Development of Algal Biomass Yield Improvements in an Integrated Process

DE-EE0007689

Final Report

January 5, 2021

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Pacific Northwest National Laboratory
Qualitas Health
Scripps Institution of Oceanography at the University of California, San Diego
TSD Management Associates
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1 Executive Summary

This project built on the success of Global Algae Innovations Algae Biomass Yield Phase 1 project to accelerate the commercialization of algal biofuels through development of an integrated, economical, photosynthetic, open raceway system to produce algal oil. Two parallel pathways to a biofuel were investigated as illustrated in Fig. 1-1. In the algal crude oil pathway, highlighted in blue, the dewatered algal biomass slurry is used as a feed to hydrothermal liquefaction to produce an algae crude oil and recycle aqueous stream. In the algal lipid oil pathway, highlighted in green, the algal biomass slurry is dried, and the oil is extracted to produce an algal lipid oil biofuel intermediate and a high protein algae meal co-product. Upgrading to drop-in fuels has been demonstrated for the biofuel intermediates in both pathways. Since the algal lipid oil pathway requires lipid accumulation, the productivity is generally lower than the algal crude oil pathway, but the required productivity for economical algal biofuel production is also lower because the coproduct value is greater. The outdoor cultivation was performed at the Kauai Algae Farm illustrated in Fig. 1-2.

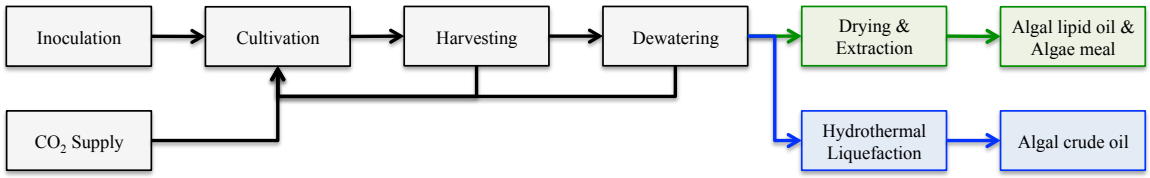

***Fig. 1-1** Block flow diagram with the two downstream pathways for biofuel intermediates*

***Fig. 1-2** Photographs of the integrated Kauai algae farm operated by Global Algae Innovations*

The project team has expertise across the full breadth strain development, advanced algal cultivation, open raceway contamination control, CO_2 supply, harvesting, dewatering, extraction, hydrothermal liquefaction, technoeconomic analysis and life cycle analysis:

- Hildebrand laboratory at Scripps Institution of Oceanography – diatom strain development.
- Mayfield laboratory at UCSD - green algae strain development.
- TSD Management Associates- CO_2 supply, algal harvesting and dewatering, and algal drying and extraction.

- Qualitas - large-scale test site for algal harvesting technology.
- Texas A&M University - algal drying and extraction technology.
- Pacific Northwest National Laboratory - hydrothermal liquefaction technology.
- GE Water and Power - membrane technology.
- National Renewable Energy Laboratory - algal techno-economic analysis.

Phase 1 of the project resulted in tremendous productivity and pre-processing improvements in an integrated, large-scale, low-cost cultivation and pre-processing process that moved algal technology closer to economic viability for biofuels than ever before. Phase 2 accomplishments further facilitate development of a commercial algal biofuel industry by adding:

- several top performing strains and strain development tools including breeding
- open-pond cultivation innovations that achieved a 30% improvement in overall productivity, an 83% reduction in cost, and a 95% reduction in energy use relative to conventional technology
- further improvements in harvesting that led to the Zobi harvester® as a universal, economical, low energy, commercially available microalgae harvesting solution
- breakthroughs in drying and extraction including a new low energy drying process and several new low energy extraction unit operations that together with the Zobi harvester® achieve a 90% reduction in cost and 97% reduction in energy use relative to conventional technology.

These improvements are sufficient to enable economical production of algae biofuel and protein meal co-product if the process were scaled up to a commercial-scale 5,000-acre algae farm. The overall projections in terms of cost and energy use savings for a commercial-scale algae farm are summarized in Figures 1-3 and 1-4.

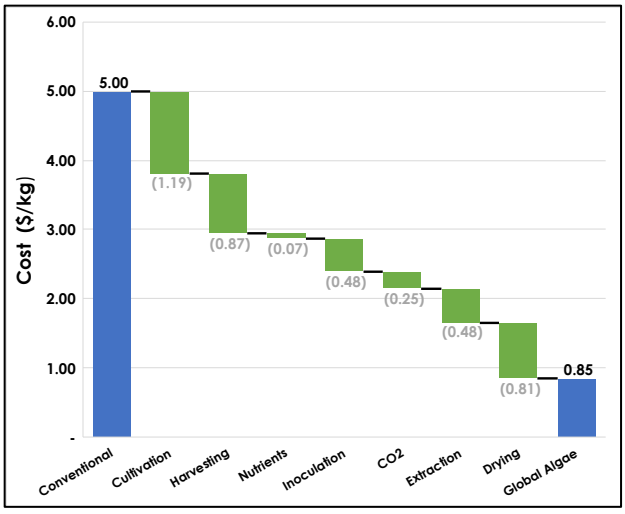

Figure 1-3 Cost reduction achievements

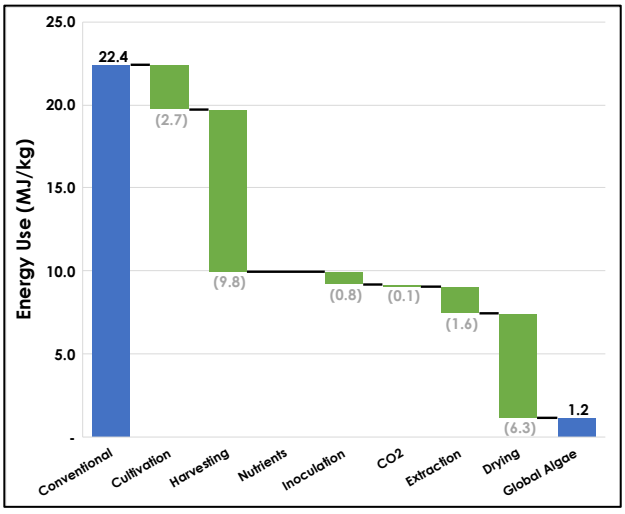

Figure 1-4 Energy-use reduction achievements

2 Accomplishments versus the goals and objectives

The program goals were to achieve a productivity of 3700 GGE/acre-year, perform preprocessing with 10% of the biofuel energy content, and demonstrate that the cultivation and preprocessing can be fully integrated in a scalable process. More aggressive goals were set for the project of 5000 GGE/acre-year, perform preprocessing with 5% of the biofuel energy content, and demonstrate that the cultivation and preprocessing can be fully integrated in an economical, scalable process with a projected minimum selling price for 15-20% algal slurry of less than $500/mt for the lipid pathway.

In the phase 1 project, the productivity in GGE/acre-year was increased 100% from 1200 GGE/acre-year to 2500 GGE/acre-year through breakthrough set of new open-pond cultivation technologies. In this phase 2 project, the goal was to obtain an additional 50% improvement with an aggressive target of an additional 100% improvement through a combination additional cultivation technology improvements and improved strains. A productivity of 3300 GGE/acre-year was achieved for the lipid oil pathway, and a productivity of 4200 GGE/acre-year was achieved for the crude oil pathway; however, only the lipid oil pathway achieved the preprocessing and integration goals. For the economical, scalable, lipid oil pathway, the productivity was improved by about 30%, but fell short of the overall project goal of a 50% improvement. The cultivation improvements translated into high biomass productivity, but the strain development did not result in the desired higher lipid content or faster lipid accumulation under outdoor cultivation conditions.

In phase 1 of the project, a breakthrough in harvesting along with improvements in drying and extraction resulted in achieving a 90% reduction in preprocessing energy, which enabled preprocessing with only 9.6% of the energy content of the biofuel intermediate. In this phase 2 project, improvements in the harvesting technology and new breakthroughs in drying and extraction achieved another 60% reduction in preprocessing energy, which enables lipid oil preprocessing with only 3.8% of the biofuel energy content. This achievement greatly exceeds both the goals for the program and the aggressive target for the project. Through the Algal Biomass Yield project, major breakthroughs have been achieved for all preprocessing operations - harvesting, drying and extraction. Each of these processes are radically different and greatly improved relative to conventional technology resulting in a 95% reduction in energy use and a 90% reduction in operating and capital cost.

The cultivation and preprocessing were fully integrated in a scalable process, and a projected minimum selling price for 15-20% algal slurry of less than $496/mt was achieved for the lipid pathway. Thus, both the program and project integration goals were achieved.

3 Project activities summary

There are four main tasks in the project – Cultivation, Strain Development, Preprocessing, and Integration. Cultivation and strain improvements were focused on increasing the lipid productivity

in terms of GGE/acre-year. Cultivation included outdoor cultivation of improved strains from strain development as well as improvements in abiotic and biotic conditions for cultivation. The strain development included attempts to improve the lipid productivity of *Nitzschia sp* (GAI-229), *Nannochloris sp.* (GAI-247), and new strains that had been bioprospected at the UCSD Biological Field Station. In addition, GAI-247 was adapted for higher thermal tolerance. The objective was to combine the improved strains with the improvements in cultivation to achieve the project goals. Unfortunately, the strain improvements other than thermal adaptation did not translate to outdoor cultivation, so only the cultivation improvements contributed to achieving the productivity goals.

Preprocessing included development of a membrane harvest system with testing in a commercial environment, and development of new extraction and drying processes. The objective was to reduce the energy use in a scalable, economic process. Material generated in the cultivation effort was used for the preprocessing tests so that the end-to-end process was fully integrated.

The integration task focused on updating the techno-economic model and providing timely information for development of the cultivation and preprocessing technologies to ensure only economical, scalable processes were developed.

3.1 Cultivation Improvements

The cultivation subtasks are:

1. Scaling down the cultivation breakthroughs developed in Phase 1 to R&D scale.
2. Improvement of abiotic conditions with lower energy use for cultivation.
3. Improvement of biotic conditions.
4. Improvement in lipid formation methods.
5. Outdoor testing of new strains from the strain improvement task.
6. Improvement in HTL yields through adjusting algae composition prior to harvesting.

Subtasks 1, 2, 4, and 5 constituted the majority of the effort. Subtask 3 resulted in more questions than answers, and the likelihood of a breakthrough before a better understanding of the microbiota composition was deemed to be low, so resources were shifted to the other subtasks. Subtask 6 was dropped because incorporation HTL test results in the techno-economic models showed that the approach was not likely to be economical or to meet the energy efficiency goals. The improvement in productivity was achieved almost solely through subtask 4, but subtasks 1 and 2 results improved the process economics and reduced the energy in cultivation. Additionally, subtasks 1 through 4 all provided a foundation and path forward that is likely to lead to productivity improvement in the future.

A non-proprietary summary of the approaches and results is presented below. Proprietary briefings on the technologies and results have been presented throughout the project to the algae team at the BioEnergy Technologies Office (BETO).

3.1.1 Scaling down the cultivation breakthroughs

In phase 1, a new cultivation technology was developed that achieved an average productivity of 23 g/m^{2}d at 0.2-acre scale and greater for 5 months using GAI-247. During phase 2, multiple approaches were tested to attempt to scale down the new cultivation technology to the smaller-scale research systems. After many tests and analyses, it was determined that there is a physical limit to the ability to scale-down this technology. This effort ended up not contributing to achieving the productivity goal of the project; however, there were many other benefits of the effort. Firstly, the understanding of the technology was developed sufficiently enough to apply for patents resulting in an issued patent as well as several patents pending (see Section 4). Secondly, while the methods attempted at smaller scale did not work for small-scale, one method did provide a 50% reduction in energy use for larger scale application, so it has the benefit of reducing the cultivation energy. Thirdly, a design to simulate effects of the technology at small-scale rather than utilize the same technology was developed. The design was developed too late for use on this project, but it is now being used by Global Algae and two universities for other BETO projects.

3.1.2 Improvement of abiotic conditions

Methods for improving the abiotic conditions with lower energy use were proposed and evaluated in several series test series. These methods achieved a 50% reduction in energy use for cultivation while maintaining the same productivity as the control, but they did not achieve higher productivity than the control. It was anticipated that the combination of these improved conditions and improved strains would contribute to the productivity goals, but none of the improved strains showed actual improvement in outdoor testing, so this hypothesis was unable to be tested. The improvement in abiotic conditions was measured and documented to facilitate development of new strains that could take advantage of these conditions to achieve higher productivity.

Additionally, the media was optimized for GAI-229 and GAI-247. Response surface method (RSM) was used to optimize media ratios between N:P:Si for the diatom GAI-229 and between N:P:K for GAI-247. Nitrogen was in the form of nitrate and urea and the nitrogen sources were used in different ratios. Experiments were conducted on microplates and cultures were transferred every 4 days. Data from the last transfer - transfer 9 for GAI-229 (Figure 3-1) and transfer 4 for GAI-247) were used for analysis to assure final cultures were in the target media ratios.

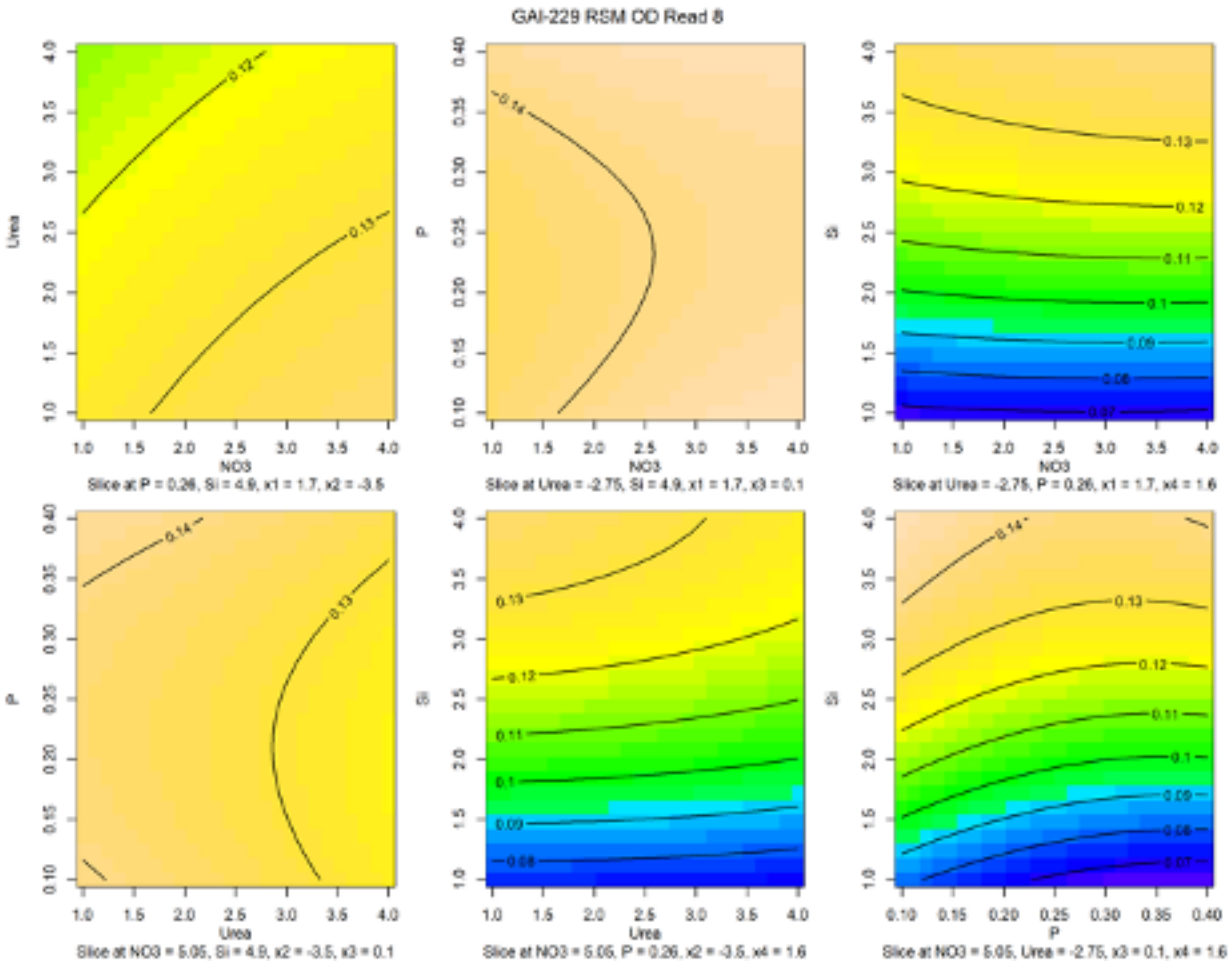

Figure 3-1 *RSM results from second order analysis for media optimization for GAI-229. Effects of urea, NO_3 and Si were highly significant. Adjusted R^2=0.74. Stationary point in original units: NO_3 5, urea -2.7, P 0.27 (all values in mM). This indicates increasing NO_3 and Si, increasing P 0.27 mM and reducing urea 2.7mM should result in largest increase in final OD as compared to the baseline of Si, NO_3 and urea at 2.5mM and P at 0.25mM. This gives NO_3 7.5mM, urea 0 mM, and P at 0.52mM. However P results is not statistically significant.*

Urea and NH_4 toxicity studies were conducted to determine if any toxic effects were occurring as the pH changed and to potentially provide a mechanism for biotic control. Algae can be cultivated using different sources of nitrogen. We tested growth of GAI-247 and GAI-229 on nitrate ($NaNO_3$), ammonium (NH_4Cl) and urea (NH_2CONH_2) in a series of microplate experiments (Figure 3-2). When cultivating algae in bicarbonate media pH can vary from 8.2 to over 10 which can affect the amount of un-ionized ammonia that is more toxic to live organisms. Toxic effect is also positively affected by temperature (Fig. 3-3).

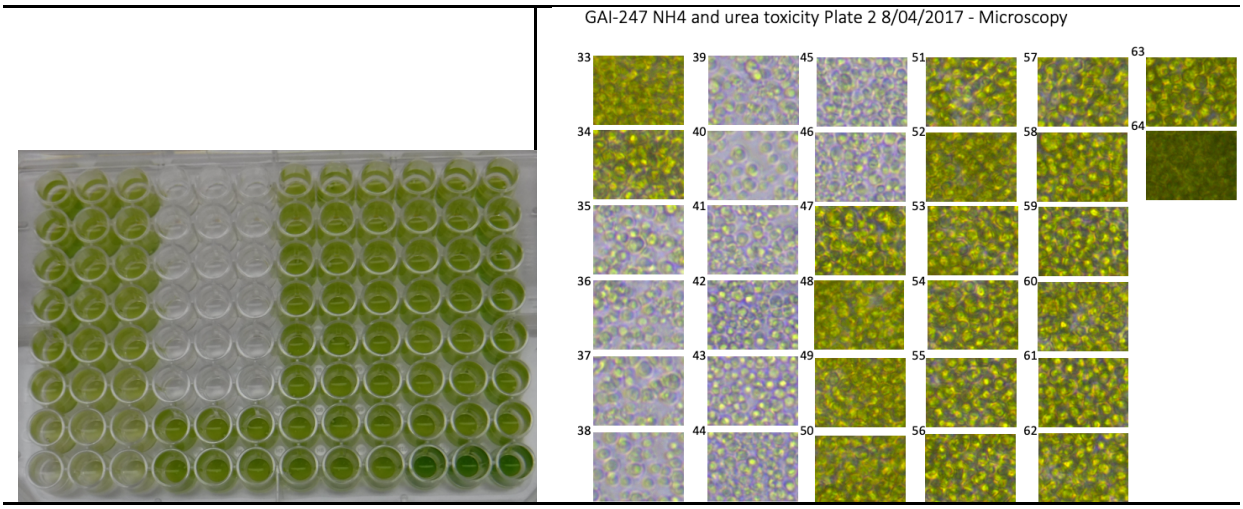

Figure 3-2 *Microplate with N toxicity test (on the left) and microscopy analysis (on the right) showing severe toxic effect of NH4Cl on GAI-247 (clear wells).*

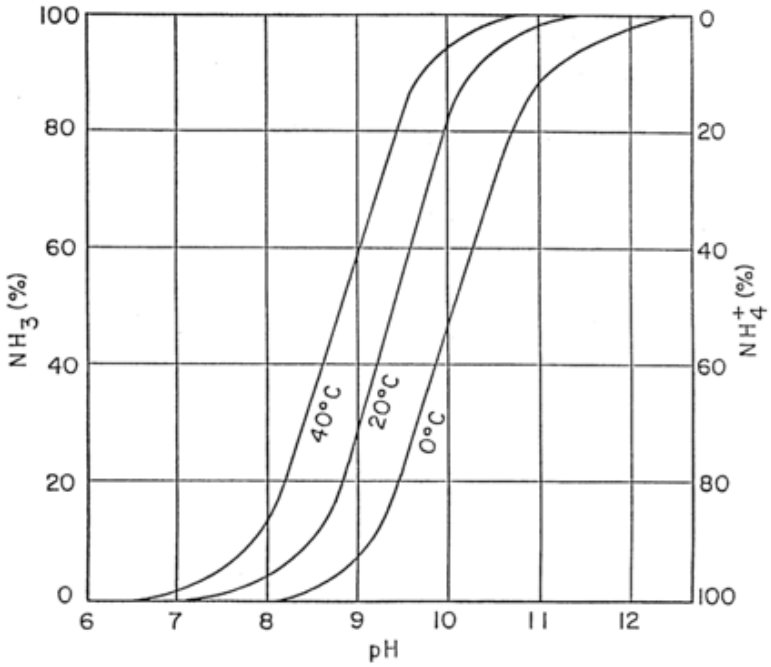

Figure 3-3 *Proportion between volatile ammonia and ammonium ion as a function of pH and temperature. Huang, Ju-Chang & Shang, Chii. (2007). Air Stripping. 10.1007/978-1-59745-029-4_2*

We conducted our experiments and winter and summer Hawaii temperatures and at pHs observed at the KAF. Our low pH was in the 8.2-9.4 range and our high pH was at the 9.6-9.9 range. Optical density measured at 750nm (OD750) was used to monitor algal growth 3 times a day. We also monitored *in vivo* fluorescence of chlorophyll *a* and we conducted microscopy analysis to confirm algae were growing or dying. Each experiment lasted 4 days and the most toxic effect was always observed on the last day. When GAI-247 was cultivated on nitrate there was no toxic effect with increasing pH and temperature. For ammonium the effect was the most severe and for urea lower

toxic effect was observed (Figure 3-4, Figure 3-5). When GAI-229 was cultivated on nitrate no toxic effect was observed with increasing pH and temperature. For ammonium the effect was the most severe and for urea lower toxic effect was observed (Figure 3-6, Figure 3-7). Overall NH_4Cl was the most toxic to both algae species and toxic effect increased with temperature and pH as expected. Nitrate was not toxic to algae at high pH and temperature and urea had a small toxic effect.

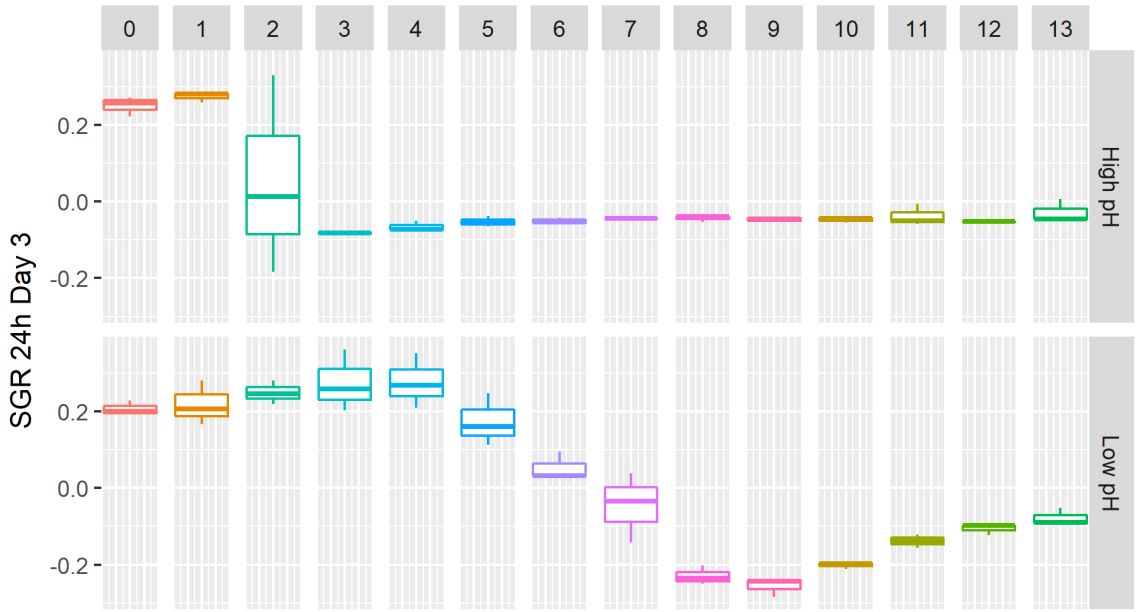

Figure 3-4 *Specific growth rates of GAI-247 at different concentrations of NH_4Cl (concentrations are in mM along the horizontal axis) at winter temperatures. Specific growth rate was calculated based on OD750. At high pH strong toxic effect of ammonia is observed at concentrations above 2 mM.*

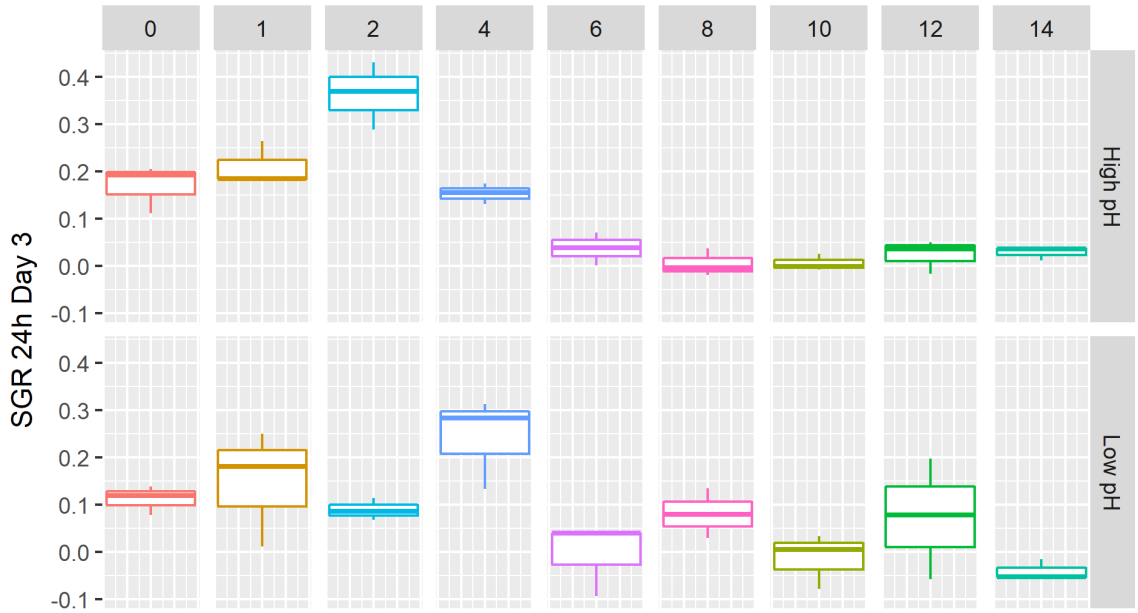

Figure 3-5 *Specific growth rats of GAI-247 at different concentration of urea at summer temperatures. At high pH decrease in growth rats was observed at urea concentration above 4 mM. Probably due to urea degradation to ammonium and ammonia under high pH conditions.*

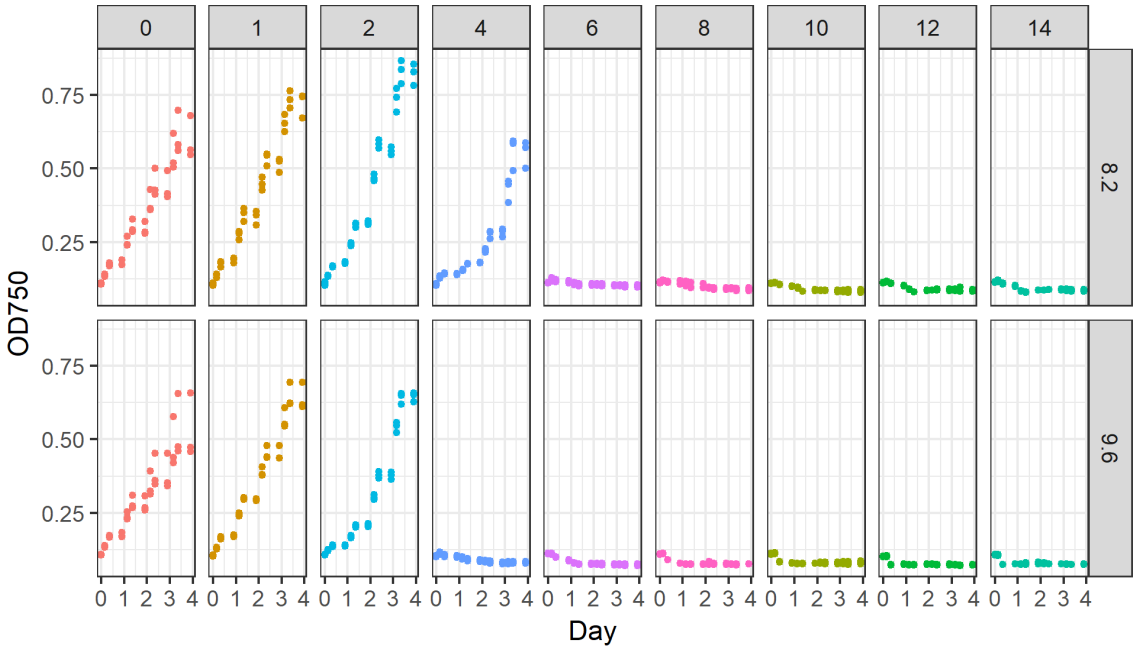

Figure 3-6 *OD750 of GAI-229 at different concentration of NH_4Cl at summer temperatures. Experiment was conducted at pH 8.2 and 9.6. Toxic effect of NH_4Cl was more sever under high pH and was observed at concentration above 3 mM.*

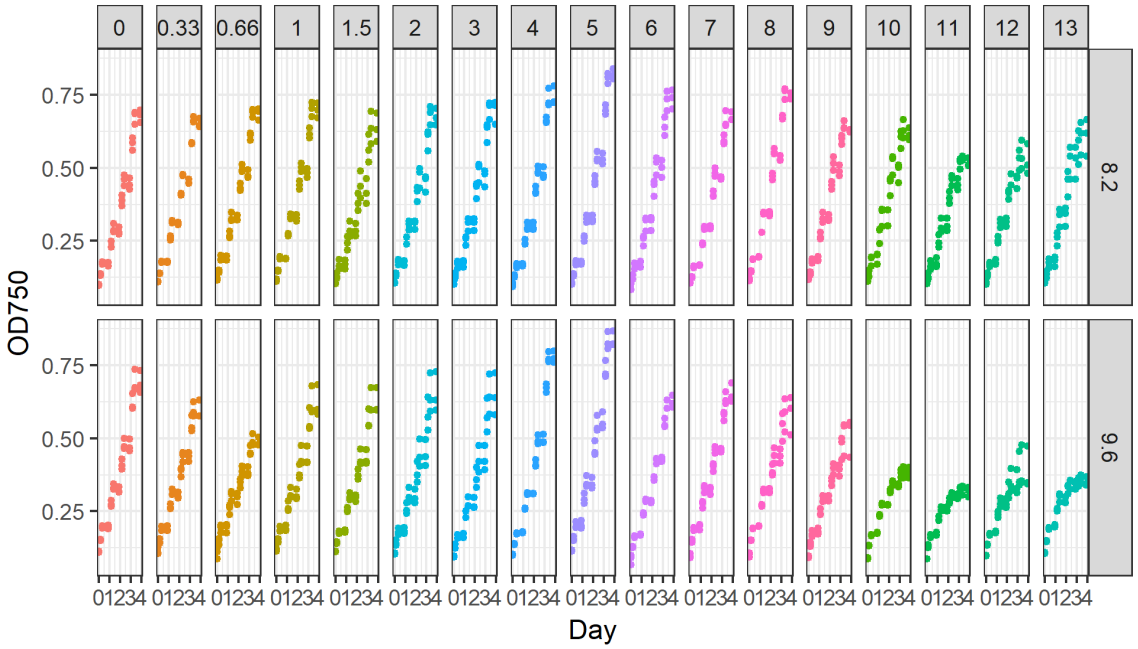

Figure 3-7 *OD750 of GAI-229 at different concentration of urea at summer temperatures. Experiment was conducted at pH 8.2 and 9.6. Toxic effect of urea was more sever under high pH and was observed at concentration above 6 mM.*

3.1.3 Improvement of biotic conditions

Methods to control the bacterial population during algae cultivation were proposed and evaluated in several series of tests. These methods worked to control the population resulting in a qualitatively "cleaner" algae biomass, but no improvement in productivity over the control was observed. There are so many options for control, that better information on the microbiota composition and effects are needed to achieve improved productivity. Therefore, this approach was terminated after the a few series of tests to free resources for use in the other more promising areas. Data was collected on the impacts of various methods on the algae and bacteria in general to provide a foundation for use to improve productivity once tools to measure the microbiota and a greater understanding of the microbiota are attained.

Near the end of the project, a method to control the algae internal state for improved productivity was proposed and tested. The results varied from no improvement to a 25-50% improvement, but consistent results were not obtained. This approach shows promise for the future, but significant additional effort is needed to fully understand the impacts on the algae needed attain a consistent improvement in productivity, especially with the variability of weather and microbiota overlaid on the algae internal state.

3.1.4 Improvement in lipid formation methods

Extensive testing throughout the project was conducted to test hypotheses for improvement in lipid formation. The testing included media optimization, traditional nutrient starvation, and novel modifications to standard cultivation and lipid formation approaches. A 25% improvement in

overall biofuel intermediate productivity was achieved resulting solely from improvements in the lipid formation phase. This result was attained through three different methods. In addition to improving productivity, an objective of this task was to increase the protein content of the harvested biomass to increase the value of the protein meal co-product. Two of the methods resulted in much higher protein content than the third, so optimization of each method in the future would likely result in greater biofuel intermediate productivity and a higher value co-product algae meal.

Figure 3-8 illustrates the time for oil formation with the improved lipid formation methods. The time to reach high lipid level was reduced from 3 days to 1 day. Because productivity during lipid formation is lower than the growth phase, reducing the time to 1 day increased the overall productivity by 25%. A second method increased the overall productivity by reducing the area required for lipid formation.

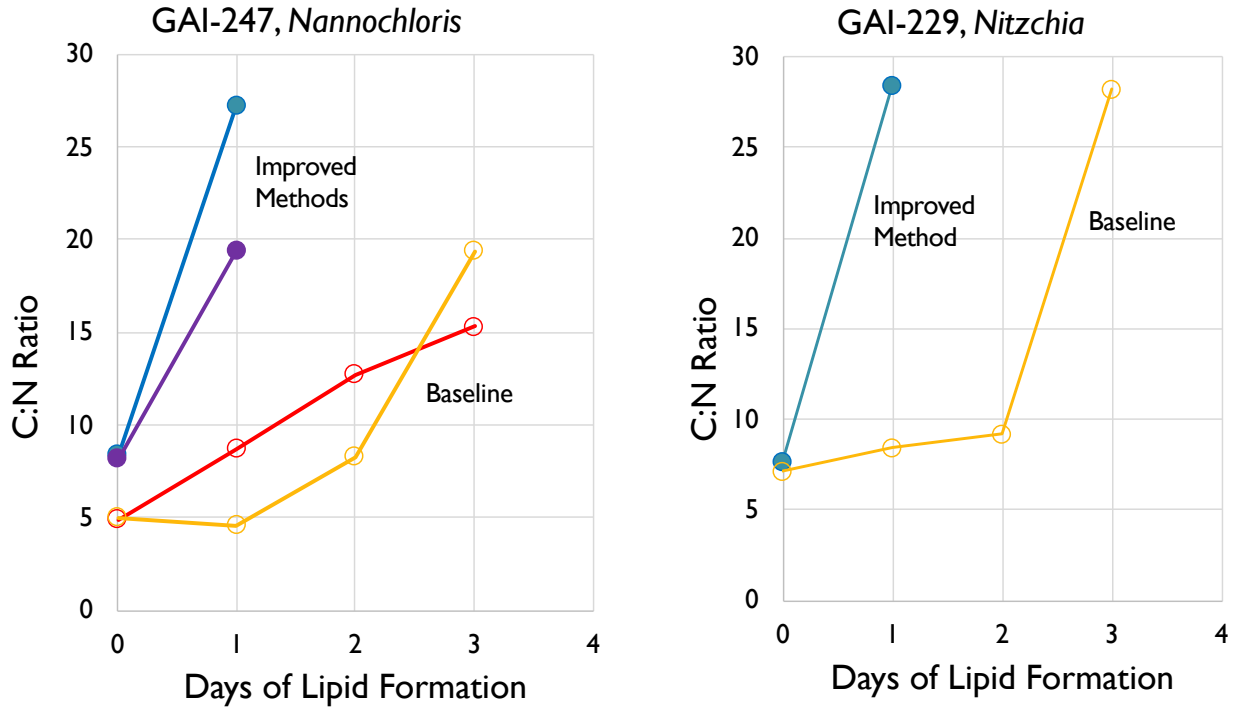

Figure 3-8 *Improved methods achieved 1-day lipid formation versus 3-day with nutrient depletion*

3.1.5 Testing of improved strains

Many of the strains developed in the laboratory were scale-up and tested outdoors. None of the strains with improved productivity or lipid content in the laboratory were better than the baseline *Nannochloris* (GAI-247) and *Nitzschia* (GAI-229) strains, Figure 3-9 and Figure 3-10. The improved *Nannochloris* and *Nitzschia* strains all achieve similar productivity and lipid content to the wild type strains when scaled for outdoor testing. Some of the *Chlamydomonas* and *Chlorella* strains were improved relative to their wild type strains, but they were far inferior, i.e. 60% greater productivity reduction, relative GAI-229 and GAI-247 in all four outdoor test trials. Both strains

clumped and appeared stressed, so media optimization could have improved the growth rates. A thermal tolerant *Nannochloris* strain (GAI-285) was developed and used outdoors.

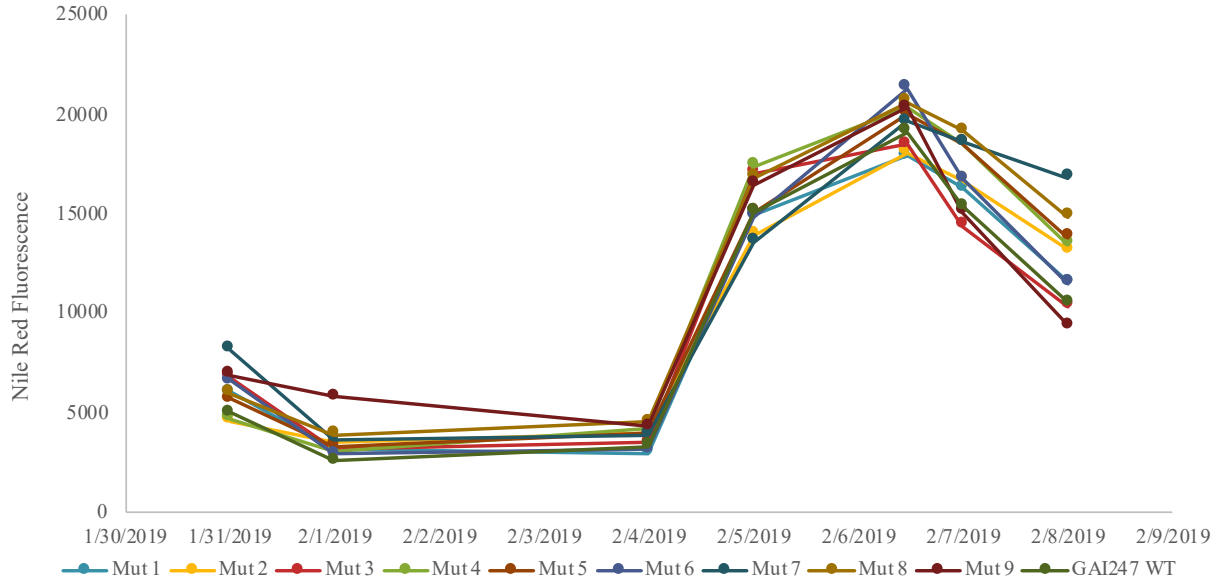

Figure 3-9 *No significant improvement over GAI-247 based on Nile Red fluorescence as a proxy for lipid content*

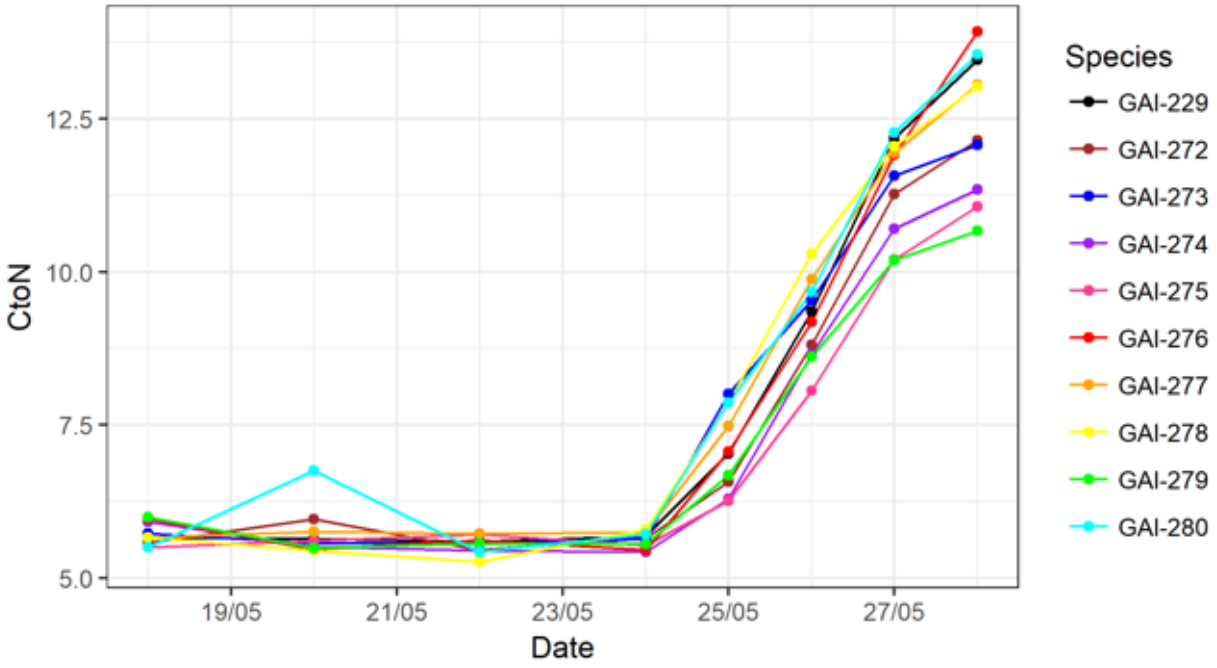

Figure 3-10 *No significant improvement over GAI-229 based on C:N ratio as a proxy for lipid content*

During the project 13 high lipid mutants of GAI-247 were tested. These mutants were obtained through mutagenesis and FACS screening of high lipid cells. The three most promising strains were screened for growth and lipid production in a microplate experiment. Strains were screened

in different N sources (nitrate and urea) and at low (8.8) and high pH (9.6). The wild type GAI-247 had the highest growth rates compared with the mutants in all growth conditions tested, and no increase lipid production was observed using Nile Red staining as a screening tool. This recalcitrance to mutagenesis and lipid production improvement may be due to the phenotypic plasticity that the genome and overall biology of the species. GAI-247 genome is diploid, resulting in multiple close paralogs of genes. The genomic redundancy in GAI-247 may explain why: (a) generating a strong phenotype was difficult in GAI-247 since most mutations would be complimented and (b) the stability of the mutations seems to be low since redundant wild-type alleles that promote fitness would likely be copied-back in to mutant loci over time.

3.2 Strain Development

With the exception of thermal tolerance, most of the laboratory strain development was conducted by UCSD.

Summary:

The objective of this task was to adapt strains from the *Chlamydomonas* genus to grow in GAI bicarbonate media. This task was successfully completed by using a variety of approaches.

(1) A species of *Chlamydomonas moewusii* was isolated through bioprospecting from the UCSD field station in freshwater media and was subsequently found to be naturally tolerant of GAI bicarbonate based media.

(2) Simultaneously a collection of freshwater *Chlamydomonas reinhardtii* strains were evolved and then bred together to produce a strain that is tolerant of the GAI bicarbonate based media.

(3) Finally, we were able to isolate a number of other species from the UCSD field station that possessed desirable traits, including the ability to grow in GAI's sodium bicarbonate containing media. Some of these species seemed to be more amenable to strain improvement through mutagenesis and screening (see the following sections) than GAI-247.

While it was unknown prior to the start of this project if this was possible for this genus, we now know there are naturally tolerant strains as well as evolutionary techniques available to generate such strains.

3.2.1 Chlamydomonas Adaptation to Bicarbonate Media

Chlamydomonas is a well-characterized genus of single celled eukaryotic green algae that has been studied in detail for several decades. Notably, the species *Chlamydomonas reinhardtii* has been a model species for genetics, evolution, and physiology making it an attractive organism for biotechnology and synthetic biology applications. Recently, *Chlamydomonas reinhardtii* has received Generally Recognized as Safe (GRAS) from the FDA making the species considered fit for human consumption. This GRAS approval opens alternative product pathways if this species could be grown at commercial scales and low cost. Therefore, we proposed to attempt to improve production qualities of *Chlamydomonas* so it could potentially be in high productivity outdoor

raceways. *Chlamydomonas* species are typically freshwater dwelling and have therefore not been grown commercially due to limitations on freshwater utilization for large scale production as well as contamination issues of raceways. We used a multifaceted approach to develop and improved *Chlamydomonas* strain that grows readily in GAI bicarbonate media.

First, we bioprospected for strains of Chlamydomonas that would spontaneously grow in the media. We established small ponds containing a range of different media formulations outdoors at the UCSD biological field station to see if naturally occurring species of *Chlamydomonas* would populate the media. This station is located roughly one kilometer inland from the Pacific Ocean, so we hoped to sample both terrestrial and potentially marine species that may be transported by aerosols into our ponds. The ponds were bubbled with air and left completely exposed to the environment to facilitate the collection of natural species of algae (Figure 3-11). Samples were taken once a week and struck out on agar plates of the same media and single colonies were isolated and species were identified via microscopy and PCR amplification with subsequent DNA sequencing of the ribosomal Internal Transcribed Spacer (ITS) region of the genome. Once strains were isolated and identified, they were screened for tolerance on GAI bicarbonate media.

Figure 3-11 *Ponds used to trap algae at the UCSD field station.*

Using the bioprospecting "trap" ponds set out at the biological field station, several species of green algae, including two species of *Chlamydomonas (C. reinhardtii* and *C. moewusii*) were isolated. However, none of these strains were isolated from the GAI bicarbonate media. We observed that the ponds containing bicarbonate were predominately populated with diatom species with essentially no green algae observed. Green algae collected were cataloged and reserved for potential future work (Figure 3-12). Of the green algae strains isolated, we determined that *Chlamydomonas moewusii, Chlorella vulgaris,* and *Desmodesmus armatus* grew on the GAI bicarbonate media.

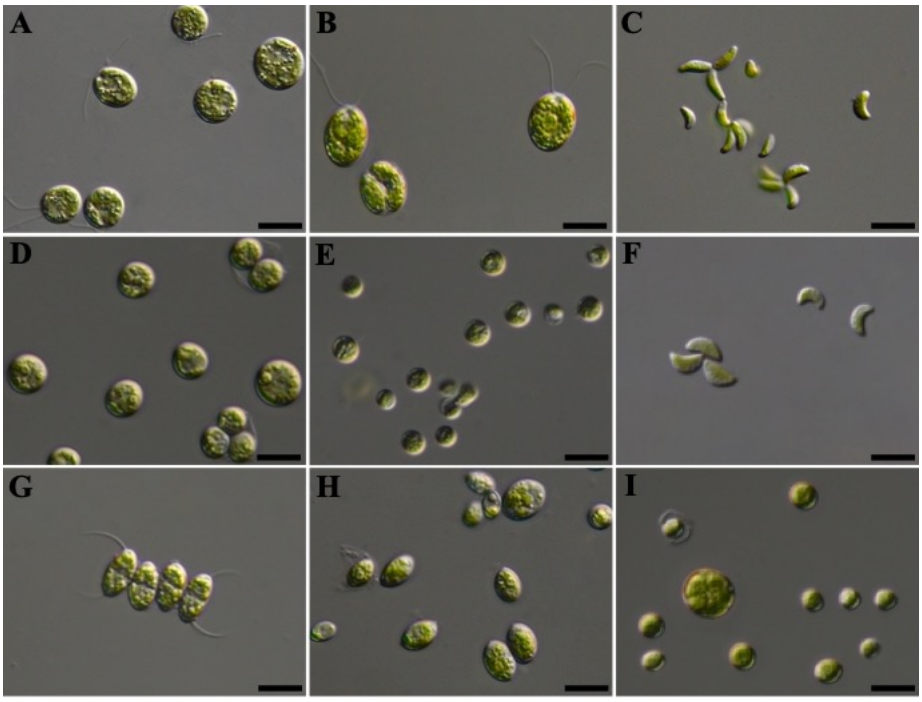

Figure 3-12 *Light microscope images of Chlamydomonas reinhardtii (A), Chlamydomonas moewusii (B), Tetranephris sp. (C), Coelastrella sp. (D), Chlorella vulgaris (E), Monoraphidium sp. (F), Desmodesmus armatus (G), Acutodesmus obliquus (H), and Parachlorella kessleri (I). Scale bars are 10 µm.*

Second, we assembled a library of wild-type species of *Chlamydomonas reinhardtii* from publicly accessible culture collections. A collection of 19 wild-type strains were assembled, including 9 strains that were mating type plus (*mt*+) and 10 that were mating type minus (*mt*-). The strains of all one mating type were mixed together then co-cultured. These co-cultures were screened for growth in bicarbonate of various concentrations. Once the limiting concentration of sodium bicarbonate was identified, cultures were restarted using inoculum from the highest concentration media. This process continued for several months in an attempt to adapt the cultures to higher bicarbonate media. Once a limit of tolerance was reached, the two cultures were mated together, and the progeny were reared in GAI bicarbonate media.

All of the 19 *Chlamydomonas reinhardtii* strains retrieved from stock centers showed very low tolerance to sodium bicarbonate. All strains died almost immediately when introduced to media containing more than a few g/L sodium bicarbonate, and even as little as 2g/L was strongly inhibitory to growth. An immediate shift in cell morphology was noted when *C. reinhardtii* cells were placed in media containing greater than 2g/L sodium bicarbonate (Figure 3-13). The cells appeared larger and would grow predominantly in aggregates of cells while becoming deflagellated when placed in greater than 2 g/L sodium bicarbonate containing media. We found that if progressively increasing concentrations of sodium bicarbonate were titrated into cultures of *C. reinhardtii* over the period of days, cultures did not die. This gradual adaptation method allowed us to generate *C. reinhardtii* strains that would grow in GAI bicarbonate media; however, these

bicarbonate adapted cells still displayed poor growth and cells clumped together and frequently settled out of solution.

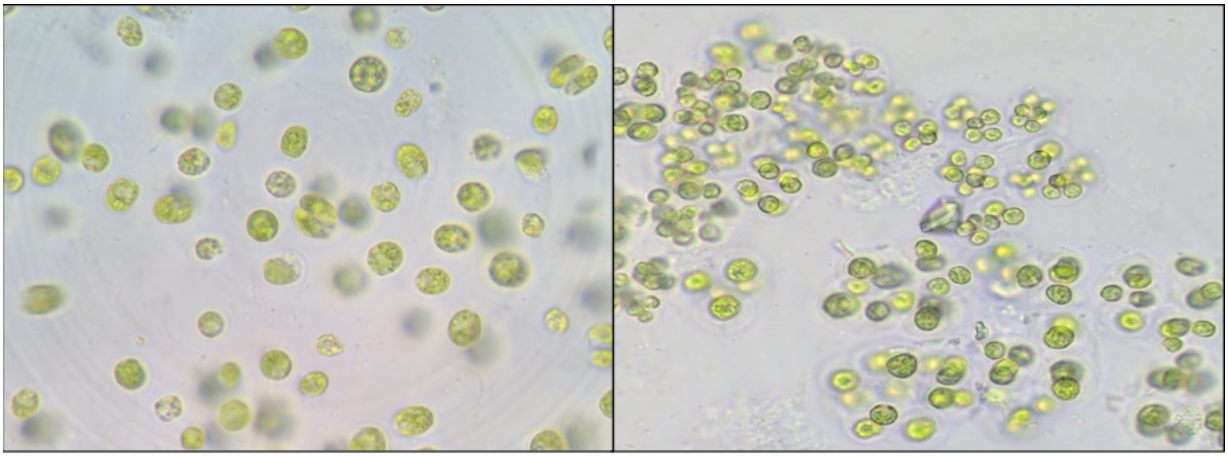

Figure 3-13 *Comparison of C. reinhardtii in different concentrations of sodium bicarbonate. Cells clumped and flagella were lost with >2 g/L of sodium bicarbonate in the media.*

Third, since the initial adaptation of *C. reinhardtii* to sodium bicarbonate containing media was slow and yielded a clearly sub-optimal strain in the lab that would be inappropriate for outdoors growth, an alternative method was required to increase genetic variability and create the potential phenotypic range. The collection of *C. reinhardtii* we retrieved from the stock centers represented strains with inherent genetic variation based on previously published literature reporting genetic polymorphisms between strain genomes. We therefore crossed our collection of mt+ and mt- *C. reinhardtii* strains together in fresh water media to permit flagella formation since flagella are required for mating in *Chlamydomonas* (Figure 3-14). The cells become de-flagellated in GAI bicarbonate media (Figure 3-13). After pellicle formation (the fusing of the haploid parents into diploid zygotes) the pellicle was placed in GAI bicarbonate media. This media was previously lethal to all *C. reinhardtii* strains we previously had tested. Some of the haploid progeny that emerged from the pellicle grew readily in this media, which was markedly better than we observed in cells that were bicarbonate adapted. This final strain was referred to as "CR25" and was used throughout the rest of the ABY2 project. Upon examination we achieved a maximum density of 1.2 g/L in media with GAI bicarbonate media in our 5% CO_2 incubator. This represents a slightly lower maximum density than in media with 1.5 g/L added bicarbonate. However, we observe that growth rate is much slower in the GAI bicarbonate media.

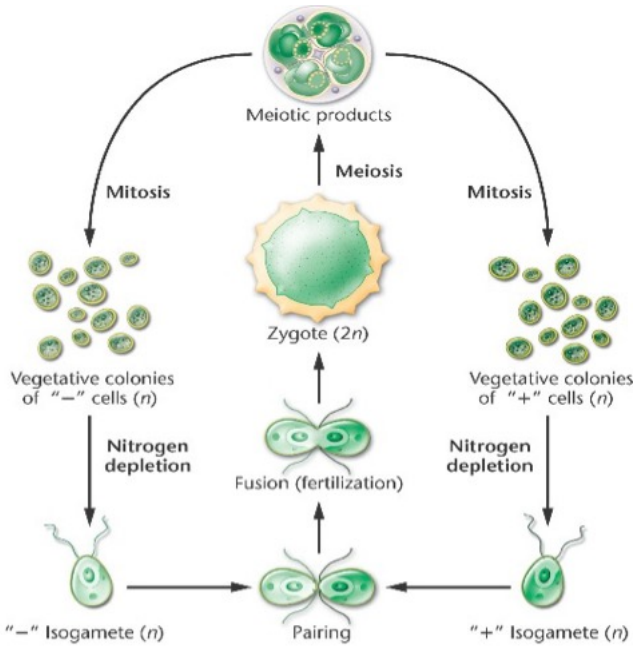

Figure 3-14 *Chlamydomonas reinhardtii sexual cycle.*

3.2.2 GAI-247 mutant generation and screen optimization

We initially performed UV mutagenesis then FACS enriched GAI-247 mutants twice for the top 5% of BODIPY505/515 cells after nitrogen starvation as described in the previous section. The ~1300 recovered clones were then screened for increased Nile Red fluorescence under nitrogen starvation conditions. We tested the top two mutants from these screens and found that the lipid improved phenotype was not apparent at flask scale experiments. The single best mutant from this initial screen GAI-247-HFLA is characterized in the large-scale grow out screen, see Section 3.2.3.

Following the long term grow-out study, we further screened for GAI-247 mutants with improved lipid production (See Figure 12 for a summary of the screening approach). In an effort to generate more diverse phenotypes we used alternative mutagenesis methods in addition to UV light. These alternative methods were Zeocin treatment (a radiomimetic agent that causes double stranded DNA breaks) with subsequent UV irradiation treatment, and colchicine treatment (causes disjunction of chromosomes during cell division and polyploidy/aneuploidy) coupled with UV-irradiation. Since there did not exist any protocols for using Zeocin or Colchicine as mutagens for green algae, we titrated each agent over several log-orders of concentrations (100 ug/mL to 0.1 ug/mL for Zeocin, and 0.5% w/vol to 0.005% w/vol for Colchicine). We evaluated the growth arrest of the culture by growing the cells in 6-well microtitre plates for 48 hours with the mutagens and measuring both the Chlorophyll fluorescence and doing cell counts. Since Zeocin damages DNA and causes cell cycle arrest until the DNA is repaired and Colchicine inhibits microtubules and the separation of chromosomes during mitosis, we sought to find a concentration of mutagen that would inhibit growth but not kill the cells. In the case of Zeocin, 10 ug/mL of Zeocin was found to be optimal. This was a remarkably high concentration, since 10 or 15 ug/mL of Zeocin is

the concentration of Zeocin we use for very strong selection of green algae carrying the Bleomycin resistance cassette when doing transgenesis. We found that 0.1% w./vol Colchicine was found optimal, which represents a range often used to mutagenize plant cells and is known to cause some mitotic/meiotic spindle formation inhibition.

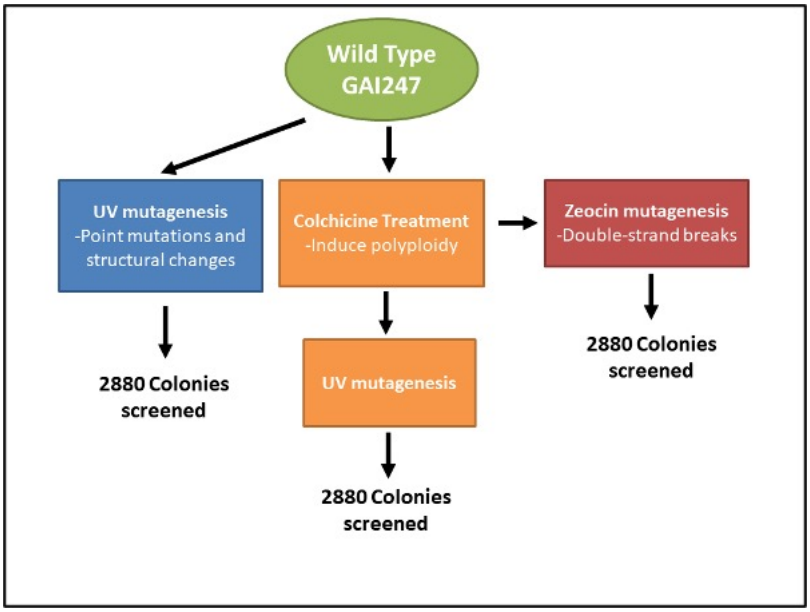

Figure 3-15 *Mutagenesis workflow used to generate GAI-247 mutants in previous quarter. The top two colonies of these multiple mutagenesis methods were used for further strain improvement.*

We screened approximately ~8000 mutants from the three different treatments in 96-well microplates. The microplates these mutants were cultivated in were rotated through different positions in our 5% CO_2 atmosphere enriched incubator in an attempt to minimize positional bias for each plate. The mutants were screened by reading Nile Red fluorescence in nitrogen replete media then switch the cells into nitrogen deplete media and reading the Nile Red fluorescence after 48 hours of nitrogen starvation. The top 10-20% of mutants were chosen as in previous screenings. We down selected to 192 "UV only" mutants, 328 "Colchicine and UV" mutants, and 274 "Zeocin and UV" mutants. Interestingly, we did not see any major differences in the variation between the populations generated by different mutagens.

These down-selected mutants were further screened in 400uL cultures in opaque deep well 96-well microtiter plates that we found to have less of a positional bias. Again, we rotated these plates through positions in our 5% CO_2 enriched grow box to minimize positional bias. We took Nile Red readings of the plates in replete media before switching the plates into nitrogen deplete media and taking readings at 48, 72, and 96 hours after the switch to deplete media. These additional measurements were done to potentially identify mutants with different lipid induction kinetics rather than simply and absolute amount of lipid after 48 hours of lipid starvation alone. Then we down-selected the top nine potential hits, seven from UV irradiation alone, and one each from the Colchicine/UV and Zeocin/UV treatments (Figure 3-16). These hits from the screen were moved into 300 mL cultures and Nile Red, OD_{750} and AFDW measurements were taken over 9 days, switching into nitrogen deplete media after 3 days. In addition, 50mL samples were taken periodically through the study for lipid extraction and analysis. Despite a highly involved effort to generate a lipid mutant of GAI-247, all the mutants had less actual lipid than the wild-type GAI-247 control (See

GAI mutants A11, B10, D9 in Table 3-2, page 28) Interestingly, when we analyzed GAI mutants A11 and B10 by flow cytometry we noticed a nearly two fold increase in BODIPY 505/515 staining compared to wild type controls (Figure 3-17).

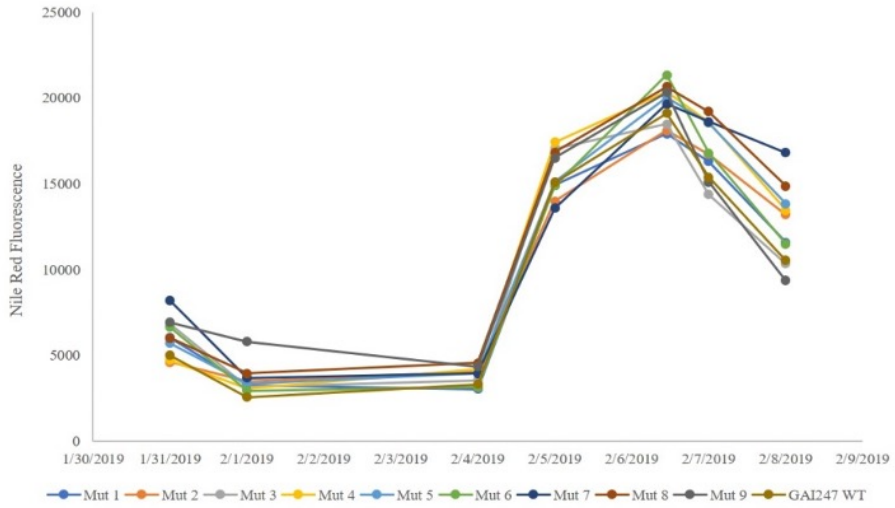

Figure 3-16 *Nile red fluorescence taken over 9 days of GAI 247 and 9 of its potential high lipid mutants. Media witch to nitrogen deplete occurred on 2/4/2019.*

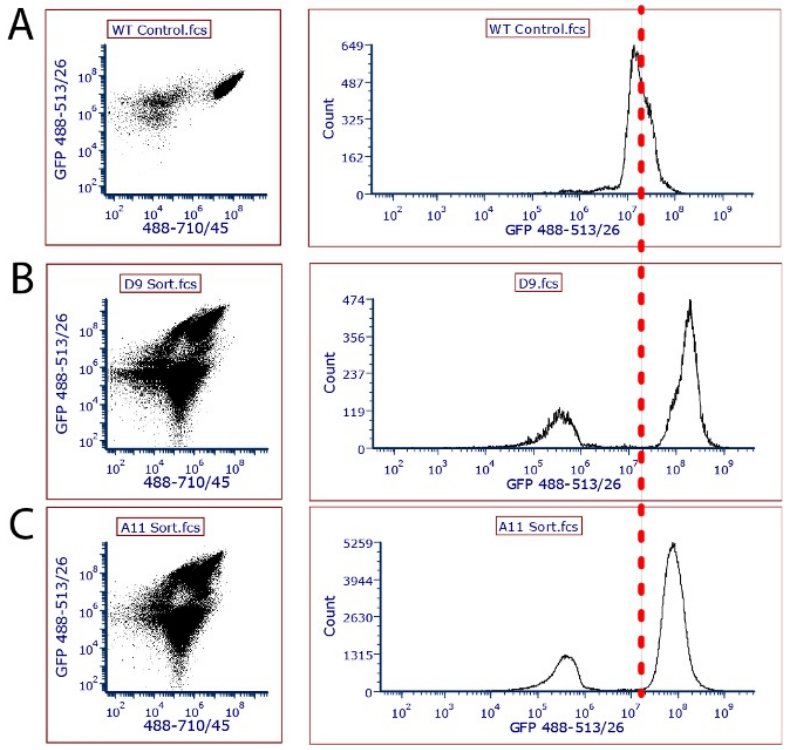

Figure 3-17 *Raw (ungated) Flow cytometry data from (A) wild type and (B&C) the two best selected GAI-247 mutants from our initial large scale mutagenesis screen. The left panels display*

BODIPY505/515 fluorescence on the y-axis and chlorophyll autofluorescence on the x-axis. The second panel displays a histogram of cell count versus BODIPY (signal).

3.2.3 Improving lipid accumulation in green algae

We originally proposed to increase lipid accumulation in *Chlamydomonas reinhardtii* and *Nannochloris sp.* (GAI-247); however, we encountered initially discouraging results in terms of low productivity in the case of bicarbonate adapted *C. reinhardtii* and very modest to essentially no recovery of obviously improved GAI-247 mutants. Therefore, we also performed forward mutagenesis and identification of improved lipid producers in other species that had been bioprospected at the UCSD field station.

This expanded lipid improvement task included these GAI bicarbonate media adapted strains from the UCSD collection: *Chlamydomonas moewusii* (CM25), *Desmodesmus armatus* (DA25), and *Chlorella vulgaris* (CV25). With these additional strains we hoped to give ourselves the best chance possible of identifying a rapidly growing strain with improved lipid production. We aimed to use random mutagenesis and high throughput screening to identify high-lipid accumulating strains.

Initially we used Ultraviolet (UV) light as a mutagenesis agent for all our strains. UV-light is a commonly used technique because it causes a range of single nucleotide and larger-scale structural DNA mutations and can be easily titrated through increasing exposure time to the light source.

Strains were subjected to UV radiation at increasing intervals and recovered in GAI bicarbonate media for 48 hours. Specifically, we used a Bio-Rad GS Gene Linker UV Chamber with an exposure intensity of 3 mW/cm2/sec. After 48 hours, the chlorophyll fluorescence and/or cell count of the cultures was measured as a proxy for living cells. As per convention of many different types of mutagenesis methods (i.e. UV, alkylating agents, gamma radiation) cultures exposed to enough UV light to cause greater than 70% death were used in the following experiments.

In the case of GAI-247, where recovering obviously improved mutants was difficult, we introduced the use a zeocin, a radio-mimetic DNA double stranded break inducing colchicine, a polyploidy inducing agent, coupled with UV- light mutagenesis, to potentially increase the magnitude and or types of mutations found in GAI-247.

UV-mutagenesis readily killed cells from each lineage with a ranging level of sensitivity. Kill-curves of *C. reinhardtii* revealed that 60s of exposure was enough to kill the virtually the entire culture (Figure 3-18Figure 3-19). For all strains besides GAI 247, we used 30s of UV exposure to generate mutants. Comparatively, GAI 247 proved to be highly susceptible to UV radiation and was only exposed to 5s of radiation (Figure 3-18Figure 3-19).

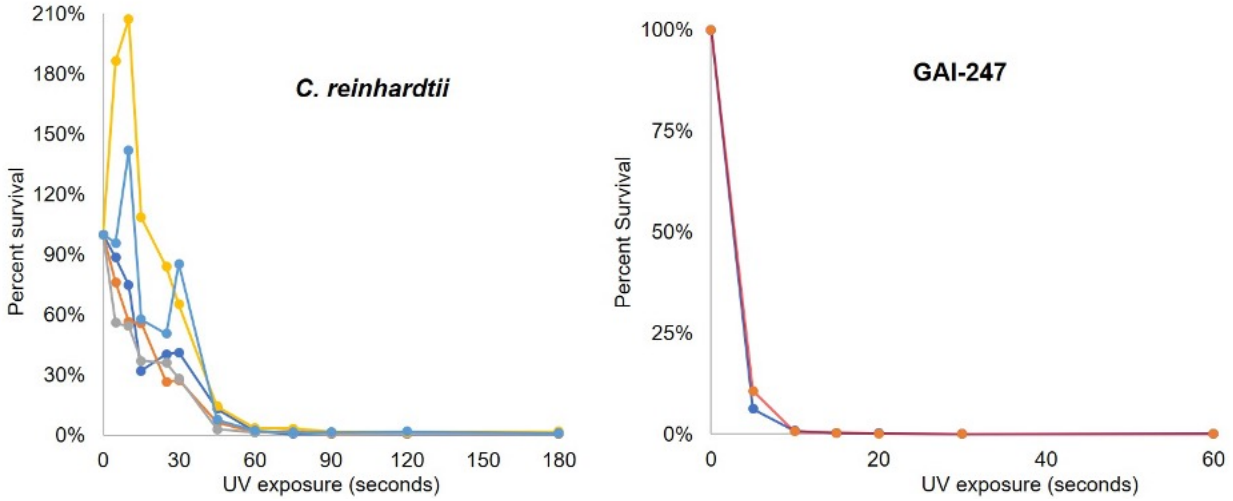

Figure 3-18 *UV kill-curves of C. reinhardtii and GAI-247*

After any of the mutagenesis methods listed above, mutagenized cultures were transferred to fresh media and grown out in a 5% CO_2 atmosphere grow box. In preparation for FACS, the cultures were diluted and split into media with and without nitrate (Figure 3-19). The nitrate-free media was used as the lipid-induction media because nitrogen-starvation is a strong inducer of lipid storage and/or carbohydrate formation in green algae. Immediately before Fluorescently Activated Cell Sorting (FACS) cultures were stained with BODIPY at a final concentration of 1 µg/mL and the top 1-10% of fluorescing cells were selected.

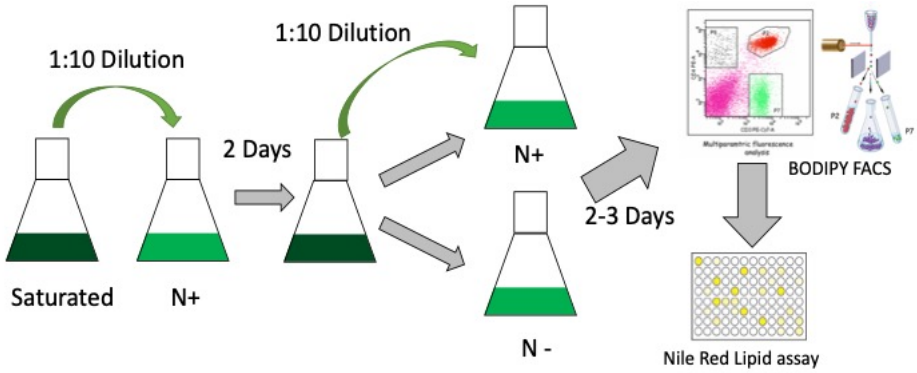

Figure 3-19 *Process overview of screening for high lipid mutants*

Strains readily recovered in fresh media, and lipids were easily detected on the flow cytometer (Figure 3-20). However, after sorting cells, only 1-5% of individuals survived and were recovered. Further, we noticed a long lag time in growth when cells were allowed to recover in either liquid media or on plates. This was a setback and often rate limiting in our strain improvement protocols. Even with this high lethality we were able to recover from FACS and screen in microplates the following number of high-lipid mutants: 192 *C. reinhardtii*, 184 *C. moewusii*, 264 *C. vulgaris*, 216

D. armatus, and an initial screen of 1,300 GAI-247 mutants using UV mutagenesis, followed by a subsequent screen of 8,640 GAI-247 generated through different methods of mutagenesis.

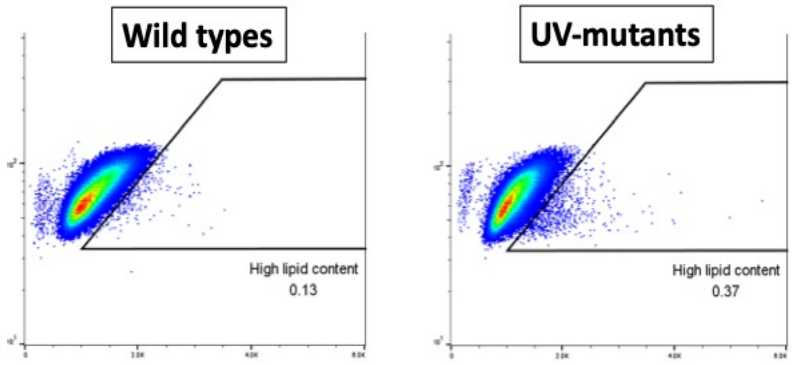

Figure 3-20 *Graphs generated from FAC sorting of wild type and UV generated mutant individuals, y-axis is BODIPY 505/515 intensity and x-axis is chlorophyll autofluorescence*

These sorted cells were plated on gellan gum media immediately after sorting to recover single colonies. Once colonies formed, they were picked into microplates and again grown in fresh media then split into replicate plates either with or without nitrogen to induce lipids. Colonies were initially picked into standard 300 ul/well volume 96-well microplates for further screening and down-selection; however, a significant edge-bias was detected, so we transitioned to opaque deep-well (600 ul/well) 96-well microplates plates to normalize light exposure and growth.

A subsample from the microplates was taken after 48 hours had passed and stained with Nile Red to compare lipid formation between mutants. The staining solution was optimized to work with GAI-247 and contained 1 µg/mL Nile Red diluted in media containing 30% DMSO as a permeabilization agent. This staining solution was added 1:1 with the cells in the microtiter plate. The plates were analyzed on a Tecan by reading nile red fluorescence at an excitation wavelength of 530 nm and emission of 575 nm, chlorophyll fluorescence at an excitation wavelength of 440 nm and emission of 680 nm, as well as optical density at 750 nm. The top 10-20% of Nile Red mutants were selected and re-examined in a second round of screening.

After several comparisons in microplates, the top mutants of each strain were identified and compared to one another in 250mL flasks in the CO_2 box, including comparisons to their wild-type parent; the suffix "-HFL" was added to the end of each final mutant to denote "High Fluorescing Lipids". High lipid mutants of *C. reinhartdtii*, *C. vulgaris*, *C. moewusii*, and *D. armatus*, were compared to their respective wild types as well as the wild type GAI-247.

These cultures were scaled to 500mL media in shake flasks and grown in a 5% CO2 box with 12:12 light:dark cycle for 10 days; nitrogen was depleted at 200 hours. Each day a sub sample was removed and the biomass/dry weight, OD750, and Nile Red fluorescence was measured (Figure ***3-21***). This study used GAI-247 as a baseline for both biomass and Nile Red fluorescence. When examining Nile Red, which estimates lipid content per cell, we found that *C. reinhardtii* and *C.*

vulgaris were higher than GAI-247. Finally, it was decided at this point that *C. reinhardtii* grew too slowly compared to the other strains and was removed from further examination and efforts were focused to improve and characterize the other strains.

After this study we rescreened the DA25 strain for a new high lipid mutant using the same protocol previously used and outlined above. We decided to further keep the DA25 strain in the study since the wild type exhibited the best growth of all cultures.

To best characterize the biomass and lipid content in the high-lipid mutants, we performed an intensive, long term, cultivation experiment. We performed head-to-head semi-continuous cultivation experiments in replicate to determine the productivity of each cell line and determine the best candidates for future work and production. In this cultivation we looked at CV25, CV25-HFL1, GAI-247 and a potential high lipid mutant (GAI-247-HFLA), as well as DA25 and a newly isolated potential high lipid mutant (DA25-MutB7). Table 3-1 provides a summary of these strains. This was carried out in 1L shaker flasks under 300uE LED lights in a CO_2 box in duplicate. We measured biomass, OD750, Nile Red, and pH daily for the duration of the experiment, see Figure 3-22. We observed the highest biomass productivity in the DA25-MutB7 strain and the highest lipid productivity by the strain, CV25-HFL1, when it was allowed to saturate naturally (versus intentionally depleting nitrogen from the media). When compared at large shaker flask scale, the GAI-247 HFL mutant did not display any obviously improved lipid accumulation under nitrogen replete or nitrogen-starvation conditions compared to the wild-type control. This is despite promising initial results from 96-well and small shake flask experiments.

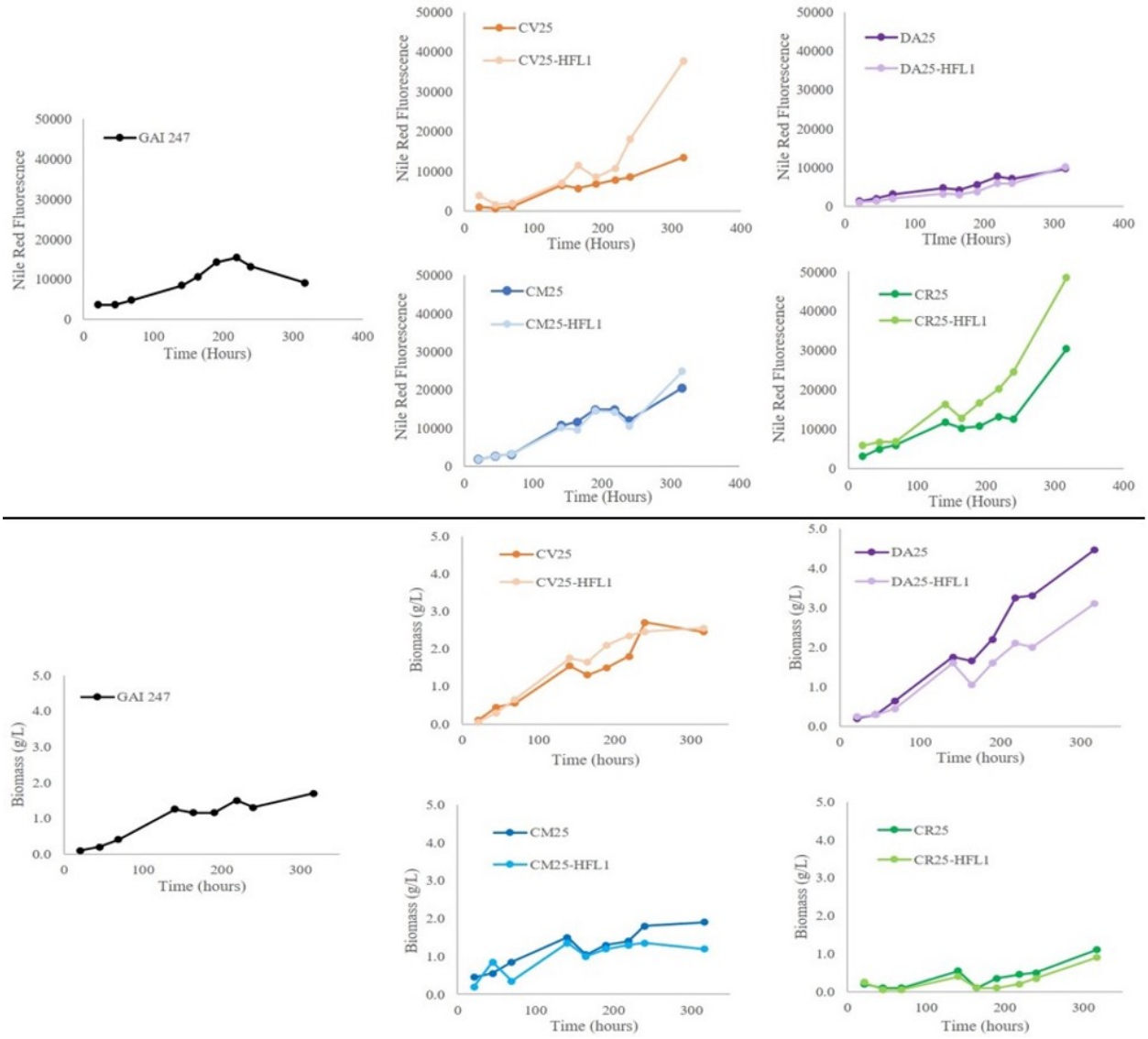

Figure 3-21 Top *- Nile red fluorescence measured over two weeks to estimate lipid content of each culture (100µL of culture). Nitrogen depletion was reached at 200 hours. CV25-HFL1 and CR25-HFL1 showed the highest levels of lipids and displayed an increase from each of their wild type counterparts.* ***Bottom*** *- Biomass measured over two weeks by taking two mL samples and filtering them to obtain the dry weight of the sample. The DA25 wild type showed the overall highest biomass accumulation, followed by DA25-HFL1, CV25, and CV25-HFL1, all of which outgrew GAI-247.*

During this long-term cultivation experiment, several samples were taken, lyophilized and sent for lipid extraction and mass spectrometry analysis. The results are summarized in (Table 2). We have included Nile Red fluorescence and actual lipid content for comparison. Notably, we found that depending on the species, Nile Red correlated differently with lipid content. For the CV25 strains, we observed more than double the Nile Red fluorescence in the CV25-HFL stain compared to the wild-type when starved of nutrients, however after quantifying lipids there was only a marginal increase of 3% more lipids in the CV25-HFL compared to its wild-type control. Conversely, in the

Table 3-1. *Reference table of species names, and the names referring to salt tolerant wild types and high lipid mutants. Generation strategy is given for each strain and their mutant.*

Species	Wild Type Name	High Lipid Mutant	Generation Strategy
Chlamydomonas reinhardtii	CR25	CR25-HFL1	UV irradiation and FACS
Chlamydomonas moewusii	CM25	CM25-HFL1	UV irradiation and FACS
Desmodesmus armatus	DA25	DA25-HFL1 (First study) DA25-MutB7 (Final mutant)	UV irradiation and FACS
Chlorella vulgaris	CV25	CV25-HFL1	UV irradiation and FACS
Nannochloris sp.	GAI-247	GAI-HFLA	UV irradiation and FACS

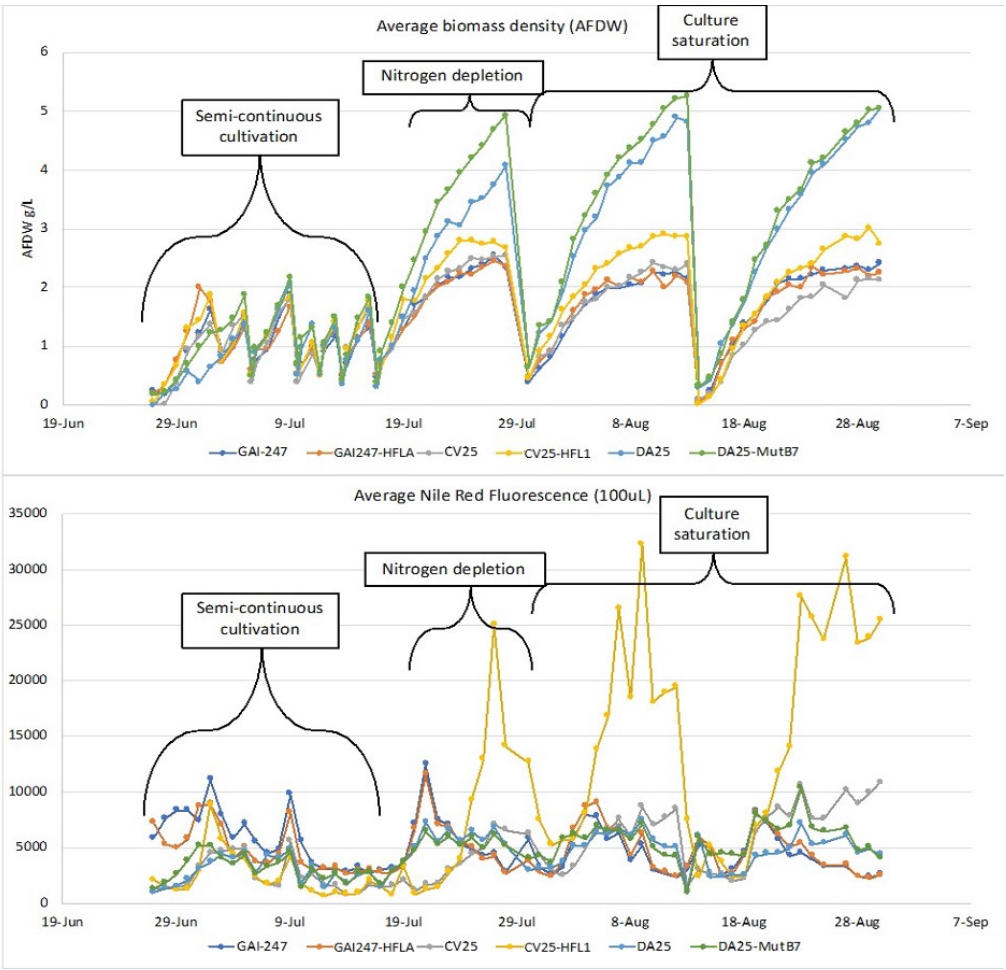

Figure 3-22 Top*—Biomass accumulation over time measured by filtering 2mL of culture and combusting the sample in a furnace (AFDW/ g/L). In the first phase, we operated in a semi-continuous cultivation condition to maximize growth rate. After this was established, we began to experiment with lipid induction by depleting the media of nitrogen (nitrogen depletion bracket) and also allowing the culture to naturally saturate and deplete itself of multiple nutrients (culture saturation bracket).*

***Bottom**—Nile red fluorescence was measured every day to estimate lipid content (100µL of culture). Both the wild type and mutant of GAI-247 produced lipids the fastest following nitrogen depletion, but the CV25-HFL1 strain ultimately had the highest lipid accumulation in any condition.*

***Table 3-2.** Nile Red fluorescence, OD750, AFDW, and lipid content values for replicate flasks of CV25, CV25-HFL1, DA25, DA25-HFL1(MutB7), GAI-247 and top three GAI-HFL mutants.*

Sample	NileRed (100uL)	OD750	%FAME	Density AFDW (g/L)	Total FAME/L
CV25-A.12.14	1633.00	1.01	11.6%	0.75	87.26
CV25-A.12.18	8257.33	1.97	22.1%	1.7	376.18
CV25-A.12.21	16025.00	1.94	31.9%	1.8	559.08
CV25-B.12.14	2014.67	1.09	10.7%	0.7	74.68
CV25-B.12.18	8136.33	2.03	21.1%	1.9	401.55
CV25-B.12.21	16074.33	2.03	32.5%	2.1	666.53
CV25HFL-A.12.14	2568.00	1.20	7.9%	1.2	90.66
CV25HFL-A.12.18	18108.00	2.20	25.3%	2.1	531.14
CV25HFL-A.12.21	43196.33	2.24	31.1%	2.5	777.93
CV25HFL-B.12.14	2563.67	1.22	8.3%	1.4	111.52
CV25HFL-B.12.18	19406.00	2.16	25.6%	2.4	601.50
CV25HFL-B.12.21	45219.00	2.25	35.3%	2.4	846.50
DA25-A.12.14	2872.33	0.51	10.3%	0.7	72.31
DA25-A.12.18	5484.67	1.14	11.2%	1.5	167.32
DA25-A.12.21	10718.00	1.85	16.0%	2.4	384.19
DA25-B.12.14	3148.33	0.69	11.1%	0.9	99.77
DA25-B.12.18	4937.00	1.44	9.0%	1.9	171.92
DA25-B.12.21	9607.33	2.08	20.1%	2.8	563.58
DA25HFL-A.12.14	3645.67	0.93	10.5%	1.0	99.75
DA25HFL-A.12.18	5957.33	2.03	20.1%	3.2	632.05
DA25HFL-A.12.21	8265.67	2.35	25.5%	3.7	930.47
DA25HFL-B.12.14	3492.00	0.90	10.0%	1.1	105.01
DA25HFL-B.12.18	5863.00	2.00	18.8%	3.2	600.48
DA25HFL-B.12.21	7658.00	2.35	24.4%	3.8	926.85
247WT-A.2.1	2570.00	1.26	10.0%	0.7	69.94
247WT-A.2.6	24560.33	1.09	22.6%	0.8	180.48
247A11-A.2.1	3041.67	0.90	11.2%	0.3	28.04
247A11-A.2.6	25061.00	1.06	22.4%	0.7	157.02
247B10-A.2.1	2935.33	1.08	10.1%	0.5	45.40
247B10-A.2.6	26349.00	1.06	20.9%	0.7	135.64
247D9-A.2.1	3941.00	0.88	10.6%	0.6	63.31
247D9-A.2.6	25670.67	0.98	22.0%	0.7	142.78

DA25 strains, we frequently observed relatively little change in Nile Red fluorescence, however after quantifying lipids we found that lipid content was nearing 25% in the DA25-HFL mutant under nitrogen depletion compared to the wild type. Thus, in some cases, sorting based on fluorescence selects strains that more readily absorb the dye rather than higher lipid content; and, in other cases, fluorescence selection misses a large improvement in lipid content.

3.2.4 Improving lipid accumulation in diatom algae

The focus of our experimentation was to improve lipid (TAG) accumulation in both Nitzschia sp. GAI-229 and *Cyclotella cryptica* by screening and characterization of mutants exhibiting higher TAG content. An iterative process to develop new strains via random mutagenesis and selection was proposed, with a major focus on isolating mutants with stable phenotypes. In addition, we aimed to critically evaluate the ability of GAI-229 to accumulate TAG during exponential growth compared to silicon starvation. Lipid content and growth rate during short-term (e.g. 24 hr) nutrient limitation would be measured in the resulting highly productive and high lipid strains.

We performed mutagenesis followed by a flow cytometric screening approach to isolate mutants with increased TAG accumulation characteristics, without negative effects on growth rate. We attempted to isolate mutants based on increased TAG content following silicon starvation and also strains that accumulate TAG during normal growth without nutrient limitation. After mutagenesis, selection for the highest TAG accumulating lines, determined by BODIPY fluorescent staining (a lipophilic fluorescent dye), was completed on regular intervals to maintain high selective pressure.

3.2.4.1 UV Mutagenesis

Nitzschia sp. GAI-229 was subjected to UV mutagenesis and selection in an attempt to obtain mutants with higher TAG accumulation ability. Cells were exposed to 0, 5, 20, 30, and 60 sec UV irradiation, allowed to recover for 24 hr, and then propagated in GAI D1 growth medium. Cultures were grown to exponential phase and then silicon starved by washing and transferring to D1 medium lacking Si. After 24 hr, cells were stained with BODIPY, and 200,000 cells were selected from the top 10% brightest fluorescence population (RFU) using a Becton Dickenson Influx sorting flow cytometer. After sorting, cells were inoculated into D1 medium and allowed to grow to mid-exponential phase. Cells were re-sorted as above, and the process was repeated (3 times total). After the final round of sorting, cells were plated on D1 agar medium to isolate single clones. We also attempted selection on non-silicon-starved (i.e. exponentially growing) cells, but never obtained a consistent population.

Twenty-four clones were isolated from each UV treatment. Six randomly selected clones were screened along with two wild-type cultures by growth to exponential phase, placement into D1 medium lacking silicon, and removing aliquots every 24 hr for 3 days. Samples were used to monitor TAG content by BODIPY staining in conjunction with imaging flow cytometric analysis.

The best performing UV mutants exhibited 4-5 fold higher TAG than wild-type GAI-229. Two other features were noted, 1) one mutant (30-B4) exhibited higher TAG during growth (0 hr) than during Si starvation, and 2) some mutants did not display a consistent increase in TAG during the

starvation experiment, with a reduction in TAG occurring during some later time points. Several strains, including wild-type, were also observed to accumulate TAG during growth. This data indirectly suggests that GAI-229 may use TAG as a carbon source in a more dynamic sense than other diatom species previously studied. Nine mutant lines (Table 3-3) were sent to Hawaii for evaluation and outdoor testing. None of these mutant lines showed improvement over the wild-type during the evaluation and outdoor testing.

Table 3-3 *Mutant strains of Nitzschia sp. selected for evaluation.*

GAI name	Isolate
GAI 272	20-A2
GAI 273	20-A4
GAI 274	30-A4
GAI 275	60-A4
GAI 276	60-A6
GAI 277	60-B1
GAI 278	30-B2
GAI 279	30-B4
GAI 280	30-C4

The stability of the mutant lines was reviewed by checking the mutants after storage and propagation in 24 well plates for 2 months. None of the UV mutant clones performed well in replicate experiments, apparently having lost their high lipid phenotype as a result of infrequent propagation. We followed up by comparing the performance of 4 clones that had been propagated in liquid in 24 well plates with the same set of clones stored on agar plates, with the idea that agar storage would reduce growth and negative selection toward loss of the high lipid phenotype. The agar-stored mutants accumulated 2-fold higher TAG on average than their 24 well counterparts, however, the agar-stored lines still did not perform as well as the original mutant isolates.

This data suggests either that mutant stability is limited or that variation in culture conditions could account for variability in response. Both factors could have important implications on yield during outdoor growth. Having stable mutants is critical, and loss of the mutant phenotype over time is be highly detrimental, considering that 2 months of cultivation is often required to generate enough biomass for outdoor pond inoculation.

3.2.4.2 Chemical Mutagenesis

Based on the apparent initial efficacy of UV mutagenesis in producing high-lipid accumulating mutants of GAI-229, we have demonstrated the strain to be capable of accumulating TAG in increased quantities; however, an alternative approach that would potentially result in a more stable phenotype was needed. Chemical mutagenesis has been previously documented for the possibility of achieving higher mutation rates with minimal effects on survival and fertility and was therefore chosen as a viable alternative to UV mutagenesis for developing lipid mutants. After exposure to the chemical mutagen ethyl methane sulfonate (EMS), we found that cell survival gradually decreased with increasing EMS concentration (Table 3-4). In addition, we observed that GAI-229 culture treated with 0.2 M EMS exhibited similar cell size and growth rate compared to the control, whereas cell size and growth rate decreased in the culture treated with 0.4 M EMS. We selected the 0.2 and 0.4 M EMS treatment cultures for further characterization of cellular lipid accumulation under silicon limitation conditions.

Following mutagenesis with N-ethyl-N-nitrosourea (ENU), we found that cell survival was not affected by ENU concentration (0.2 – 1 mM ENU). This may be attributed to the concentration of bicarbonate present in the growth medium and the resulting high pH. ENU is easily denatured in

an alkaline environment and therefore may not be functional in standard D1 liquid growth medium. In response, ENU exposure was re-attempted in D1 growth medium lacking bicarbonate, but we again observed that cell survival was not affected by ENU concentration even with a 10-fold increase in concentration range from the previous trial (Table 3-5). Due to the inability to produce mutants by ENU mutagenesis, further sorting and analysis for high lipid accumulating mutants was performed with mutants produced via EMS exposure.

Table 3-4 Cell survival of *Nitzschia* sp. GAI-229 following chemical mutagenesis using varying concentrations of ethyl methane sulfonate (EMS).

EMS conc. (M)	Cell Survival
0	++++
0.2	+++
0.4	++
0.6	-
0.8	-
1	-

Table 3-5 Cell survival of *Nitzschia* sp. GAI-229 following chemical mutagenesis using varying concentrations of N-ethyl-N-nitrosourea (ENU).

ENU conc. (M)	Cell Survival
0	++++
2	++++
4	++++
6	++++
8	++++
10	++++

Preliminary analysis after the first round of sorting of EMS exposed mutants did not yet show a significant increase in lipid level (Figure 3-23), though mutants generated with 0.4 M EMS showed a slight increase in lipid after 48 hr of Si starvation. Both mutant lines (0.2M and 0.4M EMS) were re-sorted (4 times total), and lipid content was measured by BODIPY fluorescence (RFU). According to imaging flow cytometric analysis, lipid accumulation was induced to a higher extent in the 0.4 M EMS exposed mutant than in the 0.2M EMS exposed mutant culture after 24 hr of Si starvation (Figure 3-24).

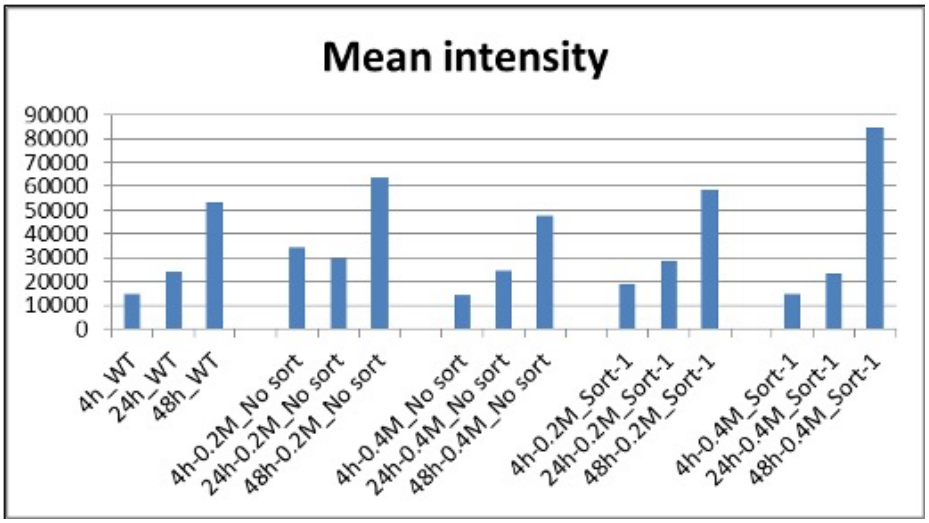

Figure 3-23 *ImageStream analysis of neutral lipid accumulation, determined by BODIPY fluorescence (RFU), in EMS exposed GAI-229 mutants after silica starvation.*

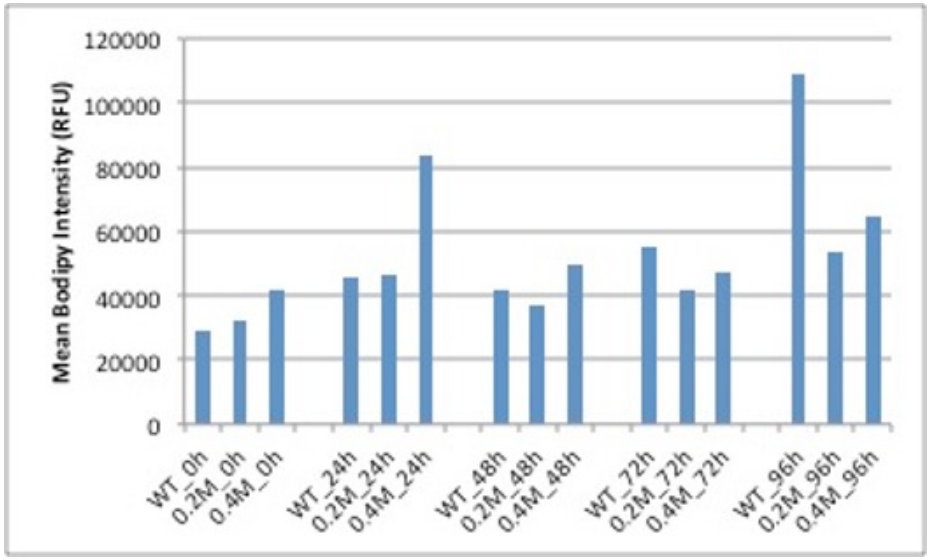

Figure 3-24 *ImageStream analysis of neutral lipid accumulation, determined by BODIPY fluorescence (RFU), in EMS exposed GAI-229 mutants after silica starvation (fourth round of sorting).*

Based on the AFDW (g/L) of the cultures calculated from the first set of processed glass-fiber filters (Table 4), the filters for lipid analysis each contained at least 20 mg AFDW, providing adequate biomass for quantification of total lipid content. GC analysis determined total lipid content (FAME mg/g AFDW) to be higher in both EMS exposed mutants than wild-type GAI-229 after 96 hr of Si starvation, with lipid comprising up to 33% of total biomass for the GAI-229 mutants. This differed from BODIPY fluorescent analysis (ImageStream) from the same experiment, which indicated neutral lipid content was higher in wild-type GAI-229 than in either mutant strain at 96 hr (Fig. 2). The lower BODIPY fluorescence (RFU) exhibited by the 96 hr Si

starvation mutants could be due to less healthy mutant cultures compared to wild-type resulting in a reduction in neutral lipid content.

Table 3-6 *Nitzschia sp. GAI-229 strain determination of ash-free dry weight (AFDW), filtered cell biomass for GC lipid analysis, and total fatty acid methyl ester (FAME) content per AFDW.*

Strain	AFDW (g/L)	Filtered Biomass (mg)	FAME (mg/g)
wild-type	0.069	24.15	274.5
0.2 M EMS	0.068	23.80	329.3
0.4 M EMS	0.086	30.10	295.4

3.2.5 Improving growth rate in green algae

Increasing growth is a complex challenge and there are few examples of repeatedly generating high-growth mutants in existing literature. However, there are examples of bacterial and fungal species that, when growth inhibited by sub-lethal concentrations of antibiotics, can be evolved to grow more quickly under antibiotic selection (develop resistance) in continuous chemostats and turbidostat culture conditions.

We proposed to increase growth of strains by mutagenizing cultures and then screening for increased growth through the use of a turbidostat and a microfluidics chip. A turbidostat is a commonly used tool for selecting for higher growth in a mixed culture by constantly adding media to the system and simultaneously removing cells through what effectively is “spill over” from the culture vessel. Over time, the slower growing cells are washed out of the system and the faster dividing cells become a dominant population in the system. A microfluidics chip works in a similar fashion, except the cells are not grown together in a large batch culture, but rather sectioned into hundreds of micro-wells that are arrayed in a grid fashion. In effect, each well works as a tiny (<1mm) turbidostat. As the faster growing cells grow in their respective wells, they displace one another and are captured in the effluent flowing over the whole chip.

A turbidostat was designed and built at UCSD specifically for this project. The system was gravity-fed and used light sensors linked to a digital microcontroller that triggered pinch valves in the media feed lines to control the flow of media into ten different sterile culture tubes (Figure 3-25). The microcontroller was configured such that as the cultures in the turbidostat vessel grew denser and the light transmitted through the culture decreased and the signal strength from the light sensor decreased, a solenoid controlled pinch valve on the media feed would open once the light intensity dropped below a certain threshold. Media would then drip in to the bioreactor vessel, diluting out the cells as the excess media flowed out of the exhaust which would increase the signal strength from the light sensor back to the set point; thus, triggering a negative feedback loop causing the microcontroller to close the pinch valve. During turbidostat operation, constant air was bubbled into the turbidostat to mix cells and provide atmospheric CO_2.

A single-layer microfluidics chip was cast based on a custom design intended to grow algae up to 10um in diameter. The chip had 1,000 wells in the array with single input and output ports. The

wells were “U” shaped, with the opening facing upstream (Figure 3-26). As culture flooded the chip, cells would settle into the wells and fresh media would constantly flow through the chip. Cells that grew the most rapidly would fill the well and spill into the flowing media and out into a collection tube. Strains were mutagenized as described above and the mixed mutant cultures were used as inoculum.

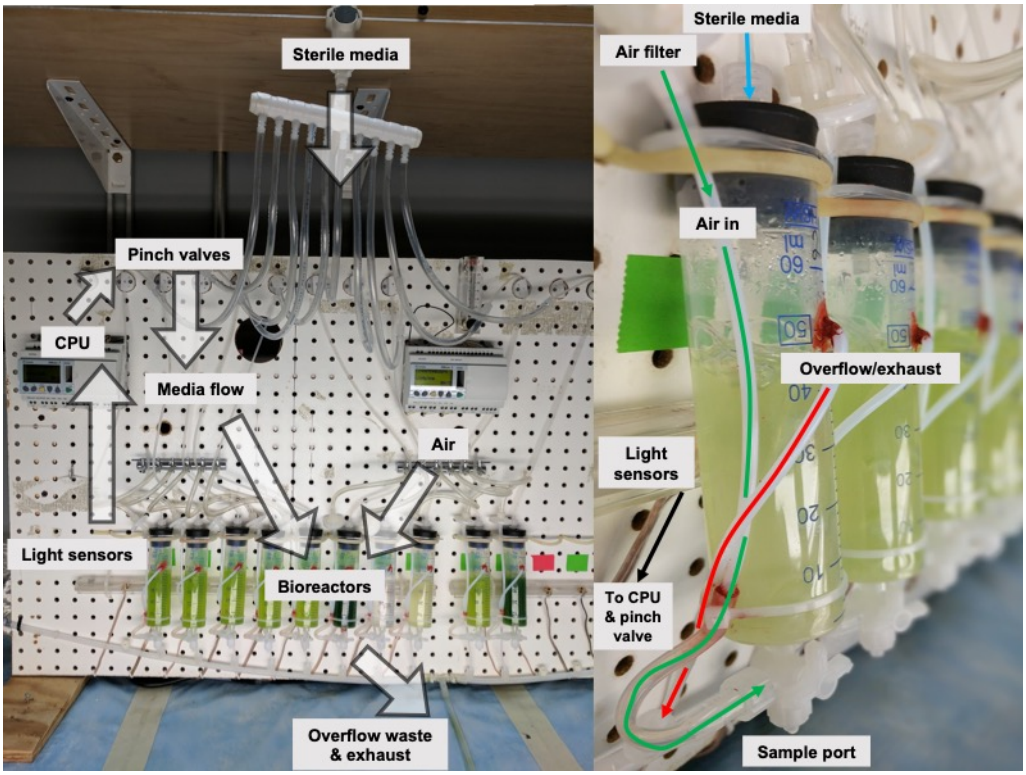

Figure 3-25 *Photograph of Turbidostat array used in this study*

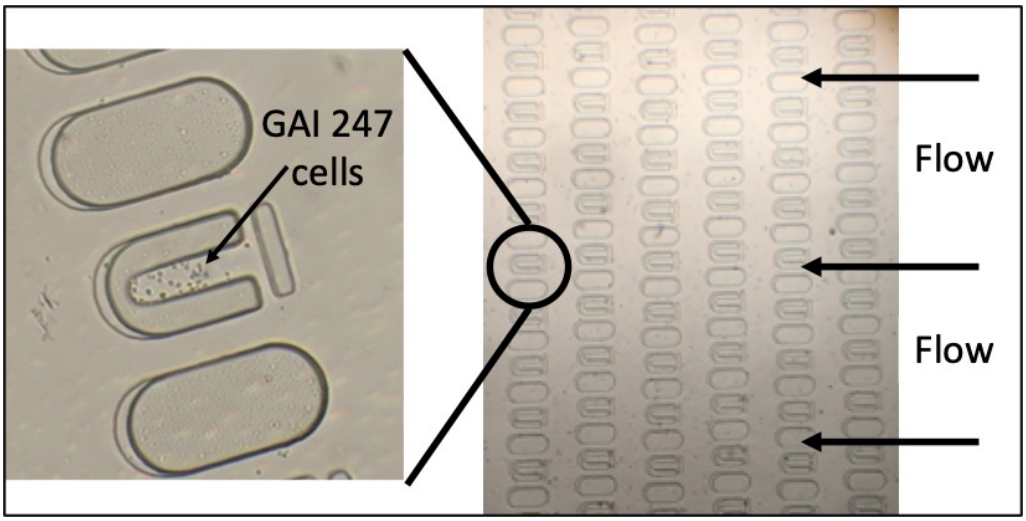

Figure 3-26 *Micrographs of microfluidics chip*

The strains used in this project responded quite differently to the environment of the turbidostat. Only the GAI bicarbonate adapted *C. vulgaris,* and *D. armatus* cell lines (CV25 and DA25, respectively) as well as GAI-247 grew successfully in the turbidostat. We were able to run the turbidostat with these strains successfully for several months and the resulting strains were re-isolated and compared to wild type cultures. Mid-log phase starter cultures were diluted 1/50 into fresh media in shaker flasks then grown at room temperature under a full spectrum light at 350-400 uE/sec/m^2 light intensity on a 12 hour On-12 hour Off light cycle. OD_{750} readings were taken with a spectrophotometer two or three times a day. The maximum specific growth rate was determined for each culture. We found that the specific growth rate was very similar between the wild-type control and all turbidostat cultures.

The microfluidics chip was successfully cast, and media flowed readily though it during initial testing, however we quickly encountered issues when working with the GAI bicarbonate media and inoculum. Salt crystals formed which clogged the narrow tubing in the chip and resulted in the total evaporation of media from the chip. Lowering the sodium bicarbonate concentration resolved this issue. However, the larger issue is that when we inoculated the chip, it was nearly impossible to get the GAI-247 cells to land into a well. While we were able to successfully demonstrate growth in some wells, it was extremely difficult to land a single cell into each well. We tried multiple different cell densities and flow rates in order to trouble-shoot the issue, but never achieved the desired result. When the cell densities were low, the vast majority of the chip's wells remained empty as the cells would flow around the wells and not into the wells. When cell densities were high, numerous cells filled the front wells closest to the inlet while the rest remained empty. In the end, we were unable to successfully implement this strategy to isolate any novel strains.

While these approaches were not successful in achieving a higher productivity strain, we screened the high lipid mutants to see if any also had higher productivity. In a side-by-side comparison, we found that the *Desmodesmus armatus* strain DA25-HFL (Mut B7) yielded higher lipid content and higher growth rates. In DA25-HFL, total lipid content as a fraction of ash free dry weight increased on average to 25% under nitrogen limitation, compared to the DA25 wild-type which yielded 18%. In the same experiment, the mutant strain DA25-HFL simultaneously produced more than 20% more biomass (Figure 3-27). So, not only does the DA25-HFL mutant contain more lipids when nitrogen starved but the overall productivity of DA25-HFL is higher than its wild-type counterpart. These phenotypes were consistently observed in other experiments.

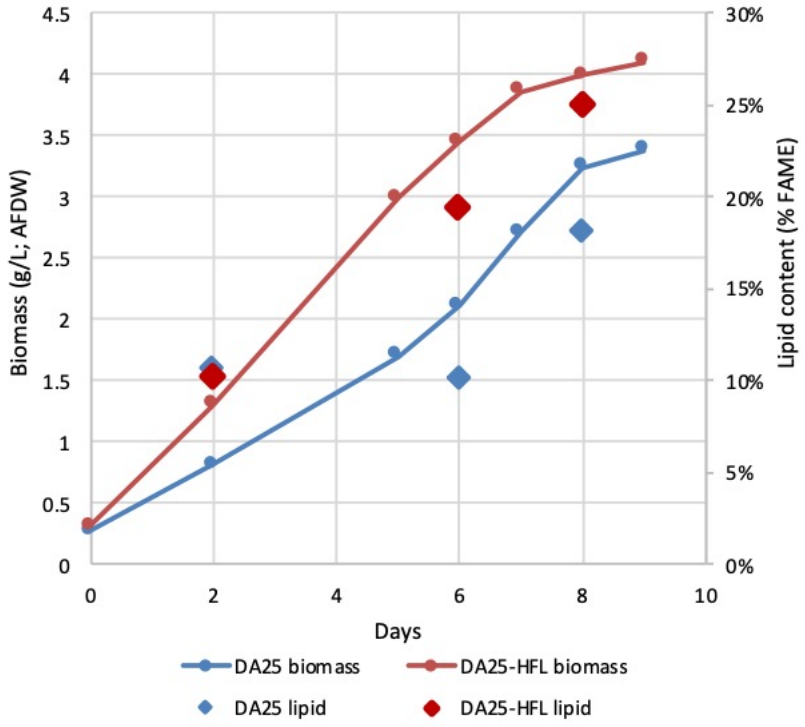

Figure 3-27 *Comparison of biomass and lipid content of Desmodesmus armatus high lipid strain (DA24-HFL) to wild type control over time under nitrogen limitation conditions (starting day 0) after initial out growth in nitrogen replete media.*

3.2.6 Increasing growth rate in diatom algae

During phase I, it became apparent that different approaches could be applied to yield high triacylglycerol (TAG) in GAI-229. The current production approach relies on increasing biomass through growth phase in nutrient replete media and switching to nutrient deplete media to induce a lipid phase. From our laboratory work, we have determined GAI-229 also has the ability to simultaneously grow and accumulate TAG. This may be partly due to a slower growth rate during these cultivation conditions.

Previous work on centric diatom species in the Hildebrand lab indicated a high sensitivity for the ability to accumulate TAG related to culture stage, with better ability as the culture entered stationary phase. A notable observation from the experiment was that the morphology of the lines, and subsequently their size, varied; the 30-A4 UV mutant had a visible area (determined by imaging flow cytometry) of 145 um2, whereas the 30-B2 UV mutant was 68 um2 and wild-type

65 um2 (46% of the size of 30-A4). 30-A4 had a typical elongated shape typical of *Nitzschia* species, but 30-B2 and wild-type had become compacted (Fig. 3).

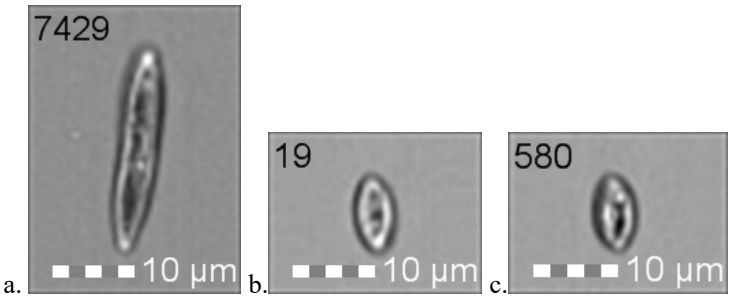

Figure 3-28 Morphologies of (a) 30-A4, (b) 30-B2, and (c) wild-type strains of GAI 229.

We initially hypothesized that the aberrant morphology came from selecting clones that formed tight colonies on plates as elongated cells are typically motile and form diffuse colonies. Comparison of BODIPY intensity vs. cell area in the three lines (Table 3-7) showed that even though more BODIPY stained lipid was accommodated in the larger cells (30-A4), the amount of BODIPY stained lipid per cell area was less than with the other two lines, indicating that on a per biomass basis, the smaller cells could be more productive. None of the three strains showed a consistent trend in TAG accumulation related to culture density, however, we discovered that none of the cultures reached stationary growth phase. Neither of the UV exposed mutant strains performed substantially better than wild type, confirming concerns about either culture density effects or mutant stability. A follow up experiment was performed to further evaluate this by ensuring that cultures entered stationary phase. We observed a substantial amount of TAG accumulation during growth in the wild-type culture (Figure 3-29), which had been growing more slowly than the two mutant lines, however, even the mutant lines show visible lipid droplets during exponential growth, which has not commonly been seen in other diatoms previously studied. The data suggests that GAI-229 may use TAG as a carbon storage form during growth, similar to what is seen in Nannochloropsis, which also stores little carbohydrate. TAG content was monitored over a 72 hr starvation period in medium lacking silicon, and TAG accumulation for GAI-229

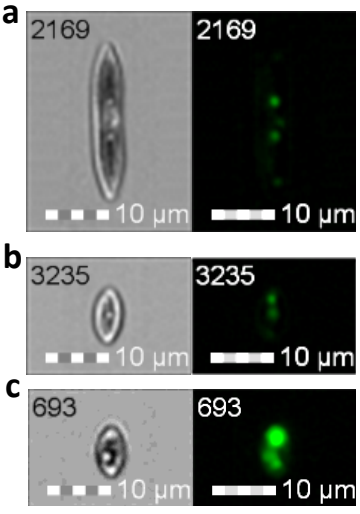

Figure 3-29 *Mean lipid accumulation during growth of (a) 30-A4, (b) 30-B2, and (c) wild-type strains of GAI-229, indicated by BODIPY staining of TAG.*

Table 3-7 *Cell area and BODIPY fluorescence (RFU) in GAI-229 strains*

Strain	Cell Area	BODIPY RFU	BODIPY /Cell area
30-A4	144.9	7423.7	51.2
30-B2	68.2	4646.1	68.1
Wt	65.2	6950.1	106.6

under light:dark conditions was comparable to levels observed in wild-type controls during our previous experiments under continuous light (Fig. 5).

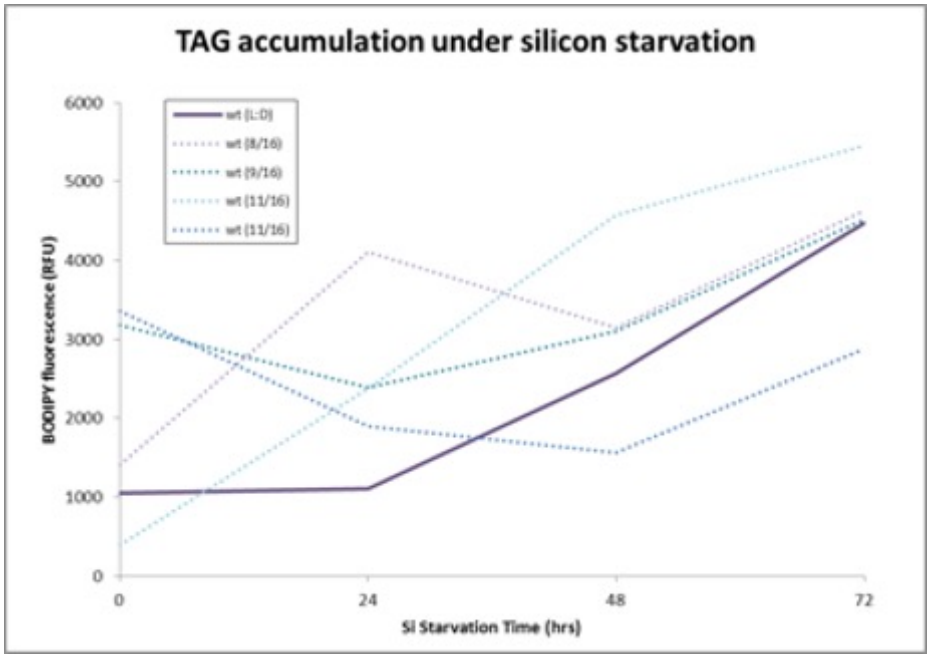

Figure 3-30 *BODIPY staining of TAG in wild type GAI-229 under light:dark and continuous light.*

3.2.7 Reducing antenna size in diatom algae

Microalgae in suspended cultures capture more light than they can process, reducing overall productivity. Calculations indicate that reducing photosystem antenna size could substantially improve light utilization and productivity. Diatoms may be particularly amenable to photosystem antenna size reduction, because their photoprotective and light harvesting pigments are distinct. We have analyzed the carotenoid biosynthetic pathway, which is responsible for both photoprotective pigments (diatoxanthin and diadinoxanthin) and the main accessory pigment (fucoxanthin) synthesis, extensively in T. pseudonana to identify particular gene targets for manipulation. We attempted to generate reduced photosystem antenna size strains of the diatom Thalassiosira pseudonana as a model for evaluating the viability of the approach. Information gained from experimentation with this species could then be applied to another diatom species, Cyclotella cryptica, which is a potential production strain.

We developed cultivation regimes that allowed for isolation of diatom pigment mutants, where cells were grown for 1 week under high light (300 uE) (to speed biomass accumulation), then transferred to low light (30 uE) to enable distinction between lines with different pigment contents. We generated 8 constructs to manipulate different steps in the carotenoid biosynthesis pathway and two transgenic lines of T. pseudonana in an attempt to reduce photosystem antenna size for improved performance in suspended cultures. However, due to cloning issues resulting from production of lethal phenotypes, efforts were focused on generating transgenic lines for two of the constructs. One line was a simultaneous knockdown of both copies of LTL (lutein deficient-like)

genes (hypothesized to convert β-carotene into zeaxanthin) in an attempt to reduce overall pigment content. The other line overexpressed a violaxanthin deepoxidase-like (VDL) gene in an attempt to alter the ratio of photoprotective to light harvesting pigments.

The VDL overexpression lines accumulated up to ~2x as much protein and ~3.4x as much TAG as wild type during exponential growth. This correlated with reduced light energy dissipation through NPQ, which in turn correlated with reduced diadino/diatoxanthin photoprotective pigment abundance. There was no significant difference in carbohydrate content based on eETR, or Fv/Fm. The overexpression lines grew up to 7% slower than wild-type *T. pseudonana,* especially as cultures reached densities at which light became limiting; however, harvesting could be timed so as to still achieve substantially higher yields of target compounds.

The LTL knockdown lines, in which total photosynthetic pigment content was reduced, resulted in cellular stress without substantial improvement in productivity. The knockdown lines appeared stressed and were 5-10% smaller than wild-type; nevertheless, there was no significant difference in overall growth rate and cell density compared to wild-type, but up to 40% more TAG, with some reduction in protein content. There were no significant changes in carbohydrate content or assessed photosynthetic parameters.

3.2.8 Thermal Adaptation of GAI-247

During high summer temperatures algal growth is lower than predicted from high solar radiation. This is likely due to low growth at high temperatures. To remedy this problem, we adapted GAI-247 in laboratory conditions to high temperature. The adaptation was conducted in the incubator in a 250mL flasks maintained on a heating pad with a thermocouple attached to the surface of the flask or inserted in the flask (Figure 3-31). GAI-247 has a temperature limit of about xx. The strain was evolved by gradually increasing the temperature over a period of 4 months to 39°C during the day and 25°C at night. After the 4 months strain was maintained at the final achieved temperatures. Attempts to further increase temperature all have caused the strain to die, so 39°C during the day and 25°C at night is the final achieved temperature. The newly established strain was designated as GAI-285 and maintained at high temperature.

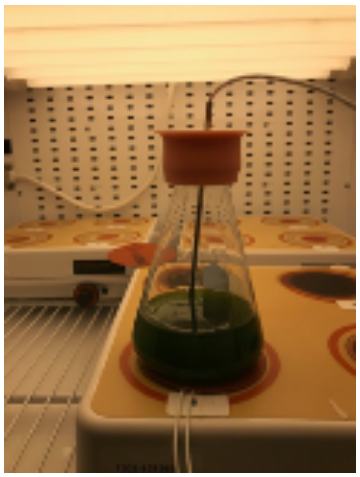

Figure 3-31 *Adaptation to high temperature with a thermocouple inserted in the flask on a heating pad in the incubator.*

3.2.9 Adapting *Cyclotella cryptica* to growth in GAI bicarbonate medium

An initial experiment was performed on wild-type *C. cryptica* to adapt for growth in GAI bicarbonate medium. It was determined that even small amounts of bicarbonate were not compatible with the artificial seawater (ASW) medium used to grow this species. We attempted to cultivate *C. cryptica* directly in GAI standard growth medium, but the cells immediately aggregated and died.

Precipitation occurred when $NaHCO_3$ was added directly to ASW, which also resulted in extensive cell clumping. We determined that the precipitate formed due to the concentration of calcium ($CaCl_2$) present in the growth medium. Reducing the calcium concentration by 50% (0.55 g/L $CaCl_2$ compared to 1.1 g/L in standard ASW) allowed incorporation $NaHCO_3$ into the medium without precipitation. We grew *C. cryptica* for several generations in ASW with reduced calcium, containing no added bicarbonate, without a decrease in growth rate compared to standard ASW. We then attempted to adapt to media containing bicarbonate.

Based on literature pertaining to bicarbonate tolerance in diatoms, bicarbonate ($NaHCO_3$) was initially added at a concentration of 0.024M (~2 g/L). C. cryptica grew at this concentration with little to no detrimental effect. Following transfer to 0.036M (~3 g/L), cultures exhibited an increased rate of cell clumping, so a median concentration of 0.03M $NaHCO_3$ (~2.5 g/L) was executed to test if *C. cryptica* would respond positively to a more gradual acclimation approach. After several dilutions, however, this approach was not successful in acclimating *C. cryptica* beyond the initial 0.024M (~2 g/L) bicarbonate-containing medium.

3.3 Preprocessing

3.3.1 Algae Harvest Technology

Zobi Harvester® systems were tested extensively at KAF and at the Qualitas commercial facility. In every case, the Zobi Harvester® system succeeded in completely separating algae from the

media,

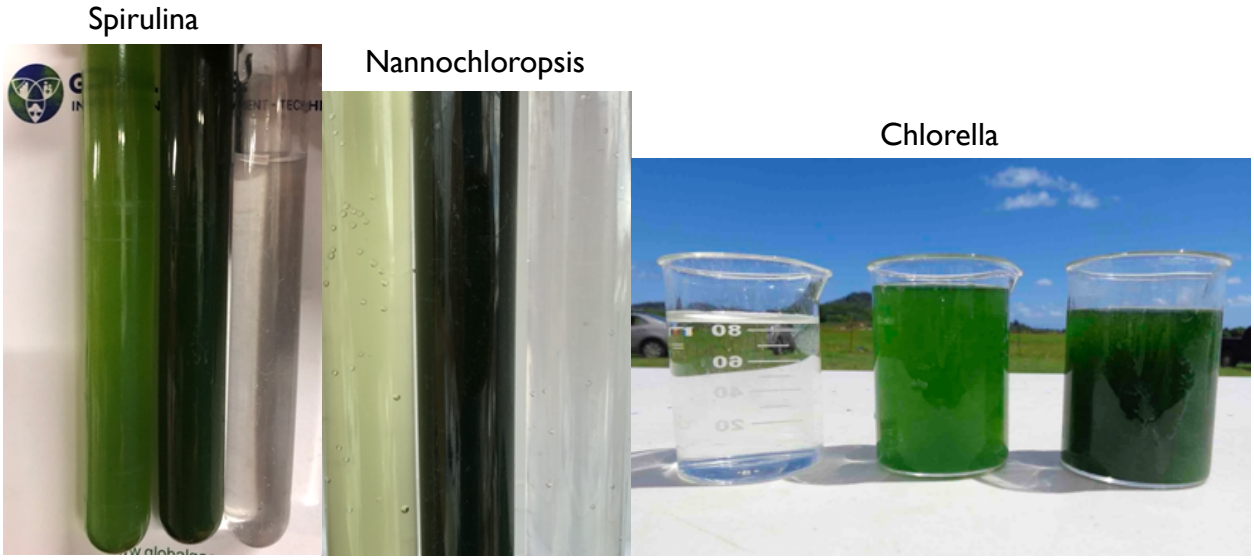

Figure 3-32, and achieved the energy use targets for the technology, Figure 3-33. The Zobi® input is algae slurry at the cultivation concentration, and the output concentration is 15-21% solids, Figure 3-34. Through the testing, modifications were made to improve the efficiency, reliability, and controls for the system resulting new patent applications and in a robust technology that is now being sold commercially.

Small scale research tools were developed to study the impact of different species, microbiota, and growth conditions on membrane flux. First, a simple blender method was used to generate a "foaming index" of a harvest batch. Attempts to correlate this with anti-foam requirements or membrane permeability were not successful. A multi-purpose Single Strand Test System was designed and fabricated to allow on-site permeability measurements of fresh algae suspensions. A number of baseline tests were completed, and the results were comparable to large-scale membrane harvest systems. A detailed SOP was written, revised and issued to standardize the test methods. This tool will facilitate further optimization and improvements to the Zobi Harvester® in future projects.

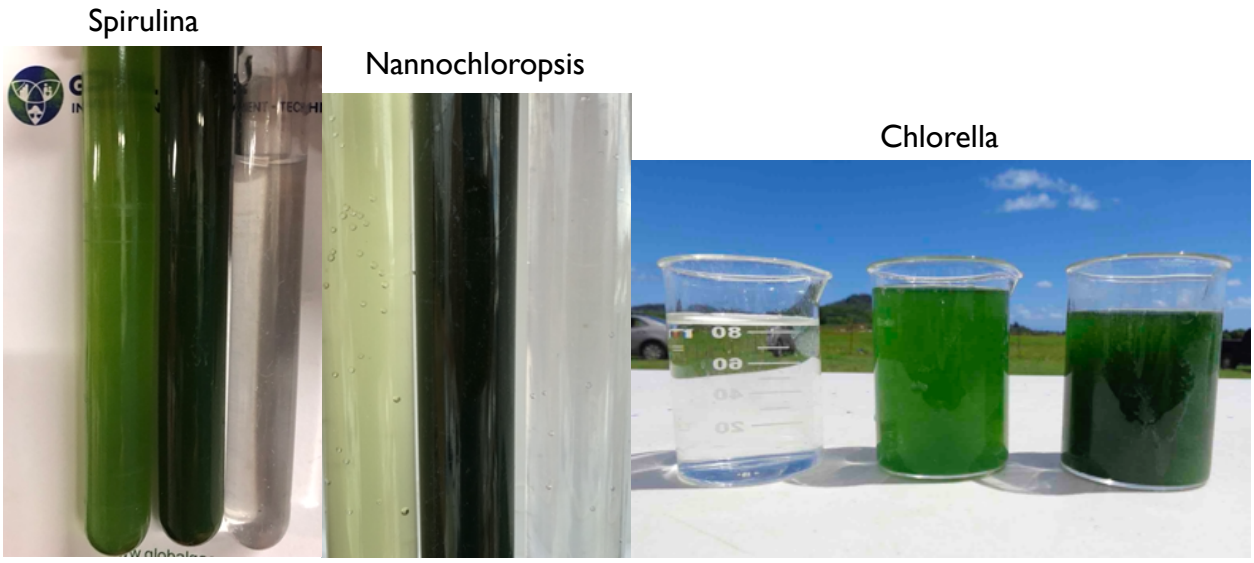

Figure 3-32 *Zobi harvester separates feed (light green) into permeate (clear), and retentate (dark)*

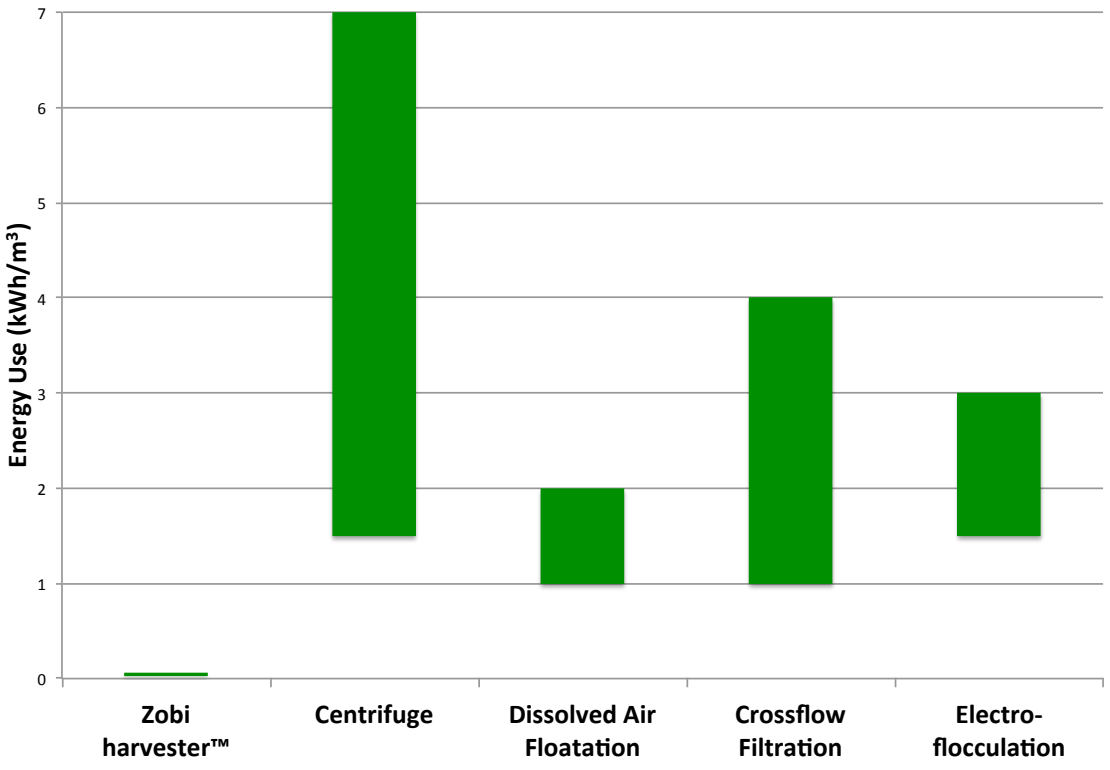

Figure 3-33 *Zobi harvester® reduces energy use by an order of magnitude*

Spirulina

Nannochloris

Figure 3-34 *Spirulina and Nannochloris 15-21% solids output from the Zobi Harvester®*

The Single Strand Test System was used for comparative permeability tests on: (a) algae slurry from cultivation, (b) supernatant from centrifuged algae slurry, (c) algae solids from centrifuging resuspended in clean water. These tests revealed that the flux is affected significantly by both the algae and the supernatant. Approaches to improve the flux by addressing both types of fouling were developed but were beyond the scope and resources of this project.

3.3.2 Algae Paste Dewatering

The original plan was to utilize a technique to chemically remove moisture from algal water while forcing above 95% of the algal cell mass to migrate to a (hydrophobic) lipid extraction solvent. The technique is based on functionalization of algal cell surface with a water-soluble cationic polyelectrolyte, Poly(diallyldimethylammonium chloride) (PolyDADMAC) that has hydrophobic ligands in its structure as described in the paper by Nalin U. Samarasinghe and Sandun D.Fernando of Texas A&M in Renewable Energy, Volume 81, September 2015, Pages 639-643. Through a series of experiments conducted in conjunction with Texas A&M, it was determined that the process couldn't achieve consistent separation and dewatering using algae cultivated in outdoor systems at the Kauai Algae Facility, so this approach was dropped.

A set of dewatering tests were conducted with five different chemicals and a lab-scale apparatus that simulates a belt press filter (75-micron opening). These tests used fresh algae paste produced at the Kauai Algae Facility. The goal was to remove additional water from the 15-20% Zobi Harvester® product paste, to reduce the evaporative duty in the dryer. Paste dewatering tests used filter aids loaded onto a filter cloth. None were successful in producing a thickened algae product.

An innovative new breakthrough process was developed for drying achieves a 97% reduction in energy use and 89% reduction in cost relative to a conventional dryer. Numerous tests were conducted to characterize and refine the process. The final process was successfully demonstrated at laboratory scale for green algae, diatom algae, and cyanobacteria cultivated in large-scale, outdoor raceways and harvested with a Zobi® harvester system. Invention disclosures were prepared, and multiple patent applications are anticipated on the overall process as well as specific innovations in the process.

3.3.3 Lipid Extraction from Algae

The baseline approach was standard hexane extraction of lipids from algae using the Crown Iron Works process. This process is effective and produces a useable oil. The costs are reasonable, but the energy use is high relative to the goals of this project. Therefore, alternative extraction unit operations were proposed and tested. The main approach in the original proposal did not work; however, seven new unit operations were successfully developed. Various process options were tested using different combinations of these new unit operations. The end result is an innovative breakthrough process for extraction that achieves a 91% reduction in the energy use and 96% reduction in cost relative to the baseline hexane extraction process. The final process was successfully demonstrated at laboratory scale for diatom algae cultivated in large-scale, outdoor raceways, harvested with a Zobi® harvester system, and dried with our innovative new drying process described above. Invention disclosures were prepared, and multiple patent applications are anticipated on the overall process as well as specific innovations in the process.

3.4 Integration

The new cultivation and downstream process technology was incorporated into the technoeconomic model. In addition, the model was reviewed with NREL's algae technoeconomic modeling team to improve the fidelity and verify the reasonableness of the cost assumptions. The model was further improved through a series of design/trade-off studies for key equipment items in the Zobi® flowsheet. This included the sump well pump, aeration blower, fin-fan aeration cooler, aeration flowmeters, product paste pump, standpipe pump, pump seal flush, and feed strainer. The model was used throughout the process to guide the research and analyze the impacts of various flowsheets and technology options. The model and results have been presented in multiple proprietary presentations to the BETO team.

4 Products developed

Three patents have allowed so far based our algal biomass yield project:

- 10,772,272 Algae cultivation systems and methods with reduced energy loss;
- 15/590,403 Algae cultivation systems and methods with bore waves (recently allowed);
- 16/507,716 Biological and algae harvesting and cultivation systems and methods (recently allowed).

Eight patent applications are pending, and we anticipate filling an additional 3 applications in cultivation and 8 applications in algae drying and extraction.

The results of the work have also resulted in multiple publications and presentations. There are four manuscripts that are either published (Smalley et al, 2020) or in review:

Smalley T, Fields FJ, Berndt AJE, Ostrand JT, Heredia V, Mayfield SP (2020). Improving biomass and lipid yields of Desmodesmus armatus and Chlorella vulgaris through mutagenesis and high-throughput screening. Biomass and Bioenergy. 142:105755. DOE report number: DOE-GAI-7689-1 https://doi.org/10.1016/j.biombioe.2020.105755

Gaidarenko O, Yee D, Hildebrand M. Enhanced triacylglycerol (TAG) and protein accumulation in transgenic diatom *Thalassiosira pseudonana* with altered photosynthetic pigmentation. *In review.* https://doi.org/10.1101/2020.01.07.897850.

Gaidarenko O, Mills DW, Vernet M, Hildebrand M. Overexpression of *Thalassiosira pseudonana* violaxanthin de-epoxidase-like 2 (VDL2) increases fucoxanthin while stoichiometrically reducing diadinoxanthin cycle pigment abundance. *In review.* https://doi.org/10.1101/2020.01.06.896316

Fields FJ, Hernandez RE, Weilbacher E, Garcia-Vargas E, Huynh J, Thurmond M, Lund R, Burkart MD, Mayfield SP. Annual productivity and lipid composition of microalgae (Chlorophyta) native to a pilot production facility in Southern California. *In review.*

Results were included in multiple presentations, including:

Multiple Proprietary presentations to DOE

Hazlebeck D, Rickman W. New low energy algal harvesting and dewatering system. Algae Biomass Organization Summit. October 25, 2016.

Hazlebeck D. Global Algae Innovations - Algae Solutions to Global Dilemmas. Advanced Bioeconomy Leadership Conference. March 1, 2017.

Hazlebeck D. Algae Cultivation for Carbon Capture and Utilization. U.S. Department of Energy, Bioenergy Technologies Office Workshop. May 23, 2017.

Hazlebeck D. Algae Solutions to Global Dilemmas. DOE Bioeconomy Conference 2017.

Hazlebeck D. UCSD MOOC on harvesting for online course.

Hazlebeck D. Global Algae Innovations. National Academies of Sciences • Engineering • Medicine. March 6, 2018

Hazlebeck D, Rickman W. Commercial Zobi Harvester Operations. 8th International Conference on Algae Biomass, Biofuels & Bioproducts. June 12, 2018.

Corpuz R. Technology Development Update. Algae Biomass Organization Summit. October 16, 2018.

Hazlebeck D. Techno-economic Analysis. Algae Biomass Organization Summit. October 17, 2018.

Hazlebeck D. Advances in Algae Technology. Advanced Bioeconomy Leadership Conference. November 9, 2018.

Hazlebeck D. Economical, sustainable algal production of protein and oil. Algae Biomass Organization Summit. September 16, 2019.

5 Lessons Learned

1. Breeding is much faster and more powerful than directed evolution or mutagenesis and sorting.
2. Directed evolution was more powerful than mutagenesis and sorting when a continuous environmental pressure could be maintained such as elevating the thermal tolerance.
3. Sorting based on fluorescence can select strains that more readily absorb the dye rather than strains that have higher lipid content.
4. Sorting based on fluorescence selection may miss a large improvement in lipid content.
5. We did not see any major differences in the variation between the populations generated by different mutagens. In future work, using mutagens that result in more dramatic DNA changes (e.g: indels) versus single point mutations may result in more stable phenotypes.
6. There seems to be large variation between species with respect to how readily high lipid phenotypes can be generated. In some cases, only hundreds of mutants needed to be screened to find a “hit” in a screen that then went on to demonstrate improved lipid content through analytical chemistry techniques. In the case of GAI-247, more than 10,000 mutants were screened in microtiter plates, many of them had been FACS enriched beforehand, and yet we were unable to derive a high lipid producing mutant.
7. Our best performing strains in outdoor cultivation are both diploid or polyploid, which (a) may make them more resilient in outdoor cultivation, (b) do make generating a strong phenotype more difficult since most mutations can be complimented and (c) lead to low stability of the mutations since redundant wild-type alleles that promote fitness can be copied-back into mutant loci.
8. Inability to get a single cell into each microfluidics chip channel was difficult in practice and requires more sophisticated control mechanisms.
9. Adaptation to GAI bicarbonate media is difficult for strains that don’t already have a fairly high tolerance for bicarbonate.
10. Open raceway design and control approaches continue to lead to improvements in productivity even without strain improvements.
11. There are many options to cause the microbiota to change and there is great potential for substantial productivity improvements; but better tools are needed quantifying the microbiota, and more knowledge is needed about the desirable and undesirable compositions.

12. Large-scale cultivation is needed to adequately develop harvest technologies because the microbiota composition, health, extracellular materials, and other components all impact the efficacy and efficiency of harvesting.
13. Larger scale and longer-term testing of drying and extraction technologies is needed.
14. Scaling down is important to enable laboratory and bench-scale studies of mechanisms and testing many options; however, scaling down can be more difficult than scaling up.