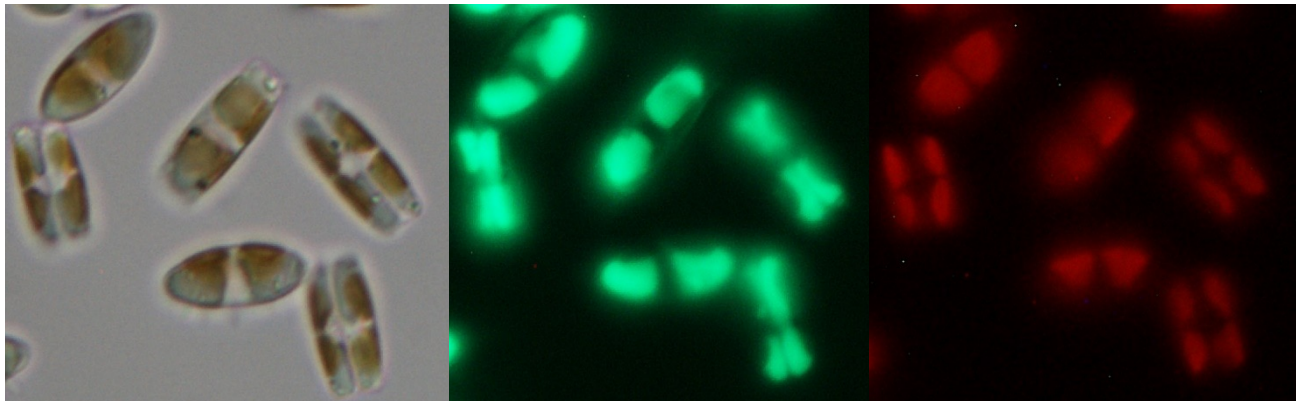
2 – Progress and Outcomes

Strain Selection Strategy



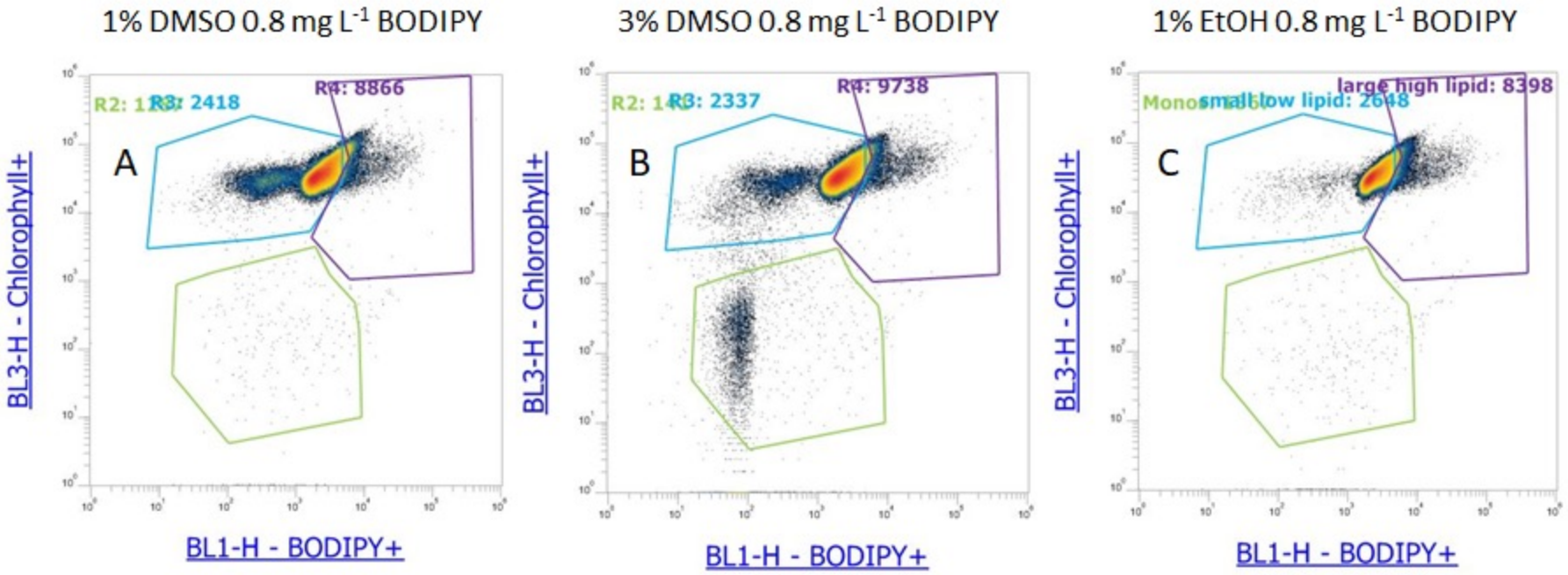
- Five iterative rounds of high productivity growth in a pond-simulating bioreactor
- Replete sorting
- Nutrient restricted sorting – fastest to lipid strains
- FACs high BODIPY and high chlorophyll cell sorting yielded a set of 260 isolates
- Four lipid tiers of the top 10%, 1%, 0.5% and 0.1% BODIPY signal

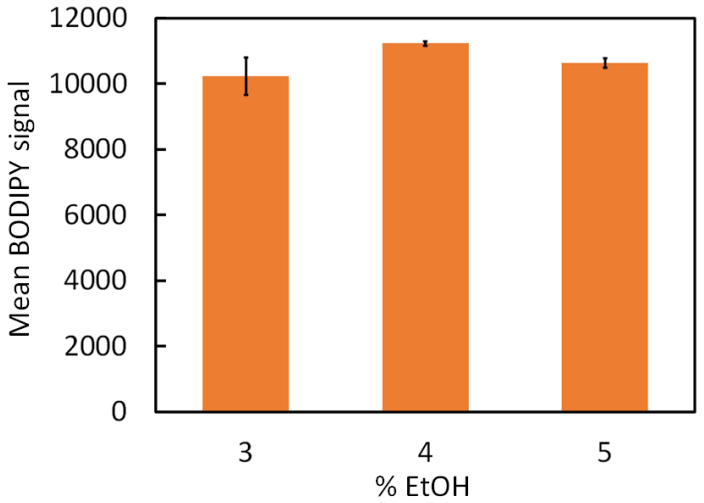




2 – Progress and Outcomes

BODIPY Staining Optimization



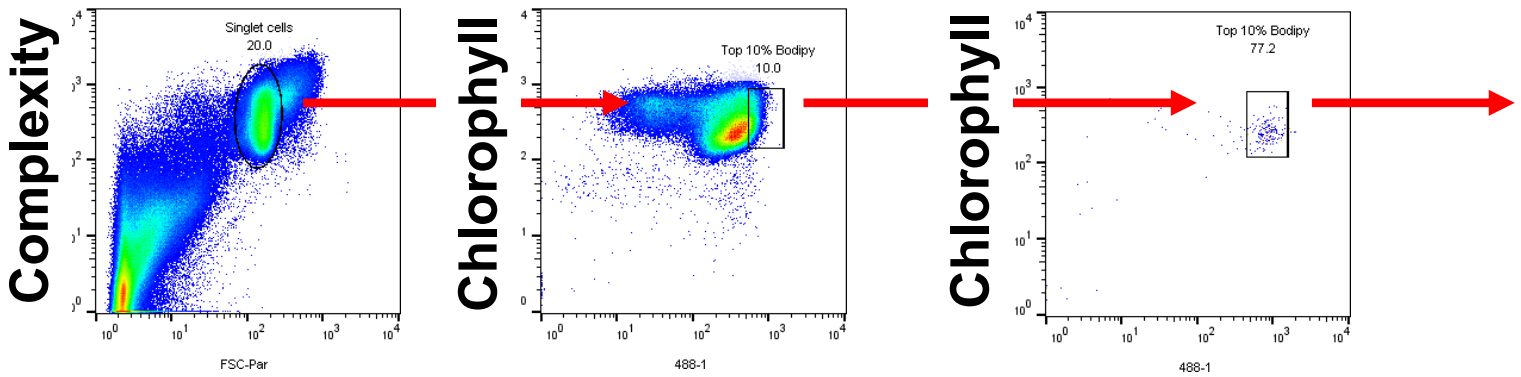


Ethanol Enabled More Uniform BODIPY Staining for *Nitzschia inconspicua*

2 – Progress and Outcomes

FACS and Cell Sorting

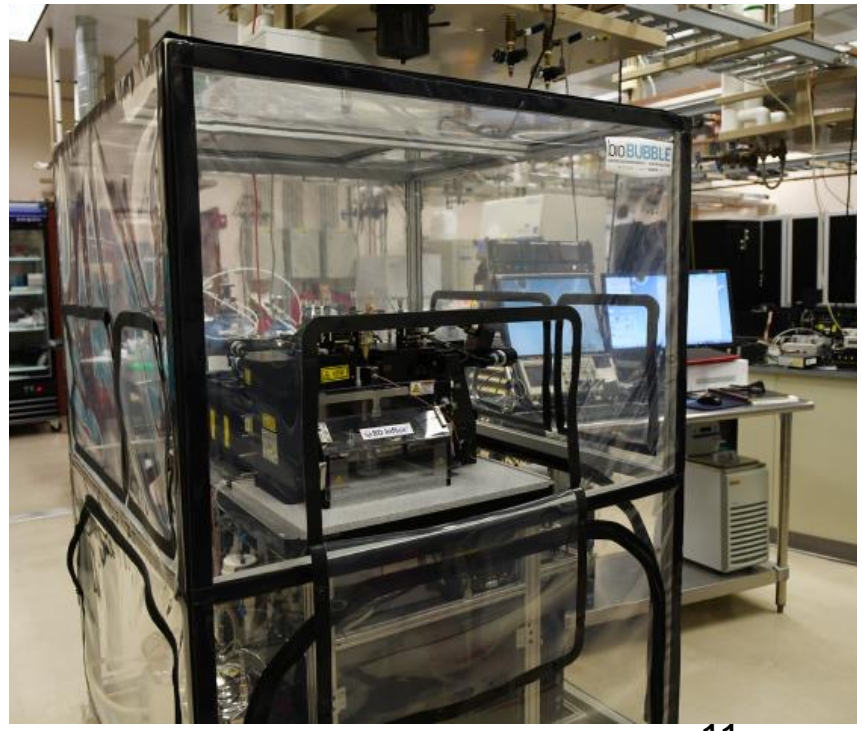
Pre-sort **Target population** **Post-sort**



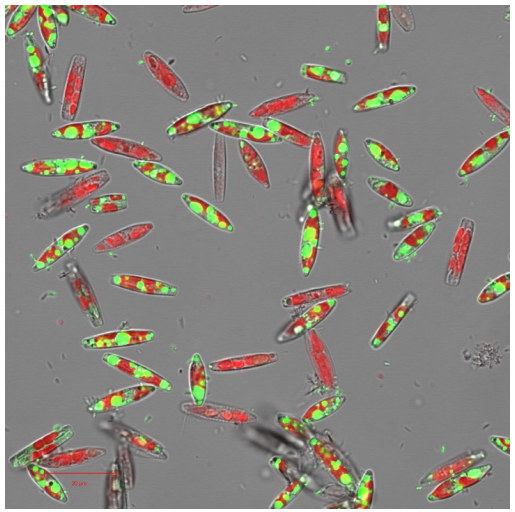
Size **Bodipy signal** **Bodipy signal**

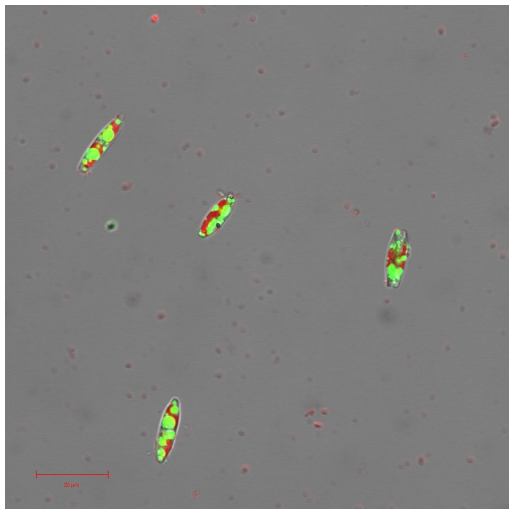
Recovery and growth

PNNL
EMSL Cell Sorting Facility



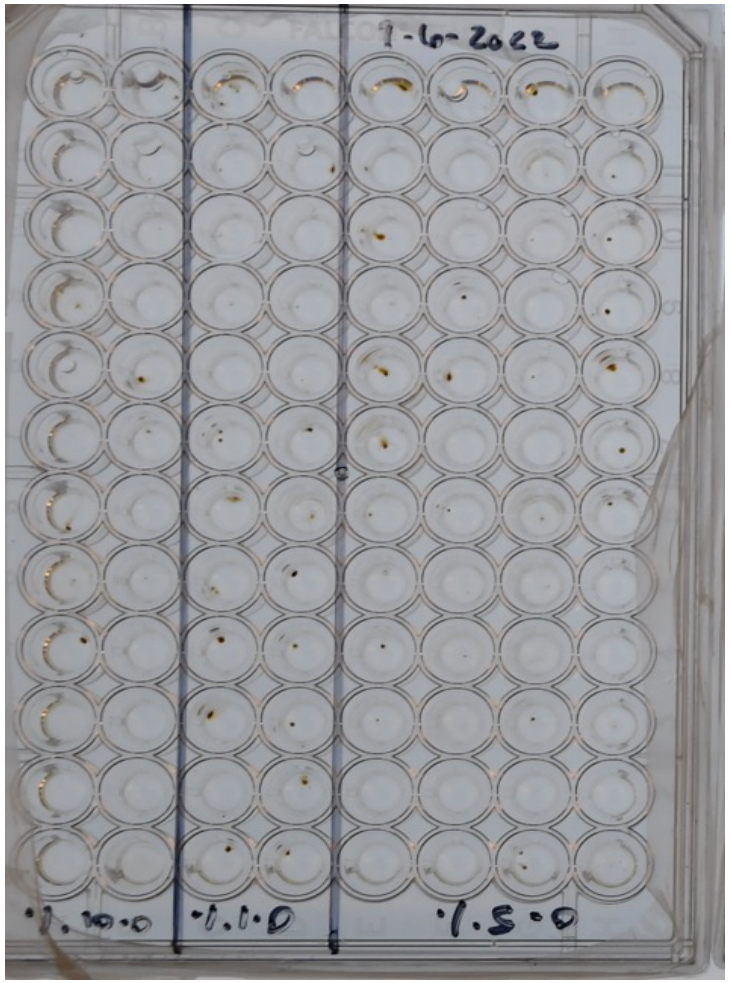
Green – Bodipy
Red - Chlorophyll



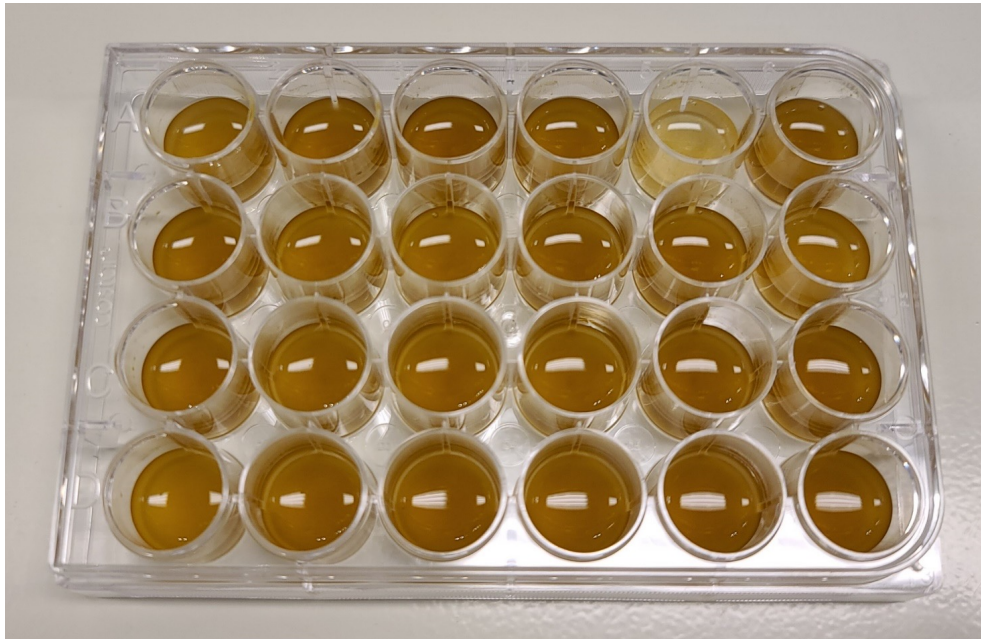


2 – Progress and Outcomes

Single Cell Sorts and Grow Out Campaigns



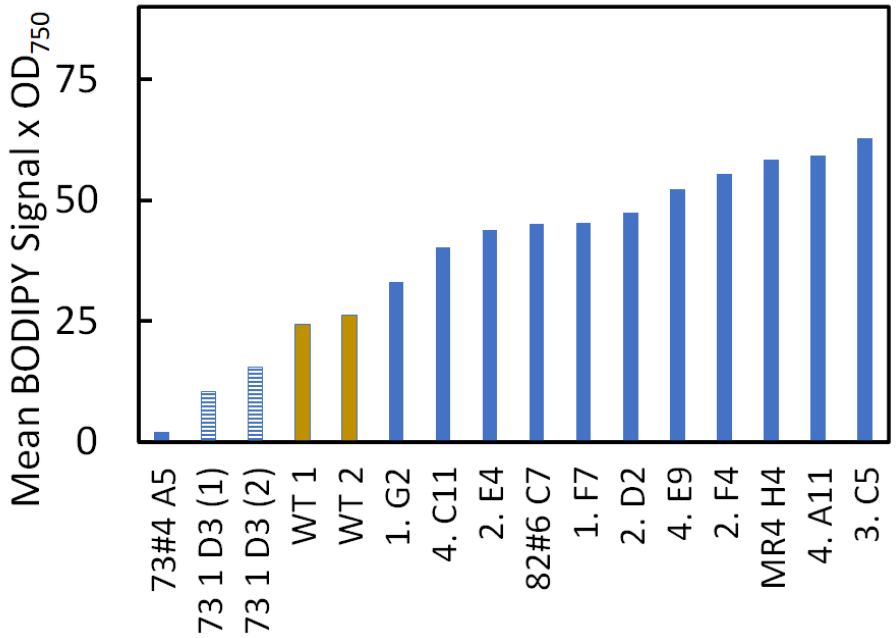
Single cells sorted into microplates for initial grow out

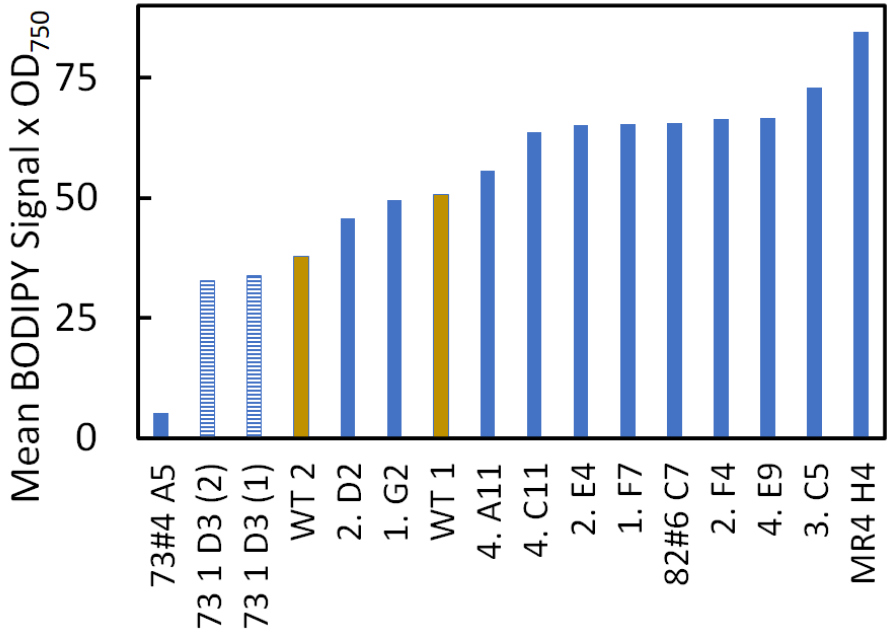


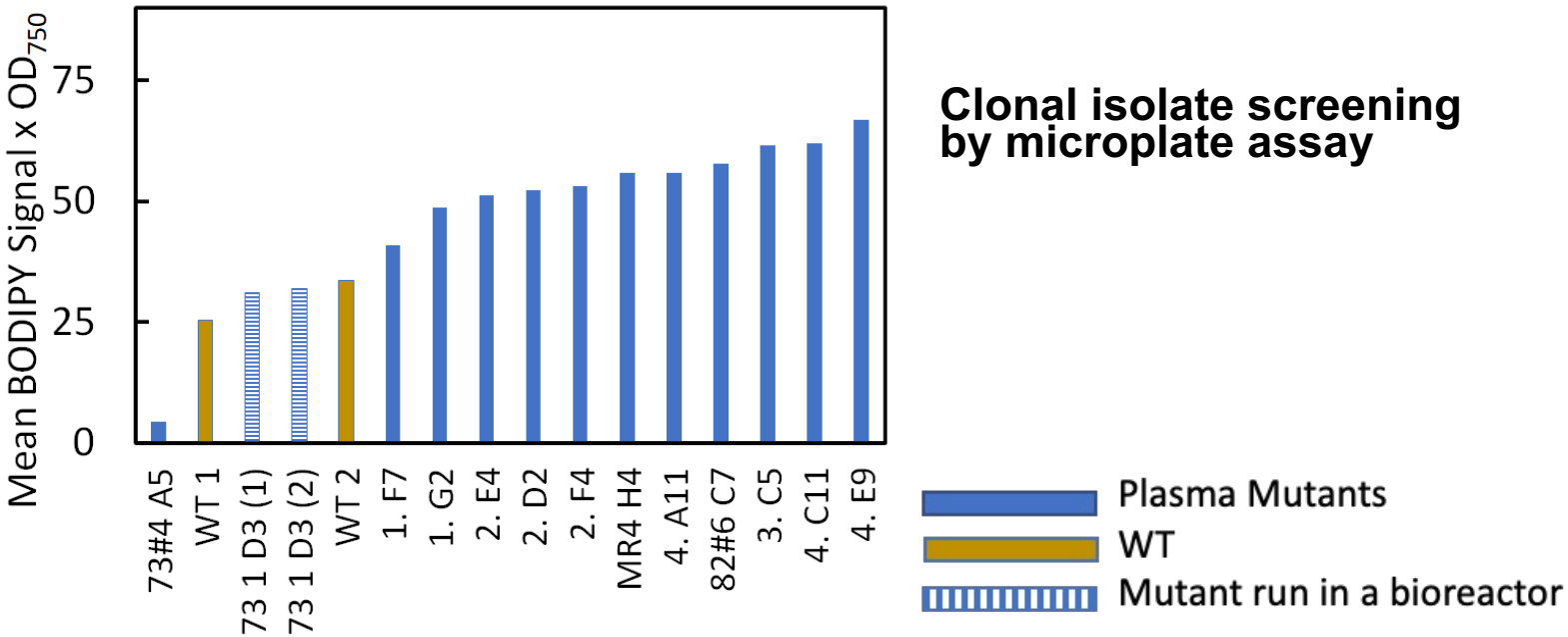
Candidates scaled in 24-well plates for initial FAME and growth assays

- Clonal isolates were obtained by cell sorting for high lipid and medium chlorophyll signals
- Isolates were grown in tubes, diluted regularly to maintain growth, then inoculated into 24 well plates at a standard density
- Plates were grown under continuous light at 28 °C on a shaker table for four days and then measured
- Measured OD_{750}, cell counts, cell size, BODIPY signal, and FAME
- Promising strains were repeated

2 – Progress and Outcomes



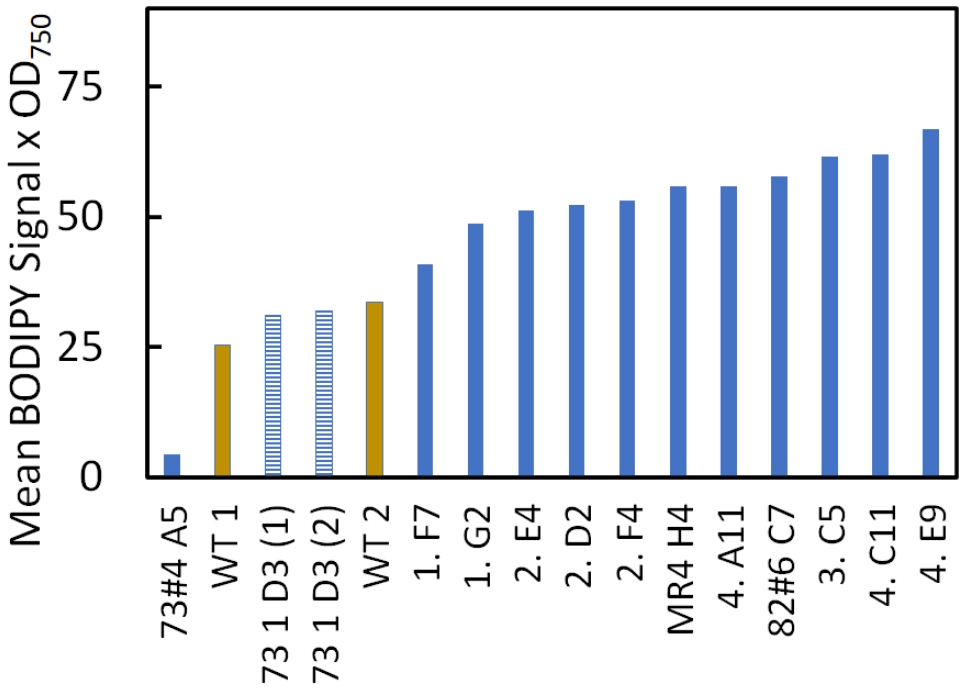


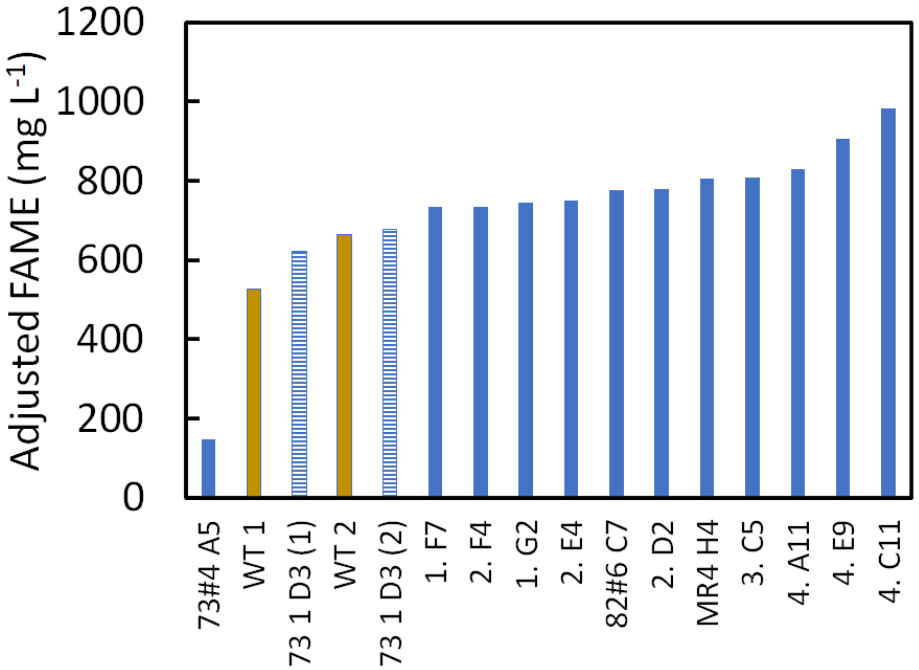


- **The microplate assay combined with flow cytometry yields consistent results between separately run plates of the same isolates**
- **High performing strains consistently outperform wildtype 337**

2 – Progress and Outcomes

Clonal Isolate Screening by Microplate Assay





Mean BODIPY signal multiplied by OD_{750} is a good indicator of lipids as tested by our FAME assay

Plasma Mutants
WT
Mutant run in a bioreactor

2 – Progress and Outcomes
Strain Down-selection

Best candidate lines in order based on relative FAME (mg L^{-1})

Isolate clone	Cell concentration (1x106 cells ml-1)	Mean cell size (FSC)	X-Mean BODIPY Signal	OD 750	Relative FAME (mg L^{-1})	Sort	% cut when sorted	Conditions
4. C11	3.24	610419	182464	3.39	982	Post #5	0.5	Deplete 24h
4. E9	2.66	659468	211391	3.16	906	Post #5	1	Deplete 24h
4. A11	3.04	615568	173357	3.22	830	Post #5	0.1	Deplete 24h
3. C5	2.49	678599	187250	3.29	807	Post #5	0.5	Replete
MR4 H4	1.99	811718	162507	3.44	805	Post #4	10	Replete
82#6 C7	2.25	667368	179701	3.21	776	Post #4		Eric's PNNL
2. E4	2.97	640450	159679	3.2	749	Post #5	1	Replete
1. G2	5.11	594117	156561	3.1	746	Post #5	10	Deplete 24h
2. F4	2.15	656215	173773	3.05	734	Post #5	1	Replete
1. F7	4.72	571452	130703	3.13	733	Post #5	1	Deplete 24h
73#1 D3	11.26	485391	91131	3.45	649	Post #3	1	Deplete 18h
3. C10	10.14	521103	99152	3.56	643	Post #5	0.5	Replete

WT	8.50	475,694	79,023	3.74	596

Best FAME and outlier on FAME/Size

Best FAME and outlier on FAME/Size

Largest cell size

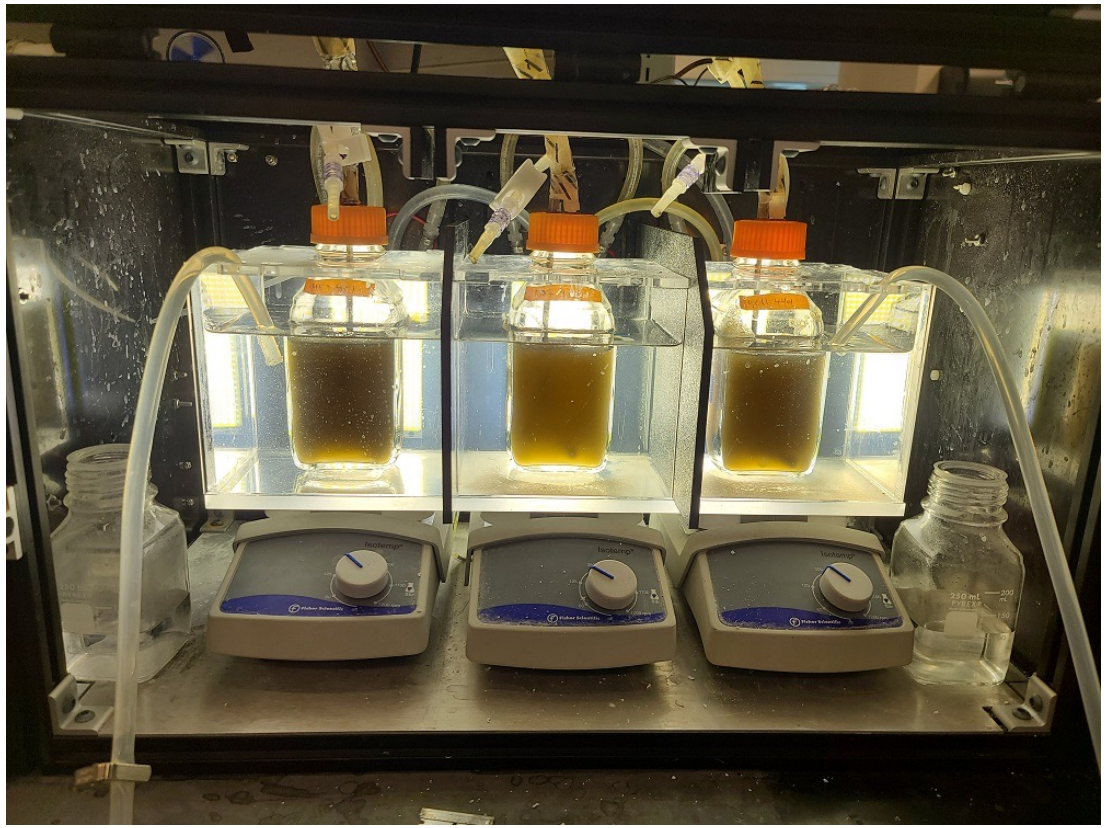
Grown in PNNL bioreactor

Strain Down-selection Focused on Four Strains of Interest

2 – Progress and Outcomes

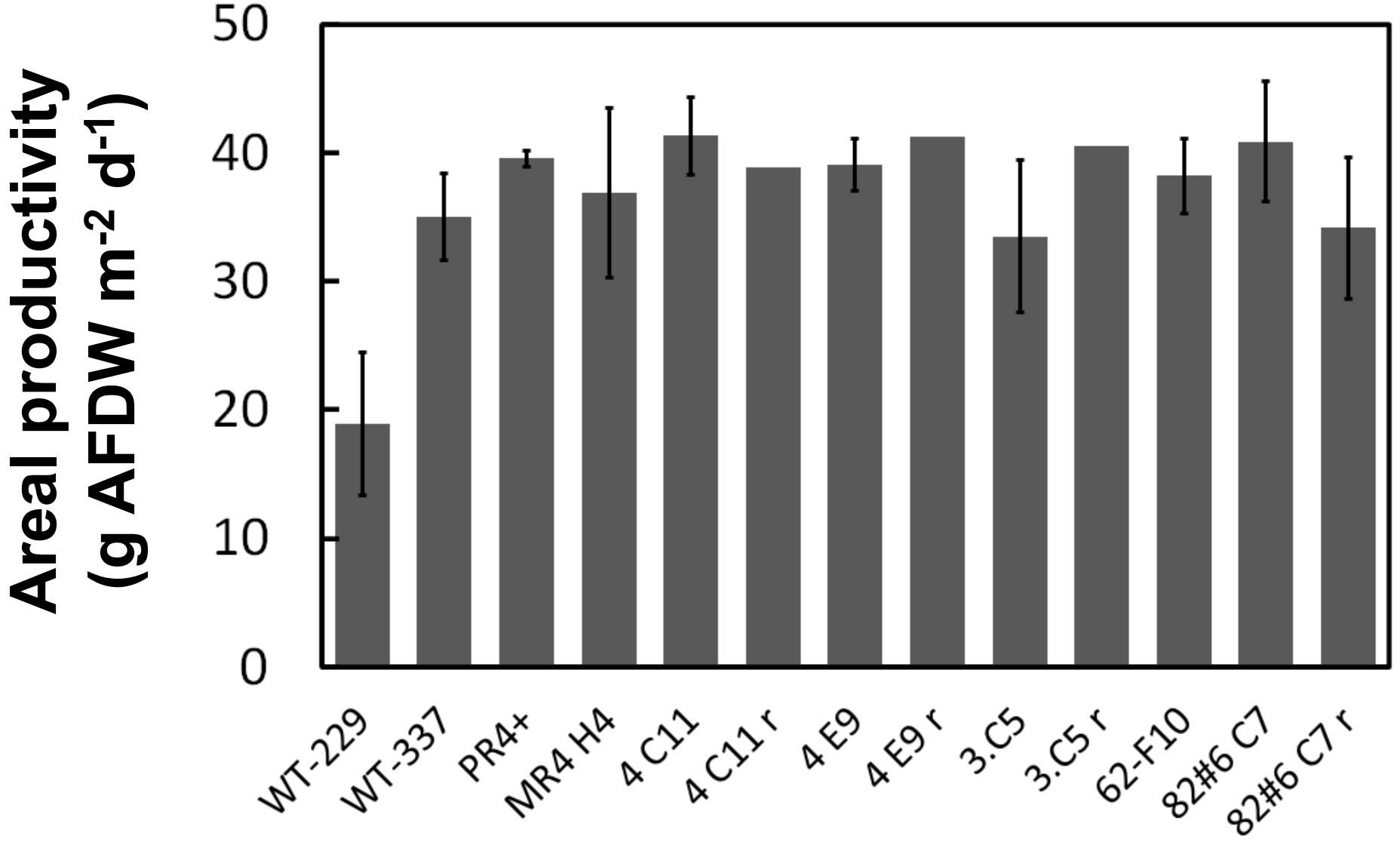
Testing of Promising Down-selected Strains Under Pond Mimicking Scripts

- Light, temperature, and oxygen simulate Kauai ponds in June
- Stable growth achieved and measured
- Lipid phase media shift
- Sampled for at least five days in replete medium and then four days in nutrient stressed medium



2 – Progress and Outcomes

Mutant Analysis Under Pond-Mimicking Conditions

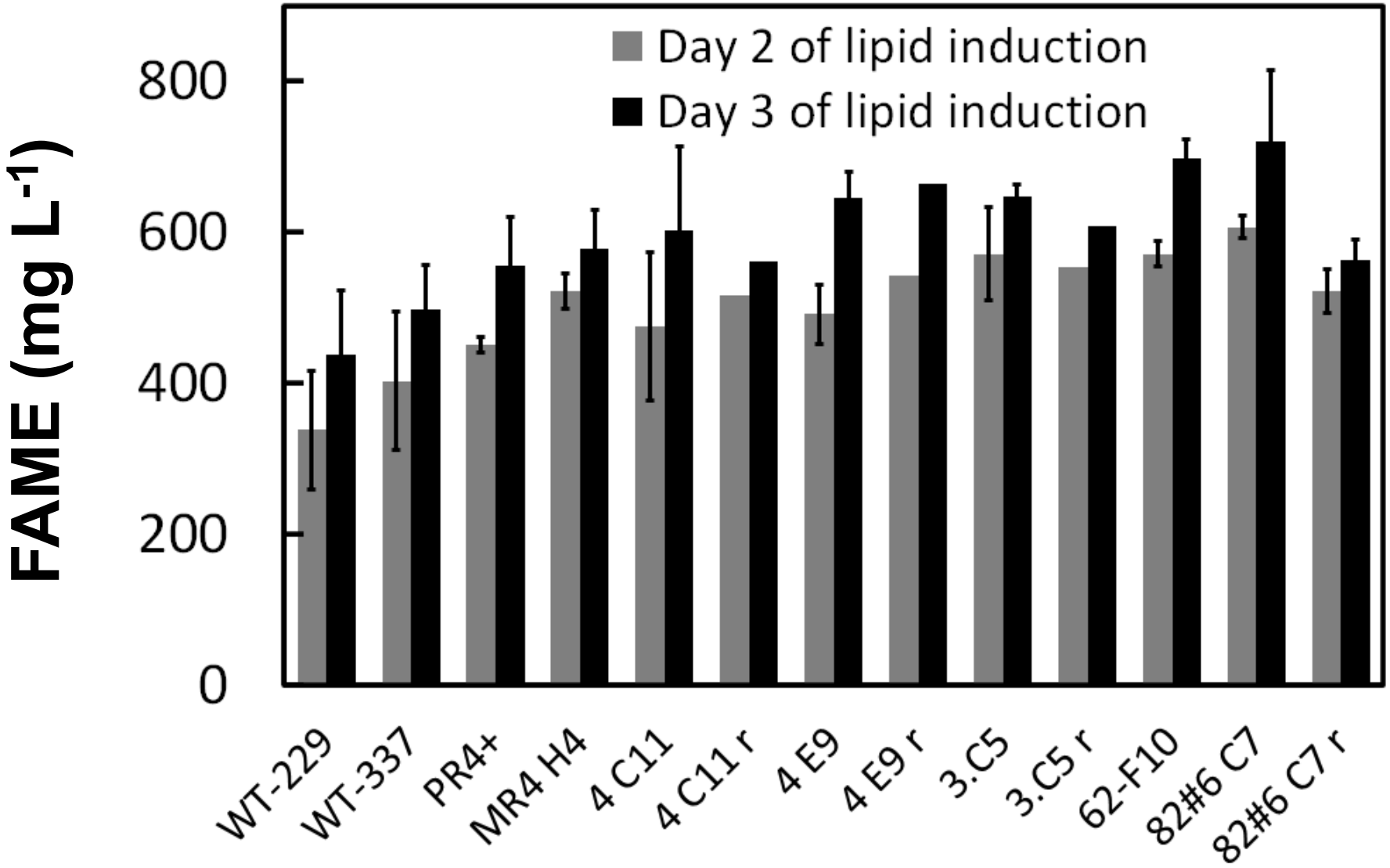


Mutants Selected Often Grow Slightly Faster Relative to WT

"r" designates cryo-revived cultures

2 – Progress and Outcomes

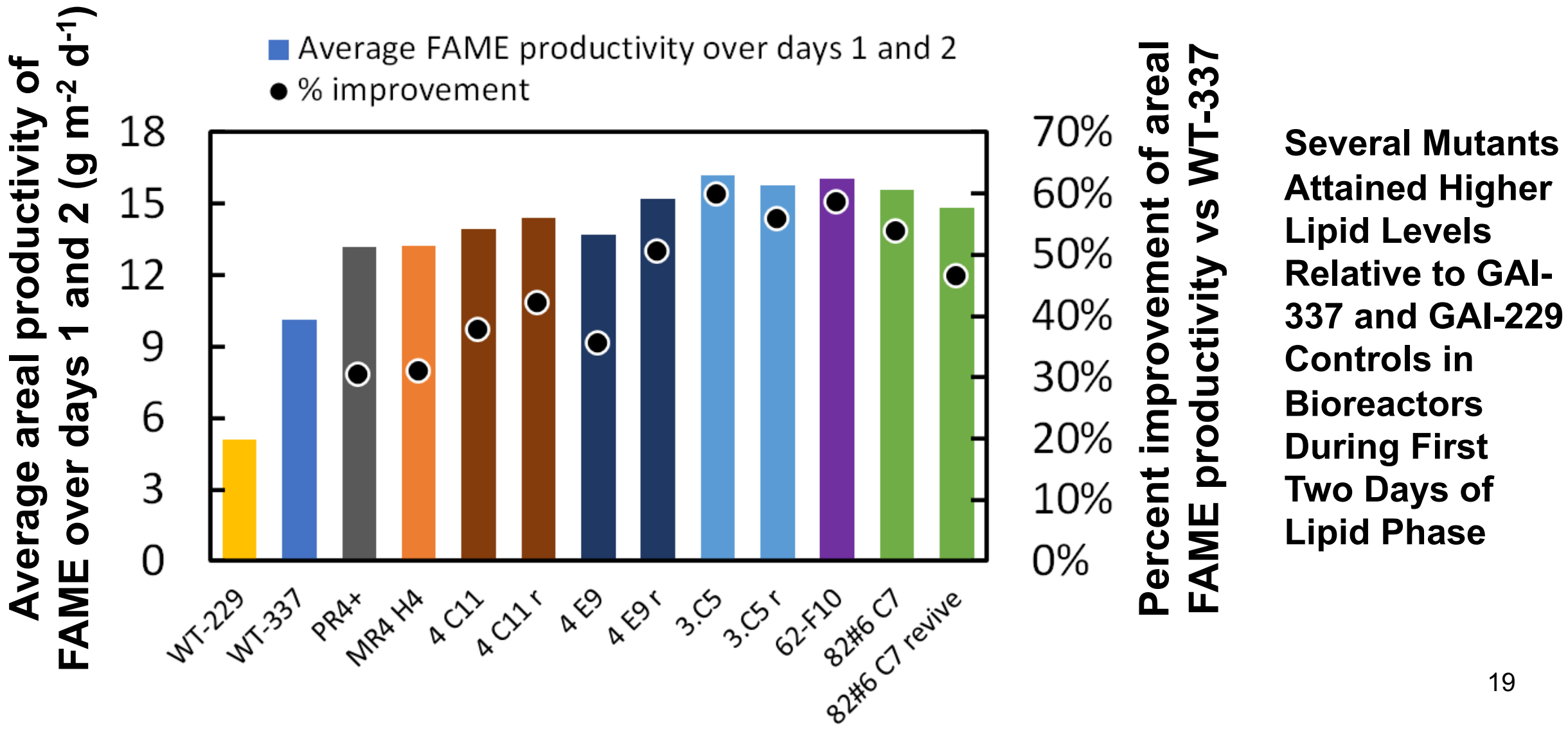
Mutant analysis under pond-mimicking conditions



Initial Screening of Mutants Indicated Higher Yields Relative to GAI-337 and GAI-229 in Laboratory Bioreactors

2 – Progress and Outcomes

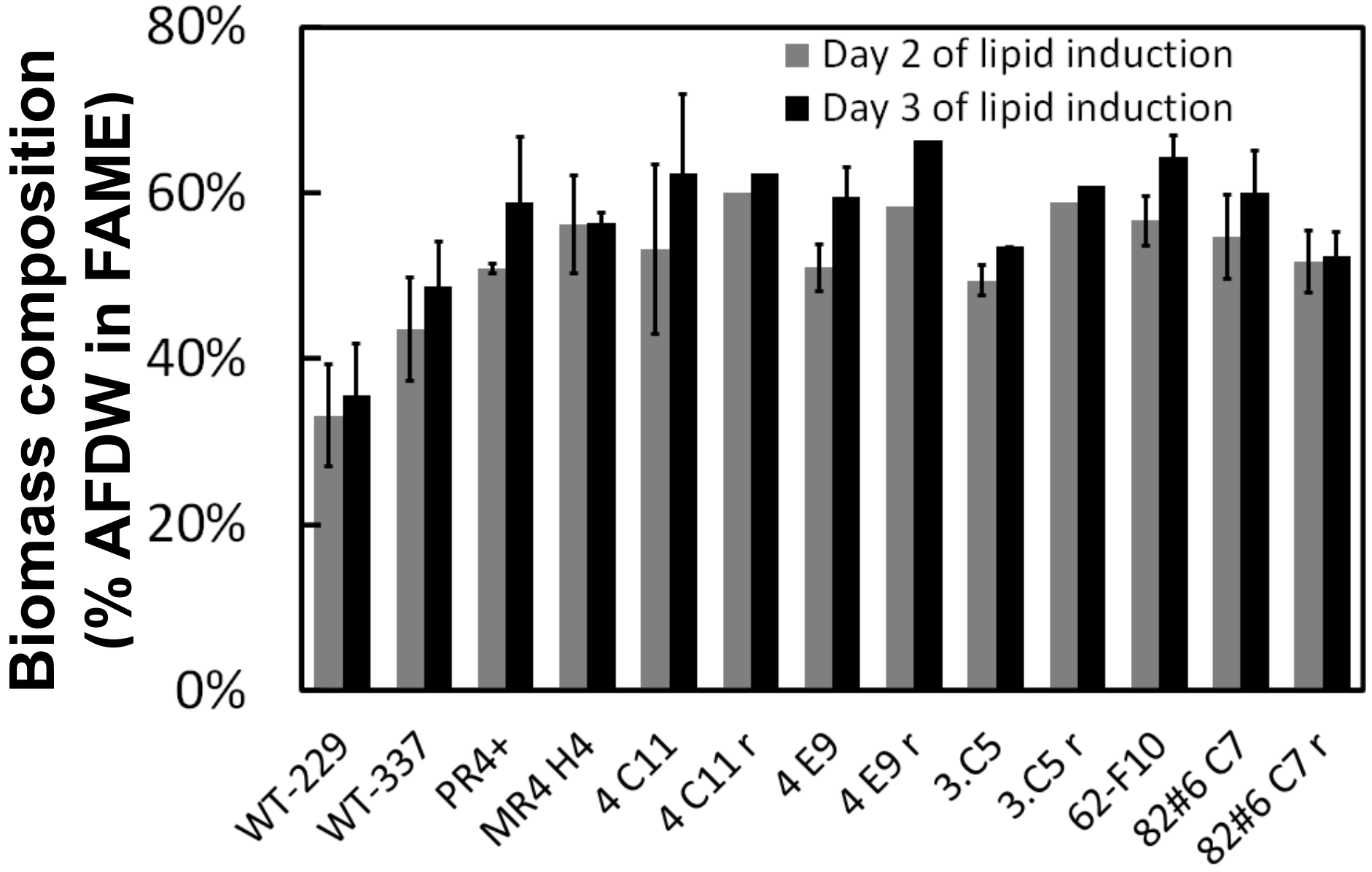
Mutant Analysis Under Pond-Mimicking Conditions



Several Mutants Attained Higher Lipid Levels Relative to GAI-337 and GAI-229 Controls in Bioreactors During First Two Days of Lipid Phase

2 – Progress and Outcomes

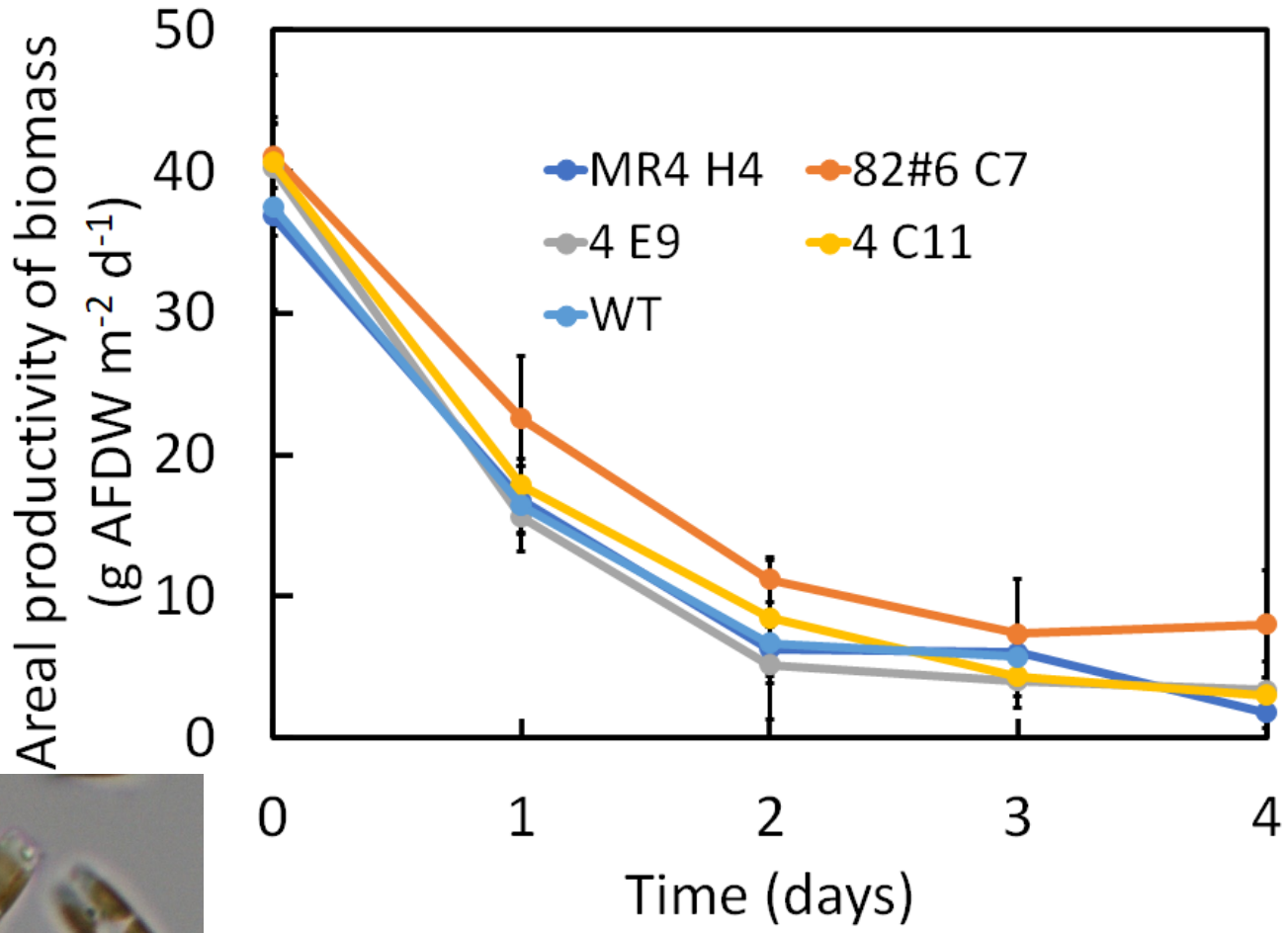
Mutant Analysis Under Pond-Mimicking Conditions

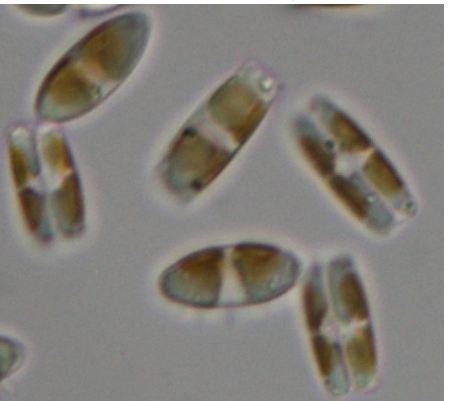


Initial Screening of Mutants Indicated Higher Yields Relative to GAI-337 and GAI-229 in Laboratory Bioreactors

2 – Progress and Outcomes

Testing of Promising Strains Under Pond Mimicking Conditions

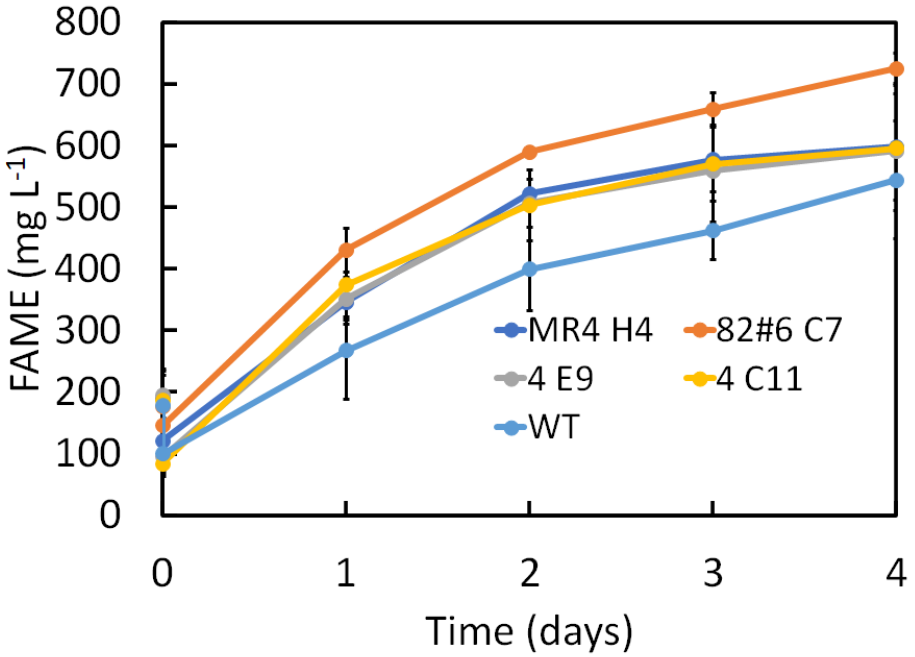


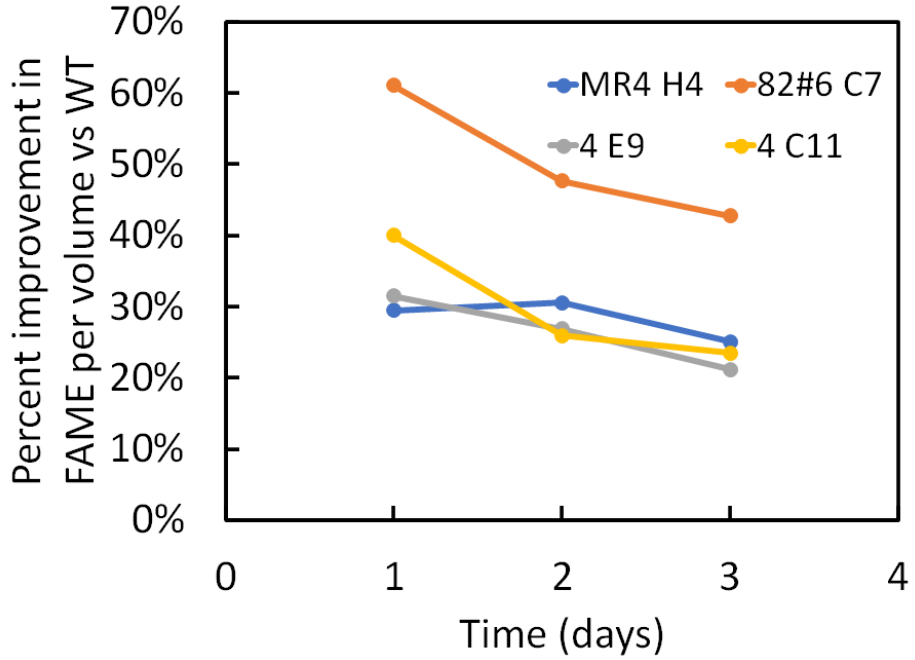


- **High performance mutants typically showed higher productivity than GAI-337 under replete conditions**
- **82#6 C7, 4 E9, and 4 C11 all achieved over 40 g AFDW m^{-2} d^{-1}**
- **Productivity substantially reduced by Day 2.**

2 – Progress and Outcomes

Testing of Promising Strains Under Pond Mimicking Conditions

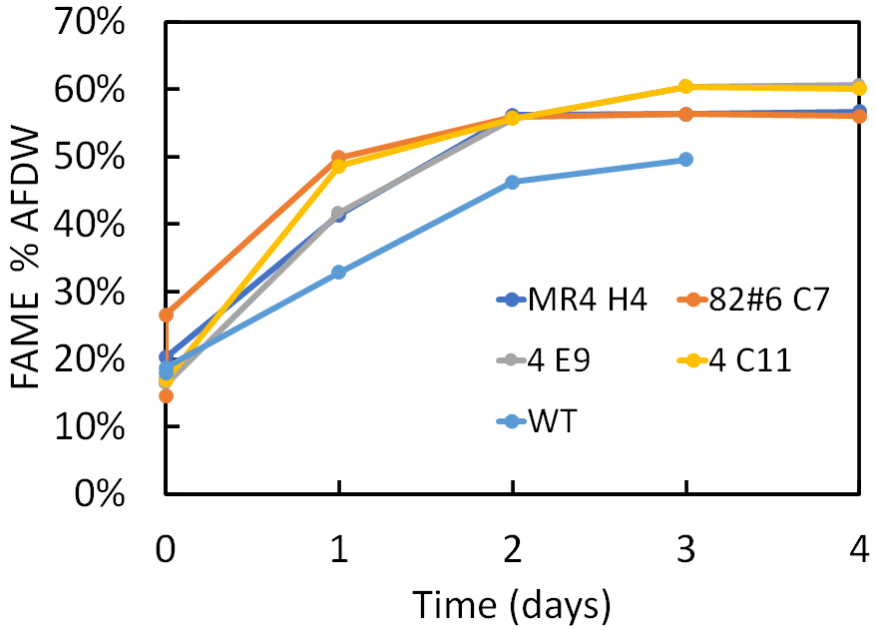


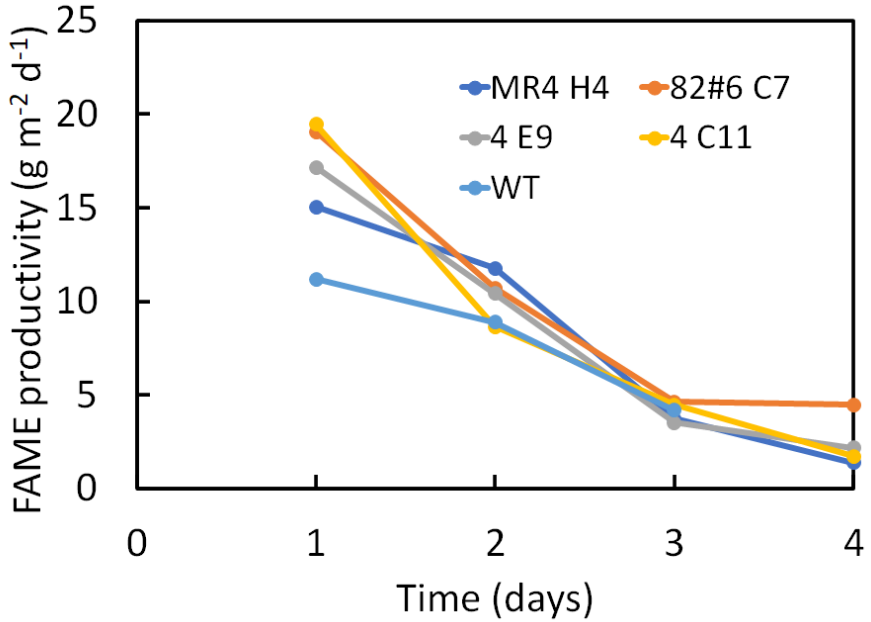


- **82#6 C7 showed the highest improvement over WT with 61% more FAME on day 1 of lipid induction and 43% on day 3.**
- **82#6 C7 achieved the same FAME per volume on day 1 of lipid induction that WT reached on day 3.**

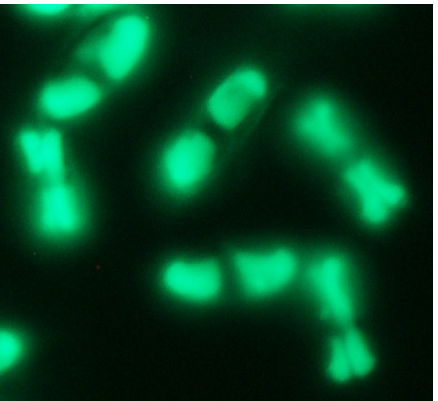
2 – Progress and Outcomes

Testing of Promising Strains Under Pond Mimicking Conditions





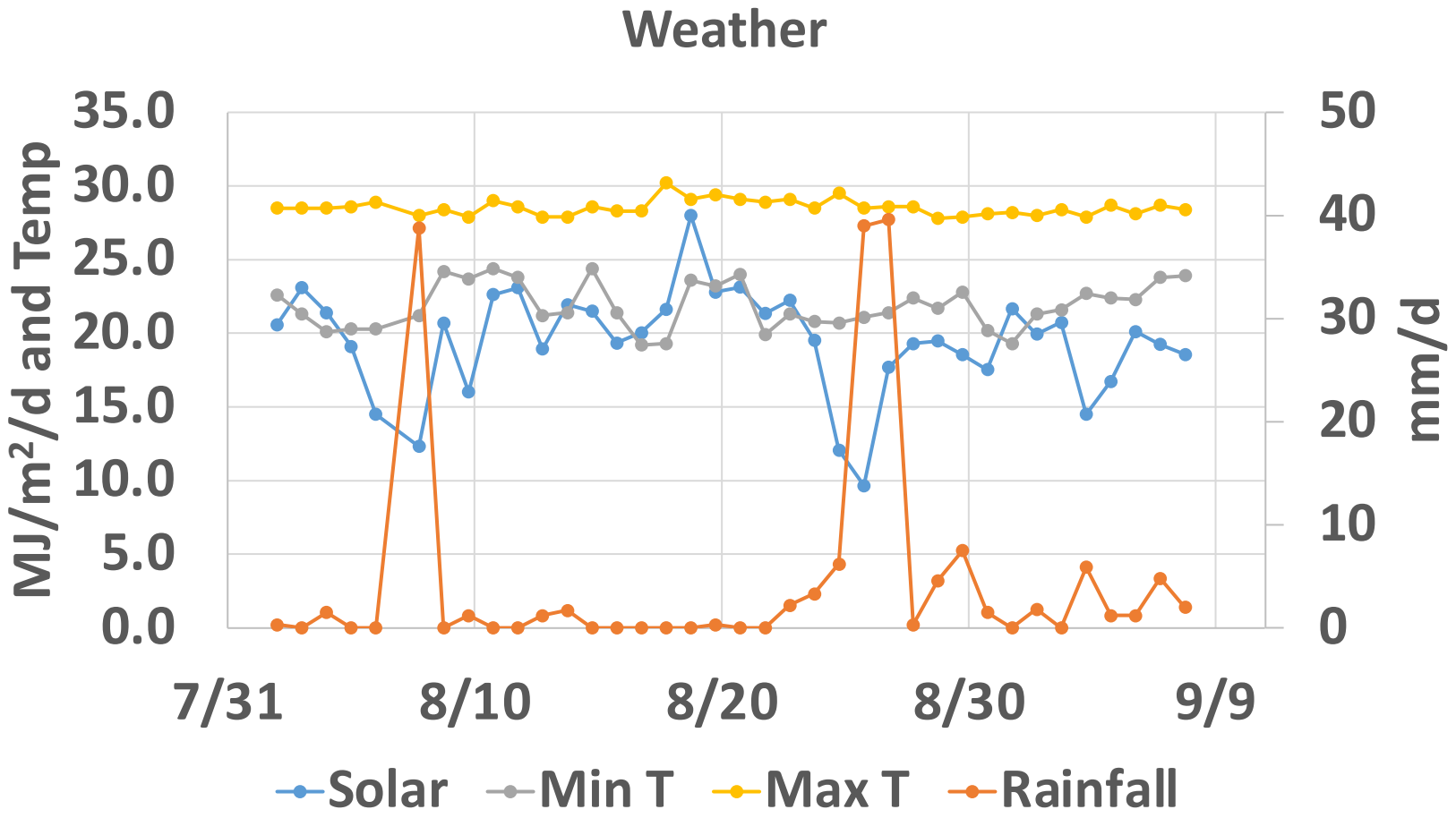
- **4 C11 and 82#6 C7 achieved 50% FAME as a proportion of AFDW by day one of lipid induction, a ~50% improvement over WT.**
- **Both 4 C11 and 4 E9 finished with ~22% improvements in FAME per AFDW over the WT on day 3 of lipid induction.**



- ***In sum attained 2022 Go-NoGo targets of >23 g m^{-2} d^{-1} biomass and >31% lipid in bioreactors***

2 – Progress and Outcomes

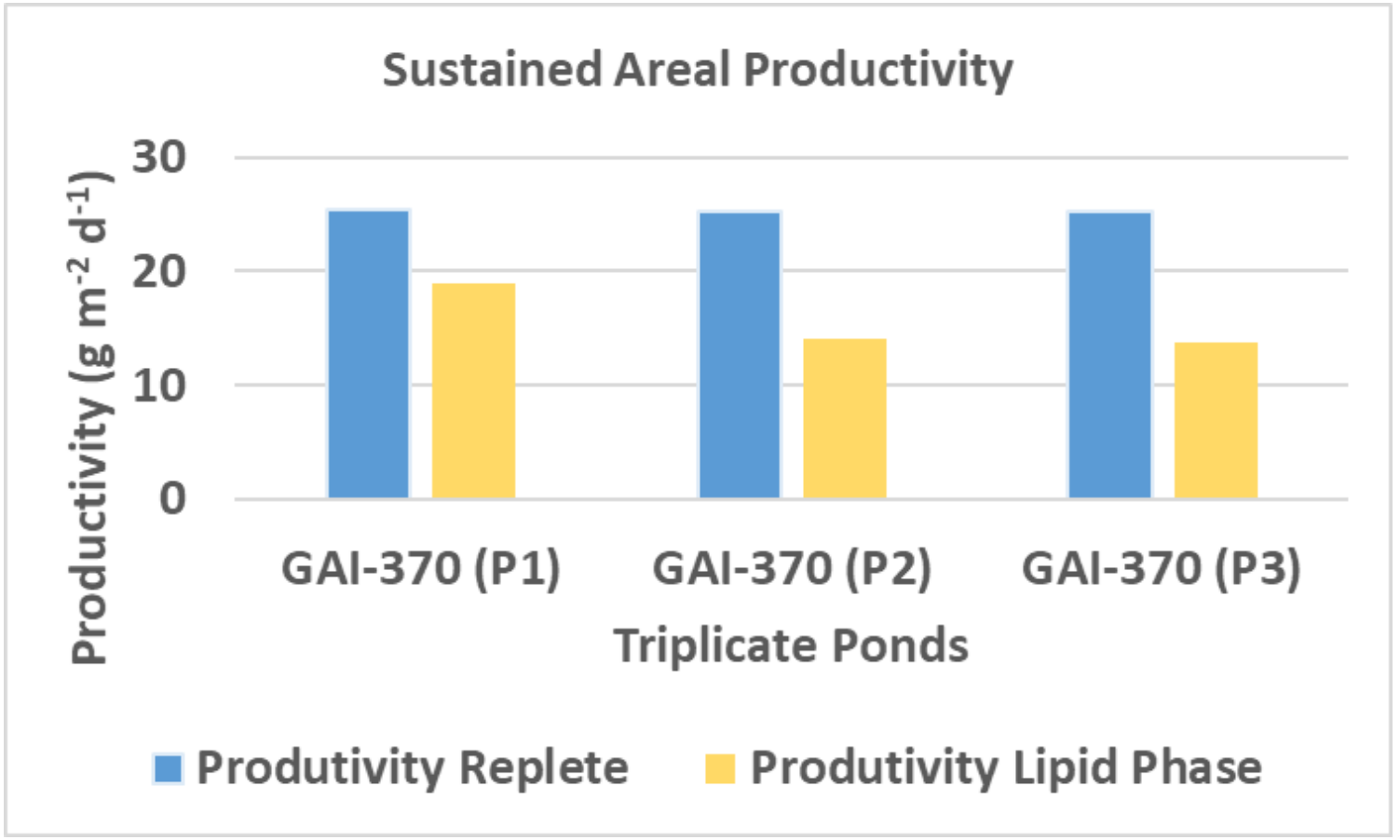
Outdoor Testing at GAI Kauai Farm



- **Strain 82#6 C7 reassigned: GAI-370**
- **Grown outdoors at GAI at end of 2022 summer**

2 – Progress and Outcomes

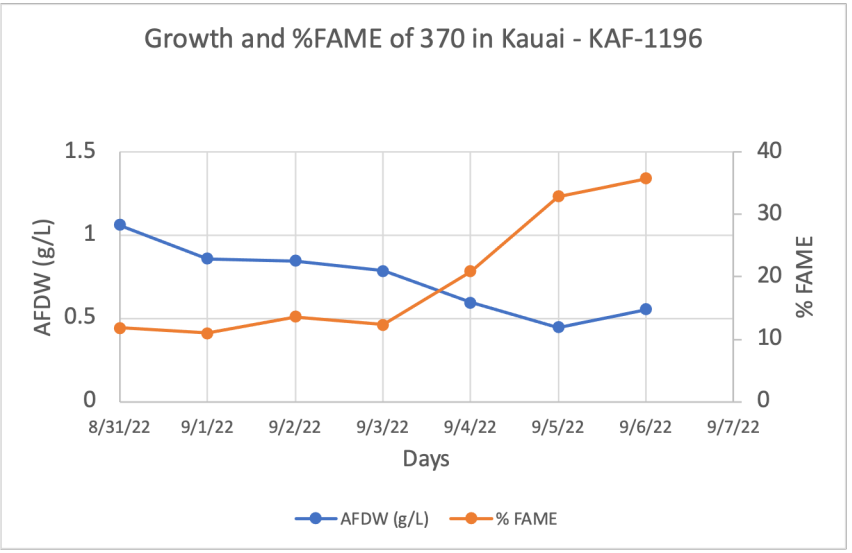
Outdoor Testing at GAI Kauai Farm

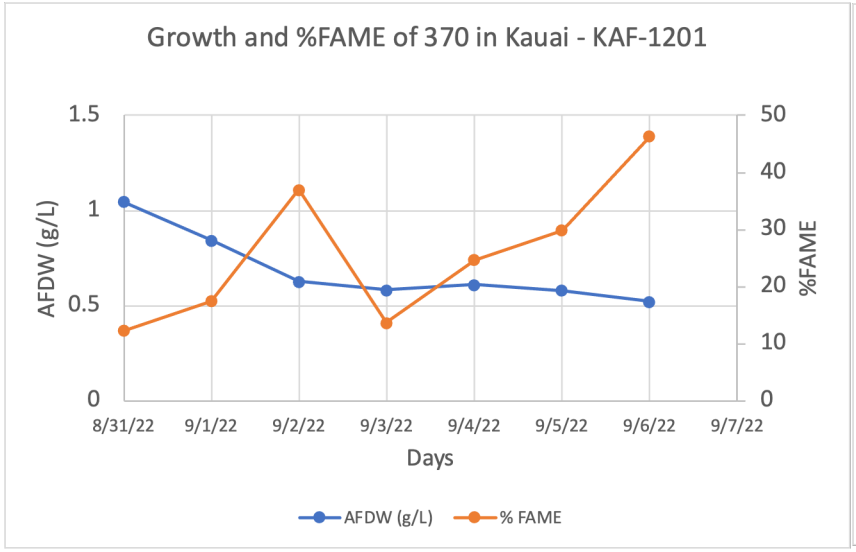


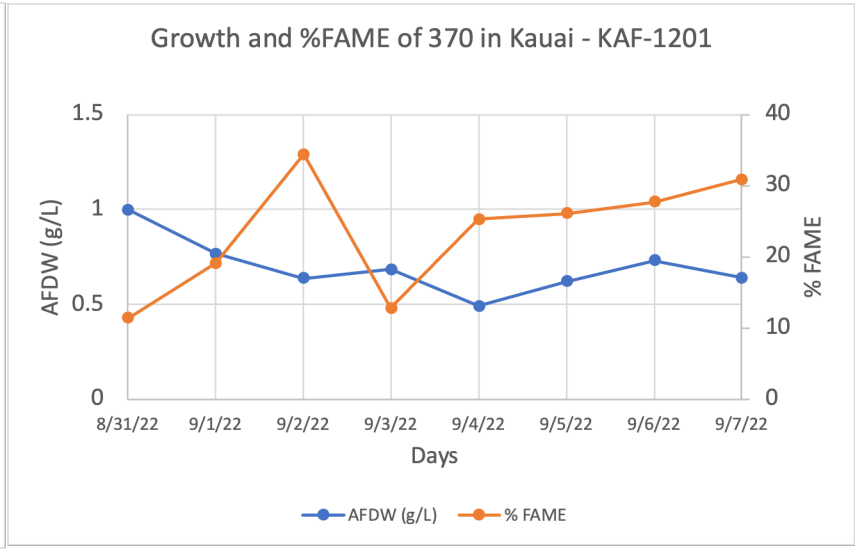
- Average daily productivities from triplicate GAI-370 ponds
- Nutrient replete growth from 8/12/22 to 9/1/22
- Lipid phase from 9/2/22 to 9/6/22

2 – Progress and Outcomes

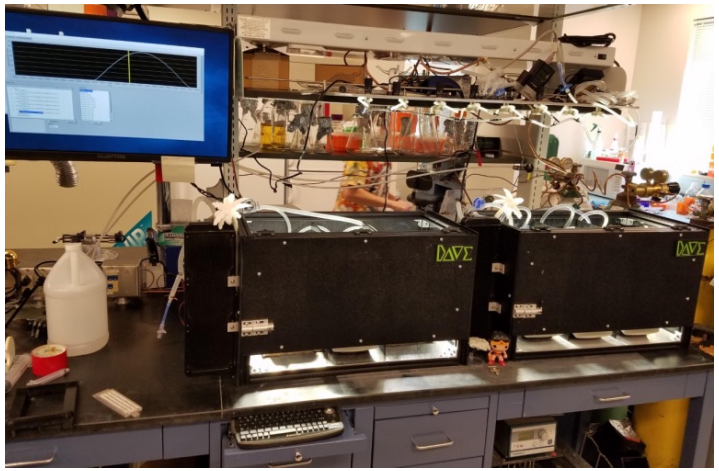
Outdoor Testing at GAI Kauai Farm





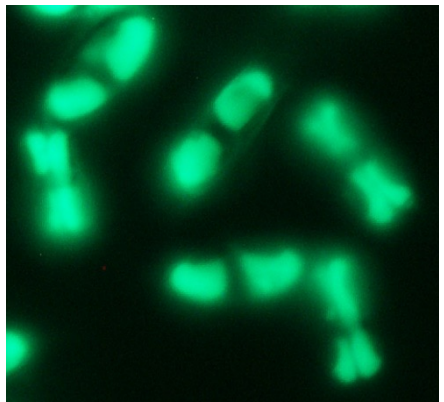


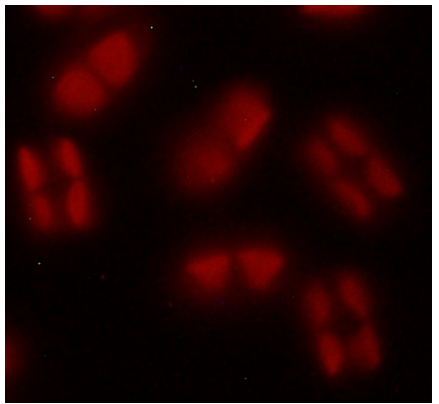
- **FAME data from lipid phase had two ponds with similar behavior and one pond that did not have a FAME dip on 9/3/2022.**
- **FAME data tracked Nile Red assays**
- **FAME levels reached >31% in all ponds**
- **Initial outdoor triplicate ponds reached FAME targets (31%) and >90% of productivity targets (23 g/m^2/d through lipid phase for project with GAI-370)**
- **Reproducibility will be assessed in 2023 and 2024 summer campaigns**

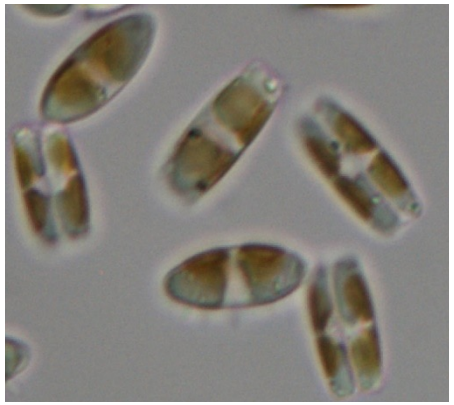


3 – Impact

- **Strain improvements leading to improved productivity and lipid yields.**
- **Viable routes to sustained biofuels require reproducible high-productivity culturing campaigns.**
- **High lipid yields provide streamlined route to sustainable aviation fuels.**
- **Project is identifying culturing mechanisms and strains to attain high productivity and high lipid yields.**







Project Risks and Mitigation

- Ability to Find Mutants With Targeted Phenotypes
  - Targeting two inherently high lipid strains for further improvement [*Nitzschia inconspicua* (diploid) and Nannochloropsis granulate (haploid)]. Additionally Using bioprospecting for new high-lipid/productivity strain isolation.
- Ability to Isolate Mating Partners for *Nitzschia inconspicua*
  - This is high risk and potentially high reward. Efforts are informing mechanisms to induce “self-mating” for increased cell size growth phenotypes.
- Strain Stability
  - We are freezing mutants of interest immediately after identification and probing phenotypes after cryo-revival prior to moving forward. Strains must pass phenotype revival check point. *Nitzschia inconspicua* has multiple morphotypes that have unique productivity phenotypes. Monitoring strain phenotypes through multiple generations.
- Productivity/lipid tradeoffs
  - Focused on high-lipid phenotypes after selecting for high-productivity growth to remove high-lipid “cripples” that do not grow well.

Management

Investigator	Roles
Matthew Posewitz Colorado School of Mines	Overall responsibility for ensuring that project obligations are realized. Coordinates routine (weekly) project meetings. Responsible for assembly and submission of DOE reports. Responsible for integrating peer-review feedback into the project. Responsible for communication of all research results among team members, identifying/mitigating risk and enabling all project participants to contribute towards project objectives. Responsible for communication and collaboration where possible with related projects and advisory boards.
Jesse Traller/Aga Pinowska Global Algae Innovations (GAI)	Responsible for managing project activities at GAI facilities. Leverages collaborative synergies with other GAI projects when possible. Shapes research thrusts and objectives to focus project on areas that are most likely to improve farm yields. Critically evaluates datasets and formulates experimental design. Overseeing bioprospecting and algal breeding efforts at GAI.
Alexander Beliaev Pacific Northwest National Laboratory (PNNL)	Responsible for coordinating mutagenesis efforts with QUT and library screening/mutant selections via cell sorting approaches. Performs strain characterization studies using mini-raceway systems in controlled environmental chambers. Responsible for helping to shape project goals and directions and ensuring open communication across the project.
Kevin Dudley Queensland University of Technology (QUT)	QUT is responsible for constructing plasma mutagenesis libraries, aspects of high-lipid screening, evaluating research progress and shaping project goals.

Summary

- **Laboratory directed evolution of *Nitzschia inconspicua* to high-O_2 stress yielded cultivars (GAI-337) with improved productivities in laboratory bioreactors.**
- **Mutagenesis of GAI-337 and FACS sorting for high lipid strains yielded four priority cultivars that grow slightly faster than WT-337 and reach high lipid levels faster than control cells in lipid formation media.**
- **Bioreactor yields using diel scripts result in productivities of ~40 g/m^2/d.**
- **Strains can accumulate lipid approaching 60% of AFDW in bioreactor experiments.**
- **Initial outdoor campaigns run using one of the down-selected strains (GAI-370) yielded sustained productivities (including lipid phase) of >20 g/m^2/d productivities and yielded over 30% lipids.**
- **Best *Nitzschia inconspicua* mutant will be mutagenized a second time and screened for high-lipid cultivars.**
- **Additional strains and replicated runs will be done in 2023 and 2024.**
- **Mutant libraries of *Nannochloropsis granulata* are being screened.**
- **Approaches to enable *Nitzschia inconspicua* breeding are underway.**
- **Project is near overarching goal of 23 g/m^2/d and 31% lipid in replicated outdoor campaigns.**

Acknowledgements

- Colorado School of Mines
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  - William Chrisler
  - Pavlo Bohutskyi
  - Soujanya Akella
- Queensland University of Technology
  - Raveendra Anangi
  - Robert Speight
  - Kevin Dudley

Quad Chart Overview

Timeline

- *October 2020*
- *September 2024*

	FY22 Costed	Total Award
DOE Funding	$1,324,654	$4,920,382
Project Cost Share *	$341,647	$984,080

Project Goal

- Apply strain improvement strategy leveraging mutagenesis, targeted selection and genome editing tools
- Deploy custom cultivation systems mimicking pond mass-production conditions to improve the success of industrial deployment through indoor-outdoor iterative process

End of Project Milestone

- Algal biomass yield targets of 23 g/m^2/day and lipid content of 31% in replicated outdoor growth campaigns

Funding Mechanism

FY19 BIOENERGY TECHNOLOGIES OFFICE MULTI-TOPIC FUNDING OPPORTUNITY ANNOUNCEMENT: DE-FOA-0002029

TRL at Project Start: 2
TRL at Project End: 4

Project Partners*

- Global Algae Innovations
- Pacific Northwest National Laboratory
- Queensland University of Technology

*Only fill out if applicable.

Responses to Previous Reviewers' Comments

- 2021 review: Bioprospecting seems to be used as a risk mitigation strategy to prepare for the chance that random mutagenesis fails to yield an optimized field strain. This effort is always worthwhile but has been done so many times by so many groups using DOE funding that it no longer seems to be a suitable risk mitigation or strain optimization approach.
  - ***Bioprospecting is being pursued primarily to search for isolates that are able to cross with GAI Nitzschia inconspicua lines. We view this as a very high-risk, yet potentially high reward approach given the importance of plant breeding. Secondarily, new isolates from diverse ecosystems may yield promising new strains that are enriched using GAI growth media.***
- This mutangenizing agent may generate more insertions and deletions resulting in less reversions but the strategy still seems equivalent to gambling when compared to a rational engineering strategy.
  - ***Random mutagenesis and strain selection/enrichment has a long history of success in industrial microbiology. Additionally, several recent publications have highlighted the power of forward genetic screens in finding phenotypes of interest. Reverse genetic techniques are not yet available for Nitzschia inconspicua and random mutagenesis is the most accessible path to genetic diversity and mutants for screening.***
- This project recently started and the project seems to be off to a good start. The management plan is not clear. How often group members communicate is unclear.
  - ***Posewitz/Traller/Beliaev lead efforts at the respective institutional sites. Biweekly video conferences are held with the PIs and scientists where results are discussed and paths articulated. These have proven to be highly effective in keeping the project on task. Quarterly reports are submitted each institution and integrate into the final report. Ad hoc meetings regularly occur among specific research thrusts.***
- A Go/No-Go review was passed in June 2022. Specific progress included attaining cultivars with >15% increase in biomass and lipid yields and able to attain >23 g/m^2/d in bioreactors while attaining greater than 31% lipid yield.

Publications, Patents, Presentations, Awards, and Commercialization

- Burch, T.A., et al. (2023) Mutagenesis, strain selection and growth regime for high-biomass and high-lipid cultivation of *Nitzschia inconspicua*, in preparation.
- Akella, S. et al. (2023) Genomic Underpinnings of High Biomass Productivity Phenotypes in Industrially Relevant Diatom, *Nitzschia inconspicua*, in preparation.
- Methods for producing and cultivating high productivity algae strains. Patent pending (2023) Burch et. al.
- GAI-370 is currently being evaluated at Global Algae as a potential outdoor production strain.