

# RAFT FINAL REPORT

## Regional Algal Feedstock Testbed

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# INTRODUCTION

In 2012, a subset of partners from the National Alliance for Advanced Biofuels and Bioproducts (NAABB) with specific capabilities in cultivation and modeling organized to form a Regional Algal Feedstock Testbed (RAFT) partnership in response to the U.S. Department of Energy's funding opportunity announcement (FOA-0000615). Through the NAABB, first generation discovery efforts, technology development and feasibility studies yielded best-of-class strains and a series of robust and unique cost, growth, process and GIS models. The NAABB members that make up the RAFT partnership are the University of Arizona (UA, lead), New Mexico State University (NMSU), Texas A&M AgriLife Research (AgriLife Research) and Pacific Northwest National Laboratory (PNNL). Collectively, the team has a unique blend of fully functional algal cultivation testbed facilities and cross-cutting support centers for detailed strain characterization, environmental simulated production and modeling.

RAFT's primary objective was to conduct long-term algal cultivation trials that are replicated across different regional, seasonal, environmental and operational

conditions consistent with the DOE FOA. The RAFT team was awarded funding for these efforts toward the middle of FY13. During the first six months, Phase 1 Planning, the team developed an R&D Plan and Data Management Plan and readied the testbeds for beginning research. After the Bioenergy Technologies Office (BETO) approved the R&D and Data Management plans in January 2014, the team initiated Phase 2, Initial Testbed Operation, which focused on long-term cultivation trials and data collection at three testbed locations—the UA, NMSU, and AgriLife Research—to provide long-term cultivation data. The team conducted Phase 2 activities for approximately eighteen months, through the end of FY15. Prior to moving forward with Phase 3, Continued Testbed Operation, a Go/No Go decision point was built into the schedule. The RAFT team met the key criteria for this decision point and BETO approved continued testbed operations for an additional two years, FY16-17, based on an updated R&D plan for Phase 3 activities. The team completed Phase 3 testbed cultivation studies at the end of FY17 and completed the final data analysis and modeling for inclusion in this final report.

# SUMMARY OF ACCOMPLISHMENTS



**272**  
EXPERIMENTS



**30**  
PUBLICATIONS  
(6 UNDER REVIEW)



**37**  
PRESENTATIONS



**3**  
PATENTS

The RAFT project completed detailed characterization of twelve potential production strains and extensively tested the three best strains in long-term seasonal cultivation trials. During these trials, the team completed 272 cultivation experiments at the three outdoor testbed locations over three years, testing seasonal effects as well as various harvest, crop protection and strain rotation strategies. The team developed a sensor to continuously monitor cell density and submitted a patent application. The team also developed and validated models for predicting strain performance, determining biomass productivity, evaluating cultivation system design and operational strategies large-scale production scenarios. In addition, the project established a detailed database for the long-term cultivation trials with discrete data from the 272 testbed cultivation experiments. This database will be maintained at the University of Arizona <https://raft.arizona.edu/cultivation-data> for public access and continued academic evaluations. The RAFT project also produced 30 (6 under review) peer review publications, 37 presentations and three patents.

# RAFT TESTBED CAPABILITIES AND FACILITIES

## PNNL Indoor Climate | Simulation Ponds and LEAPS Photobioreactor Systems

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To reduce the risks associated with initial outdoor pond cultivation trials, accurate laboratory-scale outdoor pond simulators are needed to rapidly screen microalgae strains for high biomass productivity. These experiments involve using simulated sunlight intensity and water temperature scripts, such as those generated by the Biomass Assessment Tool, or BAT (Wigmosta et al., 2011) for any geographic location of choice where meteorological data are available. To address this research need, PNNL developed two different outdoor pond culture simulators: indoor LED-lighted and temperature-controlled raceways and Laboratory Environmental Algae Pond Simulator (LEAPS) photobioreactors.

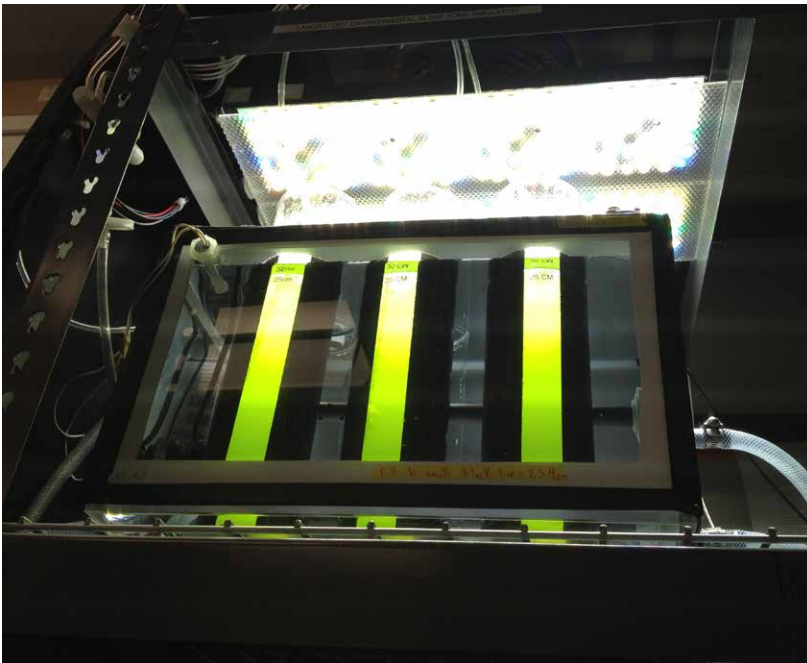
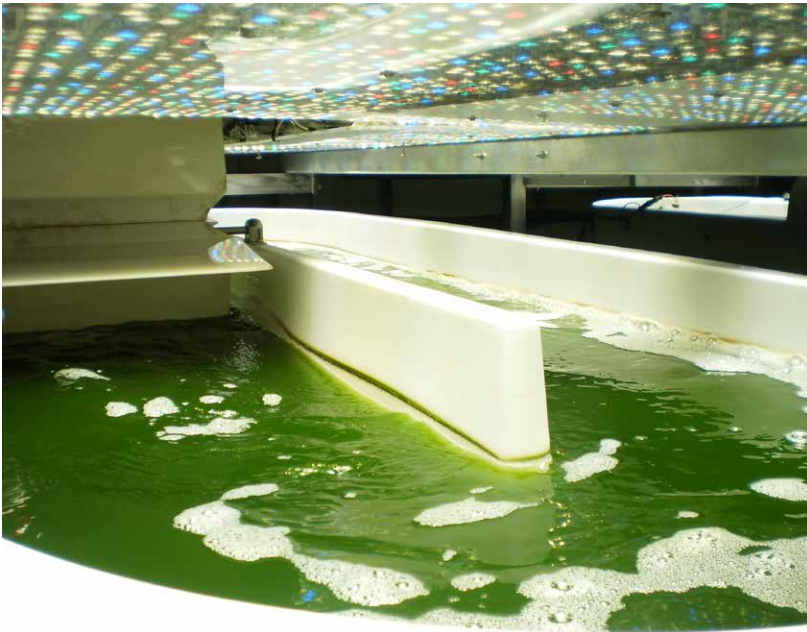
PNNL's four 800-liter indoor fiberglass raceway ponds, temperature-controlled via Labview™ software, are of a similar design to the outdoor raceways at the University of Arizona and New Mexico State University. A panel containing 4,500 multi-colored computer-dimmable LEDs illuminate the ponds to perform climate-simulated culturing

experiments (see Figure 1a). The team successfully validated the growth performance of these against outdoor ponds and conducted climate-simulation experiments to estimate the annual biomass productivity of *Chlorella sorokiniana* using Southern Florida scripts. These ponds were operated with selected RAFT strains using light intensity and water temperature scripts obtained from PNNL's Biomass Assessment Tool (Wigmosta et al., 2011).

PNNL's LEAPS consists of six well-mixed glass column photobioreactors submerged in a temperature-controlled waterbath (-2 °C to >60 °C) and illuminated from above by a programmable multicolor LED lighting system (0 to 2,500  $\mu\text{mol}/\text{m}^2\text{-s}$ ) (Figure 1b). Measured incident light intensities and water temperatures were demonstrated to deviate from the respective light and temperature setpoints on average only 2.3% and 0.9%, respectively, demonstrating accurate simulation of light and temperature conditions measured in outdoor ponds. The growth performance of *C. sorokiniana* and *N. salina* LEAPS cultures was successfully validated against outdoor ponds in another BETO-funded project. The LEAPS was used to evaluate new RAFT strains prior to outdoor pond deployment and to troubleshoot problems observed in outdoor RAFT experiments.



## PNNL Climate-Simulated Culturing Capabilities



**Figure 1.** Current climate-simulated culturing capabilities at PNNL: (a) LED-lighted and temperature-controlled indoor raceway ponds; (b) LED-lighted and temperature-controlled Laboratory Environmental Algae Pond Simulators (LEAPS).

Several publications, below, resulted from this work.

### Citations

Huesemann, M., T. Dale, A. Chavis, B. Crowe, S. Twary, A. Barry, D. Valentine, R. Yoshida, M. Wigmosta, and V. Cullinan. 2017a. Simulation of outdoor pond cultures using indoor LED-lighted and temperature-controlled raceway ponds and Phenometrics™ photobioreactors. *Algal Research* **21**:178-190.

Huesemann, M.H., P. Williams, S. Edmundson, P. Chen, R. Kruk, V. Cullinan, B. Crowe, and T. Lundquist. 2017b. Laboratory Environmental Algae Pond Simulator (LEAPS) Photobioreactors: Validation Using Outdoor Pond Cultures of *Chlorella sorokiniana* and *Nannochloropsis salina*. *Algal Research* **26**:39-46.

Huesemann, M., A. Chavis, S. Edmundson, D. Rye and M. Wigmosta. 2018. Climate-Simulated Pond Culturing: Quantifying the Maximum Achievable Annual Biomass Productivity *Chlorella sorokiniana* (DOE 1412) in the United States. *Journal of Applied Phycology* **30**:287-298.

Wigmosta, M.S., A.M. Coleman, R.J. Skaggs, M.H. Huesemann, and L.J. Lane. 2011. National microalgae biofuels production potential and resource demand. *Water Resources Research* **47**.



# University of Arizona | Cultivation Testbed

Testbed lead: Kim Ogden | [ogden@email.arizona.edu](mailto:ogden@email.arizona.edu)



**Figure 2.** Cultivation systems for UA testbed: (a) Traditional paddlewheel systems and (b) ARID Cultivation System

The UA RAFT testbed, located in Tucson, AZ, is equipped with a set of three 1000-liter open raceway growth systems for small-scale cultivation experimentation and two larger scale ARID raceway (Ryan et al., 2012) cultivation systems, as shown in Figure 2, for large-scale experiments. The ARID design allows for mixing by low head pumping and gravity flow over a channel baffle. The pumps are powered by solar panels. In addition, these systems have canals or sumps to store cultures at night to conserve heat and increase winter productivity. Newer alternative designs for the ARID system have incorporated paddlewheel mixing with the storage canal. The UA RAFT testbed also has production and processing capabilities to harvest biomass and recycle media.

In addition, the UA has specialized capabilities in molecular monitoring, real-time optical density (OD) monitoring and cultivation modeling that were employed as part of the RAFT project. Applications of these capabilities resulted in the development and application of molecular monitoring methods to test strategies for culture contamination control and improved culture stability as well as the development and testing of real-time monitoring and control strategies for large-scale algal cultivation. The team also developed various cultivation models to assist with cultivation system design and operations.

# New Mexico State University | Cultivation Testbed

Testbed lead: Wayne Van Voorhies | [wvanvoor@nmsu.edu](mailto:wvanvoor@nmsu.edu)



**Figure 3.** NMSU Testbed Culture Systems: (a) traditional paddlewheel systems and (b) Solix PBR system

The NMSU RAFT testbed, located at 3,900 feet above sea level in Las Cruces, NM, has the coolest climate of the RAFT testbeds. The testbed includes three 600-liter open raceway growth systems for cultivation and several photobioreactor (PBR) systems, shown in Figure 3. The open raceways and Solix PBR system with multiple individual reactor bags (the Solix System was used to grow some algae used for hydrothermal liquefaction (HTL) by PNNL) were employed for long-term growth studies of selected RAFT strains. The NMSU testbed also includes cultivation systems operated inside enclosed plastic bags fitted with an internal paddlewheel for mixing and berms created with sandbags. The “closed-bag” raceways were

used for RAFT-supported algal cultivation experiments with an extremophile algal strain using municipal wastewater and/or dairy wastewater.

In addition, NMSU applied specialized capabilities in two areas: developing chemical crop protection strategies and evaluating polyculture cultivation methods that were employed as part of the RAFT project. The crop protection efforts resulted in identifying methods that could improve culture stability with selected RAFT strains. The polycultures studies provided data on the performance of structured and natural polycultures versus the selected RAFT strains.



## AgriLife Research | Cultivation Testbed

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**Figure 4.** AgriLife Research Testbed Medium-Scale Raceways

The AgriLife Research RAFT testbed, located in Pecos, TX, is the largest of the RAFT testbeds and has a diverse set of cultivation capabilities for scale experimentation, large-scale biomass production and processing capabilities to harvest biomass and recycle media. As a RAFT testbed, it used a set of eight medium raceways (MR), shown in Figure 4, for long-term growth studies of selected RAFT strains with various treatment

replicates. In addition, the AgriLife Research Testbed also conducted scale-up production of selected RAFT strains in the 10,500-liter production ponds, shown in Figure 5. Additionally, AgriLife Research provided expertise in the development of low-cost media formulations, use of alternative water sources and development and evaluation of cultivation strategies and operational methods to reduce costs and maintain stable high-productivity cultures.



**Figure 5.** AgriLife Research Testbed Large-Scale Cultivation Pond

# OBJECTIVES OF RAFT

The objectives for the RAFT project were consistent with those required in the DOE FOA, with the major focus on ***collecting long-term algal cultivation data in outdoor pond testbeds***. RAFT also pursued other objectives as part of collecting long-term algal cultivation data:

- Develop best management practices
- Monitor real-time culture health and productivity
- Develop molecular diagnostics tools
- Develop “open-access” data management
- Improve and refine cultivation models
- Develop pond and model feedback-enhanced systems
- Increase sustainability of algae biomass production
- Evaluate and implement strain selection and crop rotation for year-round cultivation

# RAFT TECHNICAL APPROACH

The RAFT R&D Technical approach is shown in Figure 6. The team leveraged internal key strengths to present a data-driven approach to conducting long-term outdoor cultivation experiments at testbeds. A few strains isolated as part of the NAABB program were screened and evaluated for production potential at a large scale. In this approach, PNNL first characterized in detail each selected RAFT strain to determine its seasonal productivity potential as described in the section below.

The resulting experimental data served as the initial input to the species-specific Biomass Assessment Tool to generate biomass productivity maps as well as light and temperature scripts for climate-simulated culturing in indoor LED-lighted and temperature-controlled raceways. This approach identified three strains (*C. sorokiniana*, *Scenedesmus obliquus* and *M. minutum*) as having the greatest potential for high productivity seasonal cultivation in the outdoor testbed systems.

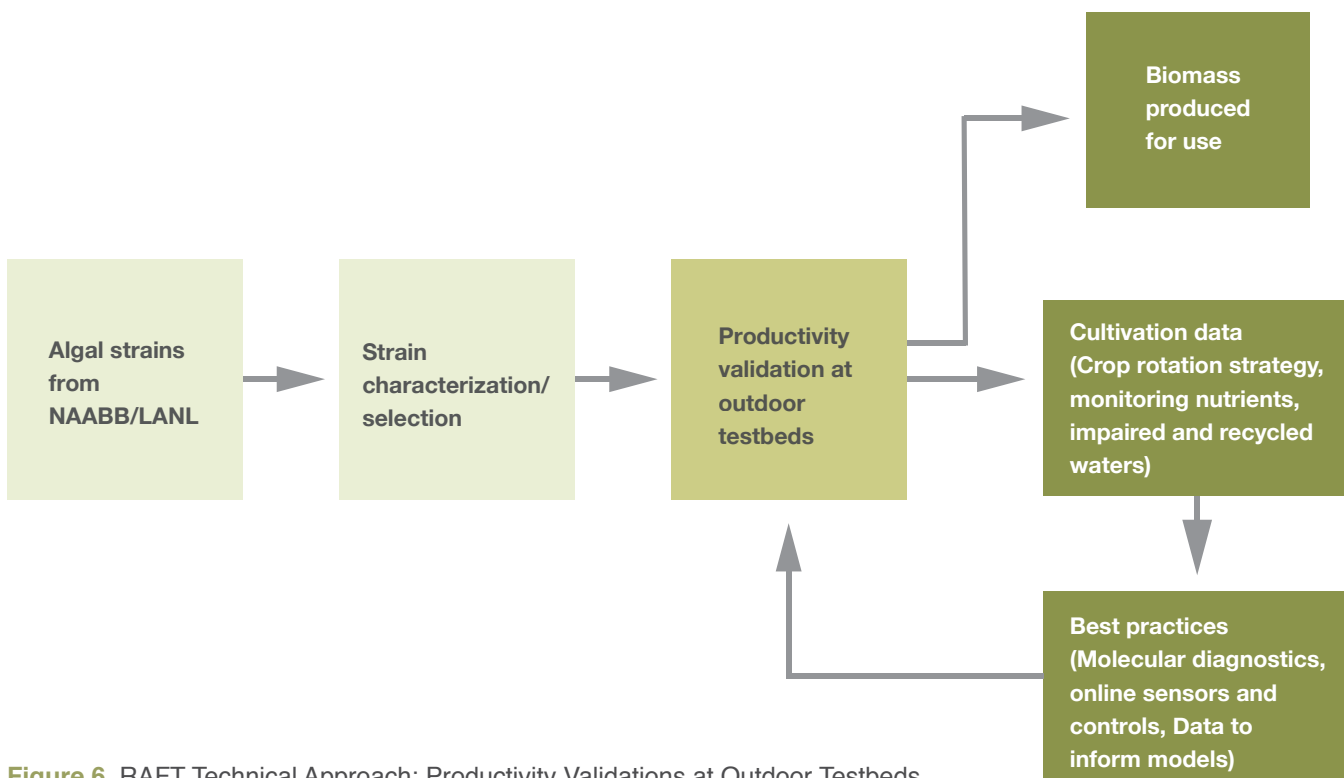


Figure 6. RAFT Technical Approach: Productivity Validations at Outdoor Testbeds

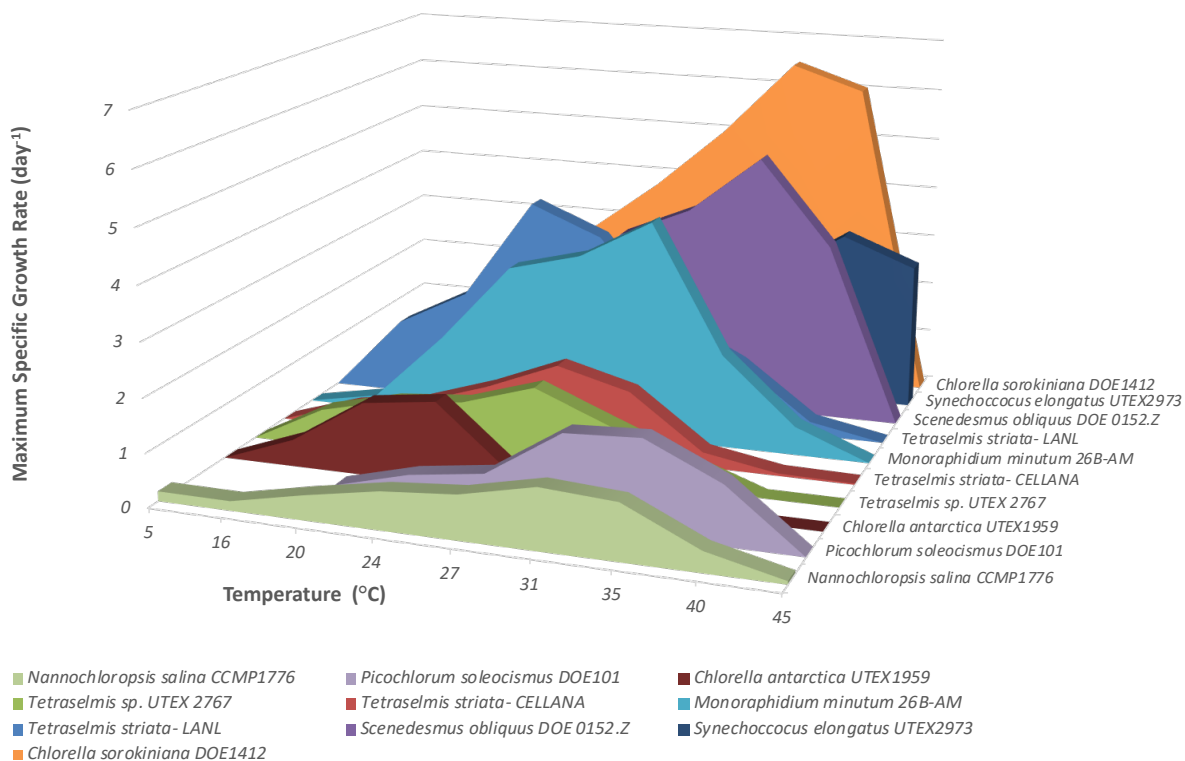
The individual RAFT testbeds have unique but complementary capabilities that the project used to test identical strains at various geographical locations and climates. In addition, each testbed used different culture systems and strategies to optimize productivity and evaluate alternative media using nonpotable water sources (e.g., saline groundwater, municipal wastewater). Biomass was supplied to groups that requested it for conversion and storage experiments. Collectively, the cultivation trials provided long-term cultivation data on system performance, culture stability and biomass productivities to the algal research community. The team fed data generated in the first years of the project into growth and BAT models, and predictions for a crop rotation strategy were made for each testbed. The testbeds then implemented the rotation strategy in the latter years of the project.

In addition to providing data for models, the project developed best practices for monitoring algal cultivation systems. These include molecular diagnostics methodology for understanding culture health, continuous online monitoring of carbon dioxide addition and

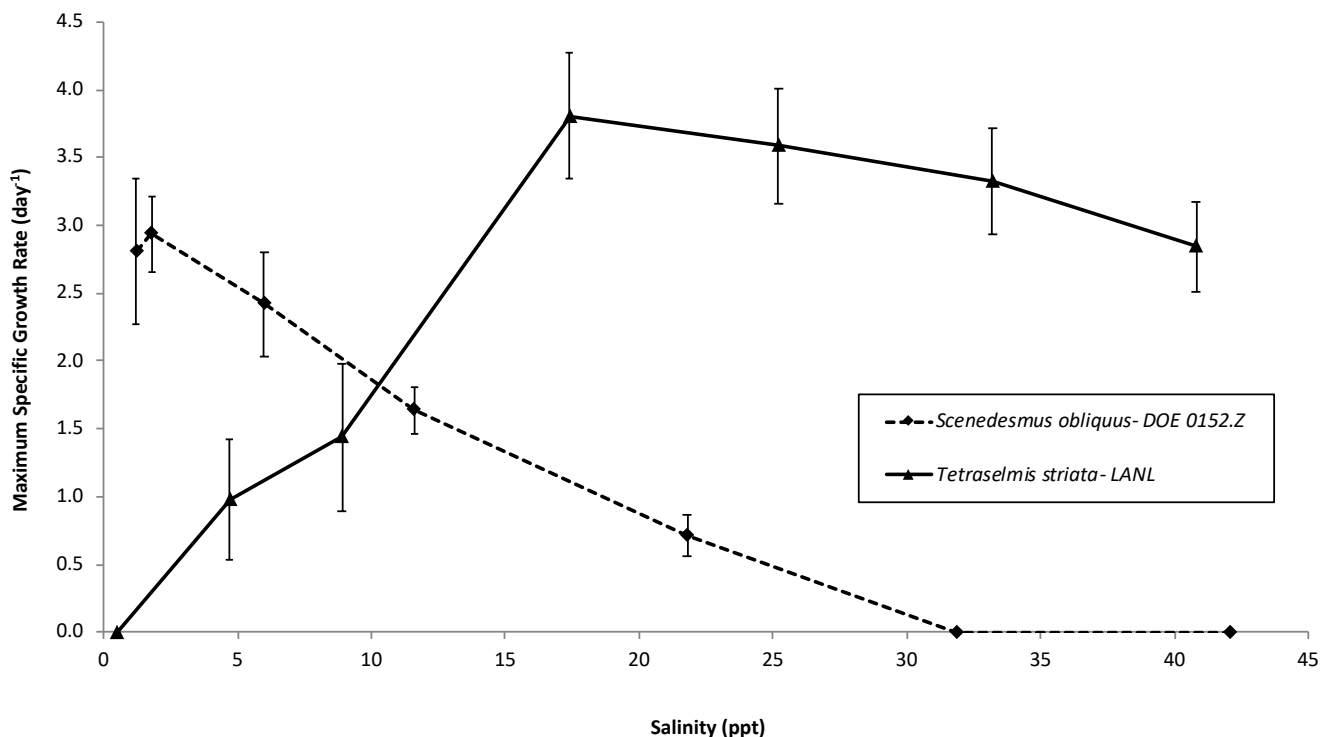
continuous monitoring of cell growth using a new sensor that was patented. Finally, the team investigated the use of impaired and recycled water and demonstrated that these water sources can be used in outdoor open pond systems.

## Strain Characterization

A major objective of the RAFT project was to identify new strains for outdoor pond cultivation and demonstrate high seasonal and annual biomass productivities via crop rotation. Therefore, to ensure the successful deployment of new microalgae in the outdoor RAFT testbeds, it was necessary to know *a priori* the following strain characteristics: temperature tolerance range, which is needed to assign each strain to the appropriate growing season (winter, summer, spring/fall) (Figure 7); salinity tolerance range, which is required to select the pond medium salinity in which biomass productivity is likely optimal (Figure 8); and growth performance and rates, which are important because unstable/unhealthy strains should be avoided and only the fastest growing ones should be chosen for pond cultivation.



**Figure 7.** Maximum specific growth rate as a function of temperature for strains characterized during the RAFT project



**Figure 8.** Maximum specific growth rate as a function of salinity for strains characterized during the RAFT project

To obtain this critical information, each new strain was first cultivated in shake flask cultures on the PNNL Thermal Gradient Incubator (TGI) to determine the maximum specific growth rate as a function of temperature, following methods described in Van Wagenen et al. (2012) and Huesemann et al. (2016). The optimal temperatures and the thermal tolerance ranges of the different strains tested were highly variable (Table 1). The peak maximum specific growth rates observed were also strain specific, with *C. sorokiniana* having the highest measured maximum specific growth rate (6.5 day<sup>-1</sup>) of all strains tested (Figure 7). The team disregarded strains that did not thrive after

repeated trials and only evaluated further strains with robust growth and superior maximum specific growth rate values over the respective seasonal temperature range. The salinity tolerance was determined on the PNNL Salinity Gradient Incubator (SGI) by measuring the maximum specific growth rate as a function of salinity (Figure 8). Based on these basic strain characterization data, a number of the best performing strains were more rigorously parameterized for predicting their outdoor biomass productivity with the PNNL Biomass Growth Model, as described in the RAFT Modeling Section.



**Table 1.** Strains Characterized During the RAFT Project

	Strain Designation	Source	Optimum Temp. (°C)	Min./Max. Temp. Tolerance (°C)	Season	Successful outdoor field trials	Respiratory biomass loss (% of total AFDW)	Approx. Salinity Tolerance (PPT) <sup>a</sup>
1	<i>Chlorella sorokiniana</i> DOE1412	CUNY-NAABB	36	18 to 41	Summer	UA, TAMU, NMSU	2-16	1-10
2	<i>Monoraphidium minutum</i> 26B-AM	TAMU-NAABB	27	<4.5 <sup>b</sup> to 38	Winter	UA, TAMU, NMSU	2-22	1-12 <sup>c</sup>
3	<i>Scenedesmus obliquus</i> DOE0152.Z	CUNY-NAABB	33	<4.5 <sup>b</sup> to 36	ALL	UA, TAMU, NMSU	3-13	1-5
4	<i>Nannochloropsis salina</i> CCMP1776	NCMA	26	11 <sup>b</sup> to 32	Transition	Not during RAFT	2-20	NM
5	<i>Picochlorum soleocismus</i> DOE101	LANL-NAABB	30	18 <sup>b</sup> to 34	Summer	Not during RAFT	2-9	NM
6	<i>Tetraselmis striata</i> LANL	LANL	23	<14.5 <sup>b</sup> to 40	Transition	Not during RAFT	1-13	17-41
7	<i>Tetraselmis</i> sp. UTEX2767	UTEX	27	16-35	Transition	Not during RAFT	NM	NM
8	<i>Tetraselmis striata</i> CELLANA	Cellana	27	16-35	Transition	Not during RAFT	NM	NM
9	<i>Stichococcus minor</i> CCMP819	NCMA	29 <sup>d</sup>	18-40 <sup>d</sup>	Summer	Not during RAFT	NM	5-50
10	<i>Tisochrysis lutea</i> CCMP	NCMA	29 <sup>d</sup>	18-35 <sup>d</sup>	Summer	Not during RAFT	NM	NM
11	<i>Chlorella antarctica</i> UTEX1959	UTEX	20	<5-20	Winter	Not during RAFT	NM	1-5 <sup>c</sup>
12	<i>Synechocystis elongatus</i> UTEX2973	UTEX	40	34-47	Summer	Not during RAFT	NM	NM

- Salinity tolerance is defined as the range of salinities in which the maximum specific growth rate was equal or greater than 80% of the specific growth rate observed at the optimum salinity.
- 14.5 °C was the coldest temperature tested for this strain.
- Salinity tolerance measured using the microwell cultures instead of shake flask cultures on the Salinity Gradient Incubator.
- Temperature tolerance measured using thermal gradient microwell cultures instead of shake flask cultures on the Thermal Gradient Incubator.

### Results for Environmental Simulated Growth

The PNNL Laboratory Environmental Algae Pond Simulator was used to determine the biomass productivity of the most promising new strains, identified in the characterization experiments described above, under simulated summer or winter season culture conditions. These indoor climate-simulation photobioreactor experiments were conducted several months prior to the arrival of the outdoor growing season, thereby enabling the selection of only the most highly productive and stable strains for outdoor deployment. In addition, the team used LEAPS climate-simulated cultures to identify and solve pond operational problems that had been observed previously in outdoor RAFT trials.

Based on earlier temperature tolerance data, *Chlorella antarctica* was identified as a promising winter season strain (see Table 1). To determine whether this strain would be able to outperform the incumbent RAFT winter season strain, *M. minutum*, both strains were cultured in duplicate LEAPS bioreactors containing standard BG-11 medium, simulating 20 cm-deep outdoor ponds in Rimrock, AZ, from February 29, 2016 (Day 0) to March 21, 2016 (Day 21). The pond water temperature ranged from ca. -0.5 to 19 °C during this period, which is representative of winter season water temperatures observed at the different RAFT testbeds. As shown in Figure 9, the rate of increase in biomass (AFDW) concentration with time was about the same in the *C. antarctica* and *M. minutum* LEAPS cultures. The average areal biomass productivity ( $\pm$  standard deviation) for *C. antarctica* and *M. minutum* during the linear growth phase (Day 0 to Day 21) was  $9.7 \pm 0.6$  and  $10.2 \pm 0.1$  g/m<sup>2</sup>-day, respectively. The difference in areal biomass productivity between these two species is not statistically significant, indicating they both could be used as high productivity winter season strains.

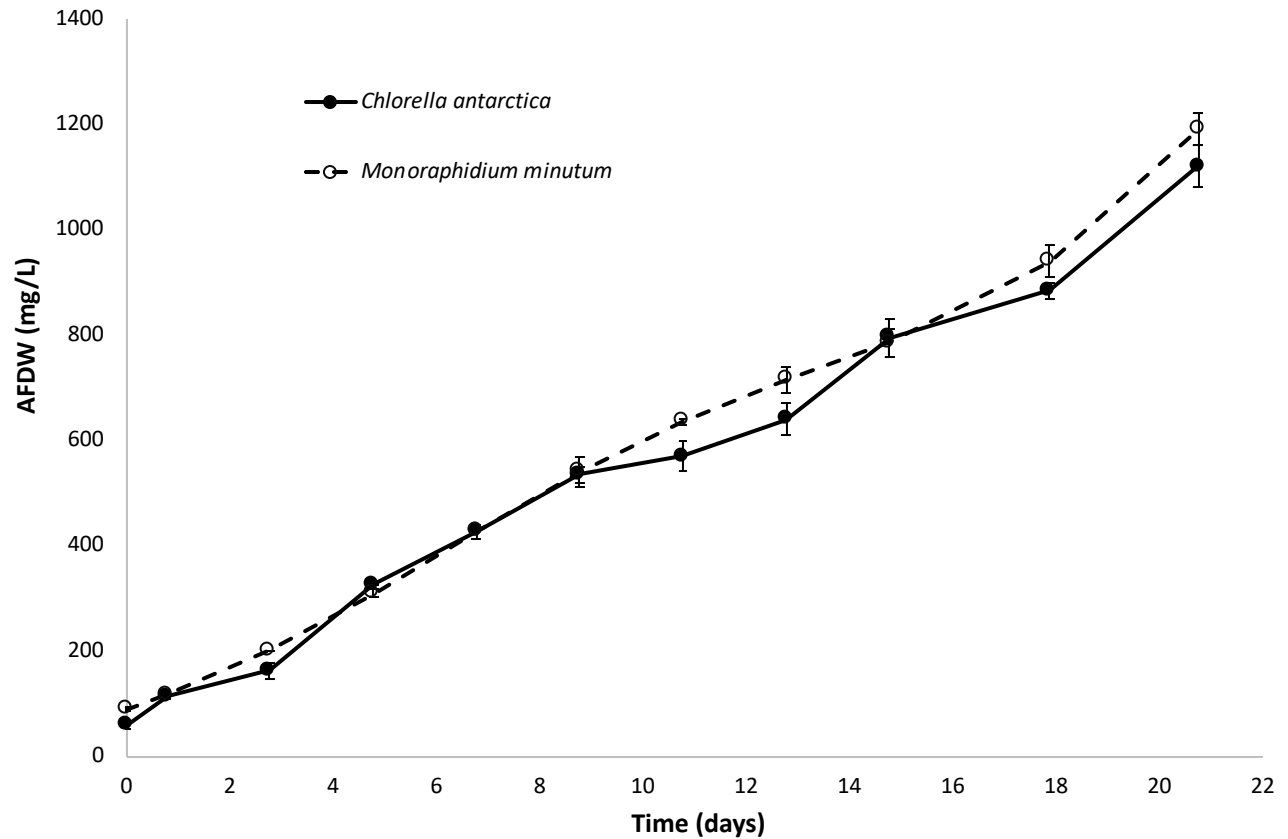
The team also used LEAPS cultivation to evaluate promising new summer season strains, such as *Stichococcus minor*, and compared this strain's biomass productivity to the two incumbent RAFT summer season benchmark strains, *S. obliquus* and *C. sorokiniana*. All three strains were cultured in duplicate LEAPS bioreactors containing standard BG-11 medium (for *S. minor*, the medium was adjusted to a brackish BG-11 at 11 PPT

salinity), simulating 25 cm-deep outdoor ponds in Rimrock, AZ, from June 10, 2012 (Day 0) to June 20, 2012 (Day 10). The pond water temperature ranged from ca. 13 to 28 °C during this period, which is representative of summer season water temperatures observed at the different RAFT testbeds. Linear growth phase productivities for *S. minor* were 11.6 g/m<sup>2</sup>-day—very similar to the 11.7 g/m<sup>2</sup>-day achieved by *S. obliquus*, but slightly lower than 12.4 g/m<sup>2</sup>-day by *C. sorokiniana*. Consequently, all three strains were considered suitable high-productivity candidates for summer season outdoor cultivation. *S. minor* is of interest because it exhibited productivities on par with the two incumbent RAFT strains in a brackish water medium and additionally can tolerate salinities as high as 50 PPT (see Table 1).

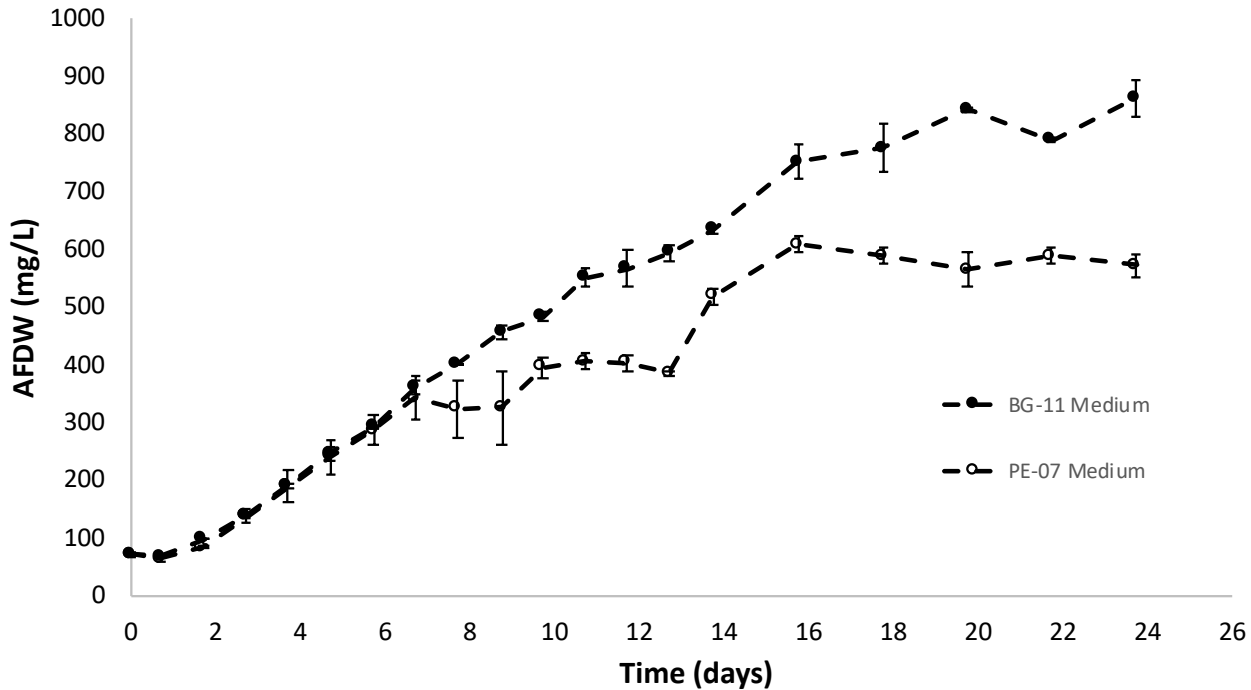
Additionally, the team used LEAPS climate-simulated cultures to identify possible causes for the unexpectedly low biomass productivities observed for *S. obliquus* in different outdoor RAFT trials. The team consistently observed that measured and modeled *S. obliquus* biomass productivities were high in indoor climate-simulation cultures when standard BG-11 medium was used but almost three times lower in the outdoor RAFT cultures with Pecos-07 (PE-07) medium. That observation led the team to postulate that the PE-07 medium was limiting growth. To test this hypothesis, *S. obliquus* was cultured in duplicate LEAPS photobioreactors using either BG-11 or PE-07 medium, simulating 25 cm-deep outdoor ponds in Rimrock, AZ, during June and July 2012. As shown in Figure 10, biomass growth proceeded at a similar rate in both media, equivalent to a productivity of about 11.7 g/m<sup>2</sup>-day, until about Day 7 (AFDW = 370 mg/L). After Day 7, *S. obliquus* growth in the PE-07 medium significantly decreased relative to growth in BG-11 medium, and it stopped completely after Day 16, indicating some type of nutrient limitation. Since PE-07 medium contains only 16% and 28% as much magnesium and iron, respectively, compared to standard BG-11 medium, it is possible that one or both of these elements were limiting biomass growth, resulting in the reduced productivities and biomass yields observed after Day 7 in the two LEAPS photobioreactors with PE-07 medium. As a result of these findings, all future RAFT testbed experiments were performed with nutrient replete media.

The team found the PNNL LEAPS photobioreactor system to be a useful and versatile tool for evaluating promising new strains under climate-simulated winter and summer season conditions prior to deployment in outdoor testbeds and for troubleshooting problems encountered during cultivation trials. The demonstrated successes of strain screening and testing under climate-simulated conditions

in the LEAPS during the RAFT project provided the motivation to further expand and apply this protocol in the recently initiated and currently ongoing DISCOVER consortium project involving PNNL, National Renewable Energy Laboratory, Los Alamos National Lab, and Sandia National Laboratories.



**Figure 9.** Average *C. antarctica* and *M. minutum* biomass concentration (AFDW) as a function of time in duplicate LEAPS photobioreactors, simulating winter season pond conditions as described in the text. The length of the error bars is one standard error (n=2).



**Figure 10.** Average *S. obliquus* biomass concentration (AFDW) as a function of time in duplicate LEAPS photobioreactors containing either standard BG-11 medium or PE-07 medium, simulating summer season pond conditions as described in the text. The length of the error bars is one standard error (n=2).

Several publications, below, resulted from this work.

### Citations

Huesemann, M.H., B. Crowe, P. Waller, A. Chavis, S. Hobbs, S. Edmundson, and M. Wigmosta. 2016. A validated model to predict microalgae growth in outdoor pond cultures subjected to fluctuating light intensities and water temperatures. *Algal Research* **13**:195-206.

Van Wagenen, J., T.W. Miller, S. Hobbs, P. Hook, B. Crowe, and M.H. Huesemann. 2012. Effects of light intensity and temperature on fatty acid composition in *Nannochloropsis salina*. *Energies* **5**:731-740.

Huesemann, M.H., P. Williams, S. Edmundson, P. Chen, R. Kruk, V. Cullinan, B. Crowe, and T. Lundquist. 2017. Laboratory Environmental Algae Pond Simulator (LEAPS) Photobioreactors: Validation Using Outdoor Pond Cultures of *Chlorella sorokiniana* and *Nannochloropsis salina*. *Algal Research* **26**:39-46.

# Productivity Validations at Outdoor Testbeds

## Strains Tested

1. *Scenedesmus obliquus* DOE0152z
2. *Chlorella sorokiniana* UTEX 1230
3. *Chlorella sorokiniana* DOE1412
4. *Galdieria sulphuraria* CCMEE 5587.1 This algae is tolerant of high temperatures and low pH (it can grow at temperatures up to 55°C and at a pH as low as 1.0) and was originally isolated from hot spring in Yellowstone National Park.
5. *Monoraphidium sp* (the identity of this species is still tentative and based on preliminary genetic analysis by J. Brown at the University of Arizona). This strain originated from the Texas A&M facility and was originally identified as a species of *Kirchneriella*.

## Cultivation data and analysis

The team obtained discrete and continuous data at each of the testbeds and generally made optical density, ash-free dry weight, water addition, harvest amount, media addition and cell counts/microscopic observations three to seven times a week. The researchers continuously measured temperature, pH and dissolved oxygen and monitored weather station data for each site, analyzing these data to determine the average linear growth rate, the maximum linear growth rate and the average and maximum total growth rates. The linear growth period was defined as the period of growth that occurred after the end of any initial growth lag period and before culture growth plateaued. The maximum linear growth was the highest linear growth recorded out of all the runs measured. The average productivity was the total productivity averaged

from when the culture was started through its time of harvest. The team excluded culture crashes from these analyzes.

## UA Testbed Results

The focus for the UA testbed was to evaluate and implement a crop rotation strategy for production of algal biomass all year. The team completed 98 cultivation experimental runs and compared three different reactor systems: traditional paddlewheel reactors, the ARID reactor and the paddlewheel ARID. In addition, the team investigated recycling water after harvest, adding alkonium chloride to delay or avoid pond crashes and varying salinity, culture depth and media composition.

## Algal species tested at the UA

1. *Chlorella sorokiniana*
2. *Scenedesmus obliquus*
3. *Monoraphidium minutum*

The UA testbed is located in Tucson, AZ (32.280069 N, 110.936256 W). The team used two traditional paddlewheel reactors for most of the experiments, operating them at depths ranging from 10 to 25 cm during the first two years of the project and at a depth of 25 cm in the final year. The team also used the 8,000-liter ARID raceway for all strains in the first two years to determine if this reactor system increased productivity. Finally, the researchers developed a new paddlewheel ARID (Figure 11) that was run at a depth of 10 cm and a total volume of 400 liters. The harvest strategy was typically set to harvest two-thirds to three-fourths of the culture after a maximum cell density was reached. The team continuously monitored the reactors for DO, pH, temperature and electrical conductivity and monitored carbon dioxide addition, adding it to control pH. The team also developed a continuous optical density sensor as part of this project and used it in many of the later experiments.





**Figure 11.** Newly developed paddlewheel ARID.

### Summarized Experimental Results

A summary of the experimental runs is found in Table 2. Productivities varied from a low of 2 to 13 g/m<sup>2</sup>/day in the winter with short days and sometimes freezing overnight temperatures, to a high of 24 g/m<sup>2</sup>/day in the spring and summer months. The longest cultivation run was for more than 100 days in the paddlewheel reactors using *S. obliquus*, while the longest run in the ARID system was 50 days using the same species. Especially

in the cooler months with shorter days, the ARID system routinely performed better in terms of productivity than the paddlewheel reactors. Typically, the productivity increases were 3 g/m<sup>2</sup>/day higher in the ARID. The ARID, however, involves many parts and requires more labor to ensure it is operating properly. For example, there are some dead zones in terms of fluid flow that require mixing by hand in the morning or evening to remove settled algal biomass. The paddlewheel ARID was developed to eliminate these issues.

**Table 2.** UA Experimental Summary – copy this table and put in alphabetical order

Strain	Number of runs	Longest run (days)	Range of linear productivity g/m <sup>2</sup> /day
<i>Chlorella sorokiniana</i>	61	66	5 to 23
<i>Monoraphidium minutum</i>	8	74	2 to 13
<i>Scenedesmus obliquus</i>	29	106	2 to 24

*Chlorella sorokiniana*: *C. sorokiniana* was the most tested strain in Arizona because it can grow at temperatures as high as 40 °C. This strain can have sustained productivities of 20 g/m<sup>2</sup>/day in April through September, when the daytime highs range from approximately 27 to 43 °C and the lows from 10 to 29 °C. This strain does not survive if a pond freezes or pond temperatures near freezing at night and therefore it cannot be used from November through February in Arizona. The issue with this strain is that it is highly susceptible to contamination to *Vampirovibrio chlorellovorus*. This obligatory bacterial parasite is specific to *C. sorokiniana* and will devastate a healthy culture within hours. The team found that the addition of 1.5 to 2 ppm of benzalkonium chloride (BAC) every four days allows for control of *V. chlorellovorus* and sustained growth of *C. sorokiniana*. Using this treatment strategy, *C. sorokiniana* was sustained in the ARID system for 66 days, from September through November. The team also investigated using increased salinity to eliminate *V. chlorellovorus*, but this did not have any effect on removing *V. chlorellovorus*. *C. sorokiniana*, like all strains, also is subject to contamination by ciliates. These organisms were observed via microscopic analysis but were not identified using molecular techniques.

*Monoraphidium minutum*: *M. minutum* is a strain that grows well in colder months and is an excellent choice to rotate with *C. sorokiniana*. Productivities in January and February ranged from 4 to 7 g/m<sup>2</sup>/day in paddlewheel reactors. Slightly higher productivities — 10 g/m<sup>2</sup>/day — were observed in November in the ARID system. The team did not grow this strain outdoors in the summer months at this testbed.

*Scenedesmus obliquus*: *S. obliquus* grows reasonably well all year in Arizona. The longest sustained runs were obtained with this strain in both reactor types (ARID and paddlewheels). In the winter, productivities were typically 4 to 6 g/m<sup>2</sup>/day, and the limited summer experiments with this strain had productivities of 14 to 17 g/m<sup>2</sup>/day. Without considering crop rotation options, this would be an excellent strain for Arizona. One of the issues with this strain is that it is very sensitive to salinity. This needs to be monitored, especially if recycled media is a strategy for long-term growth. This strain is also susceptible to *Aquamonas* bacterium contamination. The team did not have the opportunity to explore methods to eliminate this contamination.

#### ***Growth of Chlorella sorokiniana in recycle spent media***

The team grew *C. sorokiniana* in both the traditional paddlewheel and the paddlewheel ARID reactor to a cell density of approximately 0.5 g/L and harvested it via centrifugation before returning the media to the reactor. Additional nutrients were added and the algae was allowed to continue to grow, doing well in recycled spent media. The team recycled the media six times using the paddlewheel ARID and observed no decrease in average productivity with each harvest cycle.

#### ***Carbon Dioxide Monitoring***

High production costs due to high carbon dioxide usage and low lipid productivity have been among the major challenges impeding the commercial production of algal biofuels. During the project, the team evaluated cell



growth and lipid content of *C. sorokiniana* at different pH in flasks and flat panel photobioreactors. Discontinuous monitoring of carbon dioxide was optimized using flow meters, micro bubble diffusers and a data logger. The team translated the laboratory-scale work to the field in terms of monitoring.

The following publications resulted from the work.

R. Qiu, S. Gao, P. Lopez, and K. L. Ogden. 2017. Effects of pH on cell growth, lipid production, and CO<sub>2</sub> addition of microalgae *Chlorella sorokiniana*. *Algal Research* 28:192-199. <https://doi.org/10.1016/j.algal.2017.11.004>

### **Nutrient Monitoring**

Compared to commonly used recipes such as BG-11, culture media used in the RAFT raceway experiments were developed and tested with different nutrient sources and at lower concentrations. The objective was to reduce nutrient cost in microalgal biomass production. The team studied microalgae growth under different nutrient statuses in both indoor and outdoor experiments and applied the indoor laboratory experiment findings to raceway operations to prevent potential occurrence of nutrient stress. The experiment results have shown that using a cheaper nitrogen source or lowering phosphorus input did not significantly affect biomass productivities of *C. sorokiniana* and *S. obliquus*.

### **Recycling media using anaerobic digestion**

One of the potential issues associated with algae as a feedstock is the large requirements for nitrogen, phosphorous and other nutrients. Therefore, it is beneficial to evaluate potential methods for recycling these nutrients. One method that has been studied is anaerobic digestion of lipid extracted algae (LEA). This anaerobic bacteria digest the LEA and release the nutrients to the aqueous phase. The digestate can then be used as a source of water and nutrients to cultivate algae. The team investigated the process of algae cultivation-extraction-digestion-recultivation using *C. sorokiniana*. The initial study focused on repeating the entire process multiple times to determine if the nutrients indeed could be recycled repeatedly. It also evaluated the use of supplementing the recycled digestate with secondary wastewater. The follow-up study focused on how the initial nitrogen concentration impacts each stage

of an integrated cultivation-extraction-digestion-cultivation process and how the nitrogen is distributed in each step. The team completed a thorough nitrogen mass balance for every step of the process.

The following publications resulted from the work.

Zhang, B., and K.L. Ogden. 2017. Recycled wastewater from anaerobic digestion of lipid extracted algae as a source of nutrients. *Fuel* 210:705-712. <https://doi.org/10.1016/j.fuel.2017.09.026>

Zhang, B and K. L Ogden “Nitrogen Balances and Impacts on the Algae Cultivation-Extraction-Digestion-Cultivation Process” *Algal Research*, under review

### **Biomass Production**

The UA testbed supplied algal biomass to several research groups. Multiple batches of harvested *C. sorokiniana* were sent to PNNL for processing in HTL. In addition, researchers at the University of Nevada, Reno, and Utah State University received 3 to 5 kg of wet biomass (*C. sorokiniana* or *S. obliquus*) to do conversion experiments. Finally, the team conducted an experiment in collaboration with Idaho National Laboratory (INL) to explore methods for preserving algal biomass. The team harvested *S. obliquus* biomass via centrifugation and sent it overnight to INL.

The work resulted in the following publication:

Wendt, L.M., B.D. Wahlen, C. Li, G. Kachurin, K.L. Ogden, and J. Murphy. 2017. Evaluation of a high-moisture stabilization strategy for harvested microalgae blended with herbaceous biomass: Part I – storage performance. *Algal Research* 25(7):567-575. <https://doi.org/10.1016/j.algal.2017.05.016>

## **NMSU Testbed Results**

During the RAFT project, five different algal species were grown under outdoor cultivation conditions. The species and culture identifier designations are listed below. The team conducted 101 cultivation experimental runs over an approximately three-year period, from May 2014 until September 2017. Each run typically comprised three to four raceways operated in parallel. The team operated the raceways nearly continuously over this period and

collected at least three seasonal replicates for algae grown in spring, summer, fall and winter conditions. All the outdoor algal cultivation was done at the NMSU outdoor testbed located at the Fabian Garcia Experimental Farm in Las Cruces, NM (32.279N latitude, 106.771W longitude).

### Algal Species Tested at NMSU

1. *Scenedesmus obliquus*
2. *Chlorella sorokiniana*
3. *Monoraphidium sp*
4. Native polyculture: This polyculture was derived from whatever local algal species could become established in a 3 m<sup>2</sup> outdoor basin and exposed to outdoor conditions for approximately nine months. The basin was originally started by placing the contents from a “crashed” raceway culture of *C. sorokiniana* DOE141 into the basin and providing minimal mixing with a submerged pump.
5. *Galdieria sulphuraria*

With the exception of *G. sulphuraria*, all of the cultures were grown in open 3 m<sup>2</sup> raceways. The raceways were mixed with a constant-speed, four-bladed paddlewheel. The cultures were maintained at a depth of either 10 or 20 cm. At a depth of 20 cm, the total raceway volume was approximately 600 liters.

The team grew *G. sulphuraria* cultures in enclosed photobioreactors constructed from polyethylene tubing

and mixed with an internal paddlewheel. The researchers were testing the culture for its feasibility to treat primary municipal wastewater. The initial design used an enclosed PBR design that both contained the wastewater to minimize biological contamination and maintained a higher growth temperature for the algae. The volume of these PBRs was typically around 600 liters.

Data collected from the cultures on a regular basis included dissolved oxygen levels, temperature, ambient PAR light levels, carbon dioxide usage and pH. These variables were typically recorded at a 15-minute resolution, with carbon dioxide usage measured at a 10-second resolution. The team determined algal growth by measuring culture density via optical density on a daily basis, with ash-free dry weight (AFDW) determinations measured on a weekly basis. Additional variables recorded at various intervals included cell number, size and fluorescence measured via flow cytometry and photosynthetic ability with pulse amplitude modulated fluorescence (PAM).

### Summarized Experimental Results

A summary of the experimental runs is found in Table 3. The average duration of a raceway run was 20.2 days with a range of 3 to 64 days. Over this period, the overall linear productivity averaged 10.7 g/m<sup>2</sup>-d and the maximum linear productivity was 35.2 g/m<sup>2</sup>-d. The average productivity over this period was 5.3 g/m<sup>2</sup>-d, with a maximum average productivity of 15.6 g/m<sup>2</sup>-d.

**Table 3.** NMSU Experimental Summary

Strain	Number of Runs	Longest run (days)	Range of linear productivity g/m <sup>2</sup> /day
<i>Chlorella sorokiniana</i>	43	41	2.9-16.2
<i>Monoraphidium minutum</i>	19	58	0.9-10.1
<i>Scenedesmus obliquus</i>	15	58	4.4-28.2
Native Polyculture	24	N/A	5.2-35.2

*Chlorella sorkiniana*: While this strain had the potential for rapid growth and high rates of productivity, it also was very susceptible to rapid crashes. Even when applying active pest control measures, it was difficult to maintain long-term cultures. It is not clear what was causing the culture crashes. While *V. chlorellovorus* was present in some of the crashed cultures, it could not be determined if this was the causal agent behind them. Because of these uncontrollable culture crashes, this strain was not a good candidate for use in outdoor cultivation in Las Cruces, NM.

*Monoraphidium sp.*: *Monoraphidium* was grown to evaluate its potential as a winter strain. It grew reasonably well during winter 2015 into the beginning of 2016 but did not grow well after this point. The reasons for this subsequent poor growth are not clear.

*Scenedesmus obliquus*: This was the most robust of the three RAFT production strains used and grew well during warmer periods. The average run time was around three weeks, with most growth experiments running from the spring through the summer. This species grew well in the summer, with linear productivities of more than 28 g/m<sup>2</sup>/day.

Native Polyculture: The simplest algal cultivation method is to use locally derived algal species that can become established on their own. To explore the viability of this approach, the team filled a 3 m<sup>2</sup> outdoor basin with RAFT Pe-08 medium and exposed it to outdoor conditions for approximately nine months. This culture was then used to start an open raceway, which maintained itself in continuous culture for more than 18 months. Over 24 runs, the overall linear productivity of the polyculture averaged 18.3 g/m<sup>2</sup>-d and the maximum linear productivity was 35.2 g/m<sup>2</sup>-d. The average productivity over this period was 6.1 g/m<sup>2</sup>-d with a maximum average productivity of 13.6 g/m<sup>2</sup>-d. Overall, the productivity of the native polyculture was typically greater than any of the other algal species used. Another major advantage of the polyculture was that it did not have any major culture crashes that required the culture to be replaced.

## Crop Protection

It has become increasingly apparent that maintaining long-term levels of high algal productivity under outdoor cultivation conditions is a challenging task. Understanding

the factors responsible for crashes—the total loss of a culture—and more importantly, understanding how to prevent or mitigate them is critical to the economic cultivation of algae on a large scale. Part of the methods employed in this study to minimize culture crashes involved using pesticides to control algae predators or competitors. The team took a three-part approach to identify methods required for algal crop protection.

First, the team used an active crop protection protocol and applied a variety of pesticides to the outdoor algal cultures. The pesticides used were benzalkonium chloride, chlorothalonil, fluazinam and ammonium sulfate. These pesticides should represent the current state-of-art use of chemicals to control algae predators. The second approach was to use a regular schedule of pesticide treatments for crop protection, and the third approach involved growing a native polyculture that was maintained without the use of pesticide treatment. Native strains already should be adapted to the field environment and therefore should be more likely to thrive in local conditions and in the presence of local pests. The use of such healthy local algal species could preclude or limit problems caused by predators or competitors and would be the simplest way to maintain long-term algal cultures.

Starting in summer 2015 and continuing through summer 2016, the team tested these different approaches to algal crop protection. The focus over this period was determining what crop protection methods were most effective under outdoor cultivation conditions. The bottom line is that none of the chemical treatment methods were very effective in preventing algal culture crashes under outdoor growth conditions. The use of a native polyculture, however, could potentially minimize such crashes.

## Use of Native Algae Polyculture

The team evaluated the performance of a native polyculture under long-term outdoor cultivation.. This polyculture was started in May 2015, was transferred to a raceway on April 2016, and has been maintained in continuous culture to date. Thus far, this polyculture has continued to grow well without interruption for more than 18 months. No pesticides have been applied and the raceway has been “mock” harvested on thirteen occasions (total harvested volume of 5,880 liters). During this period, the team has noted three distinct species transitions

and has collected samples for subsequent genetic analysis. In contrast, similar experiments performed with *C. sorokiniana* (April to October 2016 and *S. obliquus* (April 2016 to present) have exhibited repeated culture interruption (*C. sorokiniana*) or demonstrated slower growth (*S. obliquus*, 3,350-liter total harvest volume) relative to the native polyculture. This experiment has demonstrated that, under these conditions, native polyculture is capable of outperforming monocultures over time and through multiple seasonal transitions.

### Correlation between Algae Photosynthetic Rate, Growth and Light Intensity

A critical component for the large-scale cultivation of algae is the development of growth models that can accurately predict the growth of different algal species under varying environmental conditions. Such models would allow the identification of factors that are critical in maximizing algal biomass production. Based on numerous outdoor cultivation trials, the team notes that algal growth is often difficult to predict, and that the factor or factors responsible for large differences in daily growth rates appear elusive.

To further examine what factors could be responsible for variation in algal growth, the team closely monitored growth, light intensity levels and oxygen flux rates in outdoor algal cultures. Oxygen production rates are closely linked to photosynthetic rates. A basic premise of the use of algae to produce biofuels is that photosynthetic processes can be used to produce biomass that can then be converted to desired products.

A logical conclusion would be that photosynthetic rates should be correlated with growth rates, but this relationship has seldom been rigorously examined under outdoor growth conditions. To determine the relationship between photosynthetic rates and biomass production in outdoor algal cultures, the team closely monitored both variables in cultures of *Monoraphidium* and the native polyculture over two ten-day time periods. The main results may be summarized briefly this way: When grown under outdoor cultivation conditions, almost no correlation exists between ambient light levels and photosynthetic activity. While this result initially appears counterintuitive, it is based on the fact that outdoor light intensities

typically far exceed the amount of light that algae can use photosynthetically. For this reason, most of the photons impinging upon algae near the culture surface cannot be utilized in photosynthesis; hence, higher light levels do not correspond to a higher photosynthetic rate.

A reasonable correlation existed between the overall photosynthetic rate of the algae and the daily biomass production of the culture. Variation in oxygen production rates correlated with around 40% of the change in daily biomass production. As expected, this correlation is a positive one — higher rates of oxygen production are correlated with higher rates of subsequent growth. This indicates that continuous measurement of oxygen production rates in algal cultures can provide a simple and relatively inexpensive way of monitoring algal culture health and productivity.

### Growth of Mixed Cultures

An important variable in the growth of algae under outdoor conditions is understanding when to adjust the algal species grown to match seasonal changes in light and temperature. The team tested whether two strains of green algae—*S. obliquus* DOE0152z and *Monoraphidium* sp.— would undergo a transition from predominantly one strain to the other under cold weather conditions. Based on previous experience, *S. obliquus* should perform well under moderate to cool temperatures but should be outperformed by *Monoraphidium* under cold weather conditions. To test this, raceways were inoculated with different starting ratios of the two species of algae.

Along with the biomass production analysis, the team wanted to determine if the relative species composition of the raceways changed over the course of the experiment. The prediction would be that the higher temperature growth preference of *S. obliquus* DOE0152z would cause an increase in the relative abundance of this algae early in the experiment. The team would then predict that *Monoraphidium* would increase in relative abundance during the lower temperatures seen later in the year. Despite these predictions, the data clearly indicate that no such transition was observed. Both Flowcam data and direct microscopic observation showed no significant change in the ratios of each strain in all three raceways over the course of the experimental run. This

demonstrates that, under these conditions, both strains remain productive individually and can thrive together as polyculture.

Cultures that were started with three different ratios of *S. obliquus*/*Monoraphidium* grew well throughout the experimental run, especially considering the low ambient temperatures and light levels. The average daily productivity over the entire 45-day period averaged between 2.7 and 3.8 g/m<sup>2</sup>-d. If only the 32-day period of consistent growth is included in the analysis, the average daily productivity averaged between 3.6 and 5.5 g/m<sup>2</sup>-d. A clear advantage was not apparent in biomass production or growth rates when the algal cultures were grown in a 50-50 growth ratio compared to when either one of the two cultures was the prominent species (i.e., there was no clear indication that biological interactions between these two species were inhibiting or enhancing algal growth).

### Cultivation of *Galdieria sulphuraria* in Municipal Wastewater

Results of this study, demonstrated at the laboratory and field studies, showed that *G. sulphuraria* can be cultivated in primary effluent of urban wastewater. During its growth, *G. sulphuraria* can remove dissolved organic carbon and nutrients to the mandated discharge standards with significantly lower energy input than the current standard wastewater treatment processes. The robust growth of *G. sulphuraria* in untreated wastewater indicates the potential of using this algae to treat wastewater in an energy-positive and cost-effective manner. This part of the project was primarily funded by the National Science Foundation.

### Biomass Production

An important variable for maximizing the efficiency and economics of using *G. sulphuraria* for wastewater treatment is determining whether *G. sulphuraria* biomass can be converted to liquid biofuels. To determine this, the team harvested around 20 kg of *G. sulphuraria* biomass and sent it to PNNL to be processed by hydrothermal liquefaction. The HTL conversion of the *G. sulphuraria* biomass resulted in high conversion yields to biocrude (approximately 37% on a weight basis), indicating that *G. sulphuraria* is good algal feedstock for fuels production.

Several publications resulted from this work.

### Citations

Henkanatte-Gedera, S.M., T. Selvaratnam, M. Karbakhshravari, M. Myint, and N. Nirmalakhandan. 2017. *Algal Research*, 24, 450-456.

Selvaratnam, T., A.K. Pegallapati, H. Reddy, N. Kanapathipillai, N. Nirmalakhandan, S. Deng, and P.J. Lammers. 2015. Algal biofuels from urban wastewaters: Maximizing biomass yield using nutrients recycled from hydrothermal processing of biomass. *Bioresource Technology* 182:232–238.

Henkanatte-Gedera, S.M., T. Selvaratnam, N. Caskan, N. Nirmalakhandan, W. Van Voorhies, and P.J. Lammers. 2015. Algal-based, single-step treatment of urban wastewaters. *Bioresource Technology* 189:273–278.

### AgriLife Testbed Results

The Texas A&M AgriLife Research facility in Pecos, TX (N 31.42291, W 103.49323), was conducted from August 2014 to the end of April 2017. The experimental goals for the research station included collecting long-term growth data, providing the data to help modelers refine their models, evaluating growth in three different types of water and implementing best growing practices. These best growing practices include the time of year a species should be grown, with special interest in months that are close to a seasonal switch, comparative growth of algae in impaired and freshwaters and optimization of nutrient addition and harvest schedule to minimize lag period.

The team equipped all raceways, ranging from 170-liter test systems at 4 inches in depth to 10,000-liter production systems at 8 inches in depth, with sensors to measure temperature, conductivity and pH in real time at 10-minute intervals. Carbon dioxide was bubbled into each of the raceways by way of a pH sensor connected to a solenoid. The pH was set at a high range of 8.40 and a low range of 8.30 for the vast majority of experiments. This experimental time frame encompassed seven growing seasons to fine tune when each of the three species are utilized throughout the year. In the final years of the project, the goal was to run the recommended algae



species for a given season at two different reactor scales – 170 liters and 10,000 liters – continuously to collect growth data and minimize raceway downtime.

### Algal Species Tested at AgriLife Research

1. *Chlorella sorokiniana*
2. *Monoraphidium minutum*
3. *Scenedesmus obliquus*

### Summarized Experimental Results

A summary of the experimental runs is found in Table 4. The team selected and evaluated species at AgriLife Research with varying media recipes established at the site and shared these recipes with other researchers in the program. The media was developed to optimize nutrient usage in a continuous cultivation scenario while also lowering cost without impacting productivity.

**Table 4.** AgriLife Research Experimental Summary

Strain	Number of Runs	Longest run (days)	Range of linear productivity g/m <sup>2</sup> /day
<i>Chlorella sorokiniana</i>	20	34	2-20
<i>Monoraphidium minutum</i>	28	65	4-13
<i>Scenedesmus obliquus</i>	25	71	2-14

#### Long-term cultivation trials

*Chlorella sorokiniana* overview: Based on strain characterization, the optimal time frame found for cultivating *Chlorella sorokiniana* was in June through August. It was observed that even though the summer months should provide the optimal growing temperatures for this species, that time frame also resulted in a high incidence of predatory contaminants. This species seemed to be more stable in spring and fall, as the cultures seemed more stable once the average pond temperature decreased below 24 °C. Culture longevity was short due to the presence of *V. chlorellavorus* in the culture, which killed cultures within 10 to 14 days. This led to a couple of experiments to try to improve culture longevity by manipulating pH (e.g., adding sodium bicarbonate to increase the pH). These experiments neither helped nor hindered the longevity of the culture when compared to the controls without treatment.

*Monoraphidium minutum* overview: *Monoraphidium minutum* was used for cultivation during the winter months at the Pecos site. During that time, the species was cultivated in both fresh and impaired waters with less than

5% variance in growth rate. The species was cultivated at an average temperature of 10 °C, where it exhibited a productivity of 6.72 g/m<sup>2</sup>/d average across three raceways over a cultivation period of 71 days during January to April. The species was subjected to periodic freezing temperatures and recovered each time. Similar growth rates were seen in cultivation runs in November, exhibiting productivity of 6 g/m<sup>2</sup>/d at an average temperature of 12 °C. Those runs carried into December and lasted approximately 42 days.

The goals for *Monoraphidium minutum* were to sustain long-term growth, compare productivities in impaired and freshwater and test nutrient additions and harvests based on ash-free dry weights targets. Multiple experiments lasted between 14 and 30 days. From a stability standpoint, this species was the most successful, based on the longer cultivation run times. This is primarily a winter species, but the team ran tests to see how far into spring/early summer the species could handle. During the spring, fall and winter, *Monoraphidium minutum* productivity ranged between 7.8 to 9.3, 4 to 14, and 2.3 to 8.8 g/m<sup>2</sup>/day, respectively. The team also ran side-by-

side experiments in late March/early April (average high temperature of 26 °C and average low of 6 °C) comparing the growth of *S. obliquus* to *Monoraphidium minutum* to determine when one species should be switched to the other. With the weather conditions at that time, *Monoraphidium minutum* outperformed *S. obliquus* by almost double in terms of productivity g/m<sup>2</sup>/day. Along with *S. obliquus*, this species also had an ash-free dry weight trigger for the addition of nutrients and harvesting between 0.6 and 0.8 g/L. Multiple experiments showed that a day or two after reaching 0.8 g/L, the culture density would plateau then decrease. After harvest, there was very little, if any, lag time prior to additional growth. These extensive experiments showed that *Monoraphidium minutum* could be run from October to April. *Monoraphidium minutum* in general proved to be a very consistent performing species in the winter at the Pecos site, with the colder temperatures helping to prevent large-scale contamination events and providing long runs of continuous data collection.

*Secnedesmus obliquus* overview: *Secnedesmus obliquus* was grown during the winter and spring. It showed a better productivity (12 g/m<sup>2</sup>/d) during the spring and a lower productivity (4 g/m<sup>2</sup>/d) during the winter. The species was a consistent cultivar, with runs during the winter of up to 65 days. Over time, the species maintained a productivity of 5-7 g/m<sup>2</sup>/d at an average culture temperature of 10 °C. The majority of the experiments with *Secnedesmus obliquus* lasted between 10 and 20 days. The experiments were conducted during the spring and fall, with a couple of experiments run during the summer and winter to examine what the productivities were compared to those of the optimal species (*Chlorella sorokiniana* in summer and *Monoraphidium minutum* in winter). The culture died rather quickly in the summer when compared to *Chlorella sorokiniana*, but productivities ranged from 3.9 to 6.7 g/m<sup>2</sup>/day when grown in November and December. During spring and fall, which is the optimal time to grow *Secnedesmus obliquus*, productivities were higher and ranged from 4.5 and 13.1 g/m<sup>2</sup>/day. Another goal was to use ash-free dry weights to trigger harvests and nutrient additions to minimize lag period. Based on the various experiments, the team added media and harvested within a range of 0.6 and 0.8 g/L. This helped reduce the lag phase usually associated with starting cultures.

### ***Alternate water source testing for potential cultivation***

The analysis of compromised waters and wastewater sources was completed using brackish water from two different wells at the Pecos site and wastewater from a local wastewater treatment facility. Nutrient profiles for each water source were determined to see if it would be possible to reduce the amount of nutrients needed to input into the current algal production system model.

The team continuously grew cultures in impaired water at the Pecos site using the 175-liter mini-pond systems and alternating between the two selected wells. It was shown that these waters had varying degrees of baseline nitrogen, calcium and general salinity, providing potential advantages and disadvantages for cultivating algae. Free nitrogen sources allowed for the decrease in the required “purchased” nitrogen, but calcium can cause media formulation problems if not properly dealt with, and brackish water, when combined with high evaporative rates, can increase salinity over time. Oilfield infiltration also proved problematic, as it can introduce foreign and sometimes unexpected chemicals into the site water supply.

The team analyzed wastewater from the Pelican Island Wastewater Plant at Texas A&M Galveston, testing it using *Chlorella sorokiniana* in small-scale assays in the lab. Those tests showed that the *Chlorella sorokiniana* could reach comparable growth rates of cultures grown in a lab-mixed replete media, without the addition of any nutrients at all. Researchers analyzed the water before and after each of the small-scale assays and observed that the culture had removed approximately 97% of the initial nitrogen and phosphorus levels within the wastewater.

The team conducted the vast majority of experiments in freshwater with media specific to the algae species but performed a small subset of experiments with different types of water to determine the feasibility of utilizing non-freshwater assets. Using non-freshwater sources for growing algae is vital to reducing the freshwater impact on algae cultivation. The team conducted side-by-side experiments to compare the productivity of *Scenedesmus* sp. when it is grown in freshwater, on-site well water with a salinity of 1.5 g/L NaCl and wastewater obtained from



the local wastewater treatment lagoon. Prior to inoculating the cultures outside, the seed culture in the lab was dosed with 1.5 g/L NaCl and scaled that way until inoculation to simulate the salinity of the well water. The goal was to acclimate the cultures prior to beginning the cultivation trials. Wastewater was not used in scale up and only used outdoors. The wastewater was filtered through a 1-micron filter sock before it was pumped into the raceways and no nutrients were added, unlike the freshwater and well water, which had the regular nutrients used throughout all the experiments added. The productivities for the experimental time period were 8.15, 11.62 and 9.05 g/m<sup>2</sup>/day for freshwater, well water, and wastewater, respectively. The team did not do nutrient profiles on well water or wastewater, but both non-freshwater assets performed similarly to, if not better than, freshwater. Further testing over multiple experiments should be explored, but the site well water and filtered wastewater could be a viable alternative to freshwater. The team completed additional experiments to compare only freshwater and well water. Productivities for growth ranged from 7.17 to 15.4 g/m<sup>2</sup>/day in well water and from 11.3 and 12.87 g/m<sup>2</sup>/day in freshwater. Again, the productivities were comparable.

The team also grew *Monoraphidium minutum* in wastewater, well water, and freshwater to compare productivities. The researchers cultivated the algae in the middle of November with average productivities of 6.7, 7.5 and 6.8 g/m<sup>2</sup>/day for freshwater, well water and wastewater, respectively. Productivity in wastewater and freshwater were identical, with a slightly better observed productivity in well water. This shows that *Monoraphidium minutum* could be grown in impaired waters and still have comparable growth rates. In another experiment with *Monoraphidium minutum* grown in wastewater, the researchers added no nutrients from the end of November to the middle of December. The team observed an average growth rate (based on triplicate reactors) of 2.7 g/m<sup>2</sup>/day, while the control culture using freshwater during the same time period averaged 2.3 g/m<sup>2</sup>/day.

### **Population dynamics and control strategies**

Contamination of cultures by a variety of ciliates, bacterial species and rotifers continues to be a problem that must be addressed. Hence, culture maintenance strategies became an important focus of the project and additives such as bleach and bicarbonate proved to be the most helpful. In many cases, properly calibrated bleach additions made to cultures with an unacceptable degree of ciliate contamination showed encouraging results in knocking back the contaminate while not severely hindering the growth of the target algal species. Bicarbonate was more problematic to assess, as effective treatments tended to take the target species above its desired salinity level. Also, an increase in alkalinity was shown to decrease the target species growth rate but, in many cases, completely knocked back the contaminate. It was shown that salinity ranges above 20 ppt can substantially reduce the onset of the *V. chlorellavorus* contaminant and increase the longevity of the culture if the target species is properly adapted to higher salinities.

To further monitor predators, the team used a Fluid Imaging FlowCAM to generate a method for rapidly screening collected samples. The researchers created a library of predators using the provided software from Fluid Imaging to assist the equipment in identifying other algal species within the cultivation medium. Through a variety of tests using *Chlorella sorokiniana*, the FlowCAM monitored the effectiveness of various culture maintenance techniques.

### **Biomass Production**

One of the additional goals of the RAFT program was to supply biomass to entities that requested it. The team collected algal biomass from each of the tested runs during dilution events or at the end of experimental runs and stored it for use in downstream assessment. The primary method for harvesting during the first two years was chemical flocculation, because the volume of the runs was too low to use the on-site centrifuge. To this end, 5-gallon buckets of *Monoraphidium minutum* and *Secnedesmus obliquus* biomass were sent to PNNL for HTL experiments.

# MOLECULAR DIAGNOSTICS

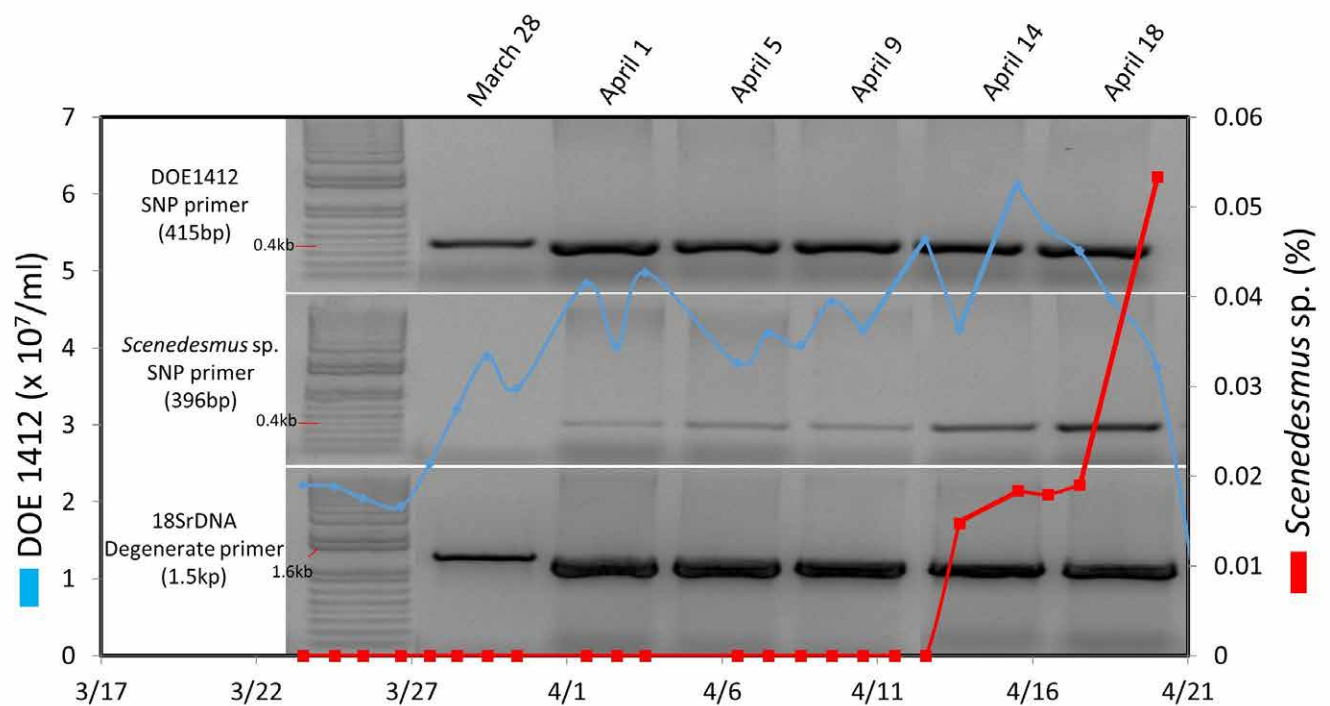
The community of microorganisms living in close association with algae cells, or phycosphere, results in a dynamic network of microscale interactions, which impinge on the macroscale accumulation of desired species and lipid products. The successful production of microalgae in the mass outdoor culture operations conducted during the RAFT project depended on accurate and expedient identification of target algal strains and associated microbes. The team augmented traditional microscopy throughout project processes with molecular diagnostic techniques, alleviating morphological ambiguity and unculturable microbes.

## 18S Methods for Identification and Quality Control Cultivation Strains

The RAFT project aimed to produce high-quality biomass of well-characterized microalgae strains, selected for their particular qualities amenable to growth in the southwestern United States. The identity of cells in culture had to be verified at each process step, including newly received strains and laboratory stocks as well as in the open pond reactors at the field site. Culture contamination by non-target microalgae species can occur in laboratory environments despite sterile transfer techniques and by invasive native species in the field. Molecular assays were developed to identify both unknown microalgae contaminants and specific project strains based on the sequences of their 18S small subunit ribosomal RNA genes (18S rDNA).

Using 18S rDNA as a barcode for species identification relies on the conservation of its sequence among organisms. Probes matching different regions of known 18S rDNA sequences were carefully designed for polymerase chain reaction (PCR) amplification of either the full-length gene with degenerate primers or particular species with specifically targeted primers. The researchers used specific primer sets to identify RAFT target algae by direct observation of variously sized PCR products, while they identified full-length products by subsequent DNA sequence analysis. Full details on the development and early implementation of these tools are contained in an in-progress publication.

Systematic implementation of these tools revealed that a native species of *S. obliquus* regularly overtook early RAFT outdoor cultures of the *C. sorokiniana* isolate, as determined by analysis of its 18S rDNA sequence. The team then designed and utilized specific primers targeting the invasive species to detect its presence prior to reaching observable concentration (Figure 12). The researchers routinely used specific primers to verify culture samples from across the RAFT project, while degenerate primers proved useful for corroborating the previously determined identity of newly obtained strains and even indicating a variety of fungi at the field site. A cold-tolerant isolate brought in for winter growth originally identified as *Kirchneriella cornuta* by morphology was re-designated to the closely related *M. minutum* based on high (97%-99%) sequence similarity (NCBI accession AY846380).



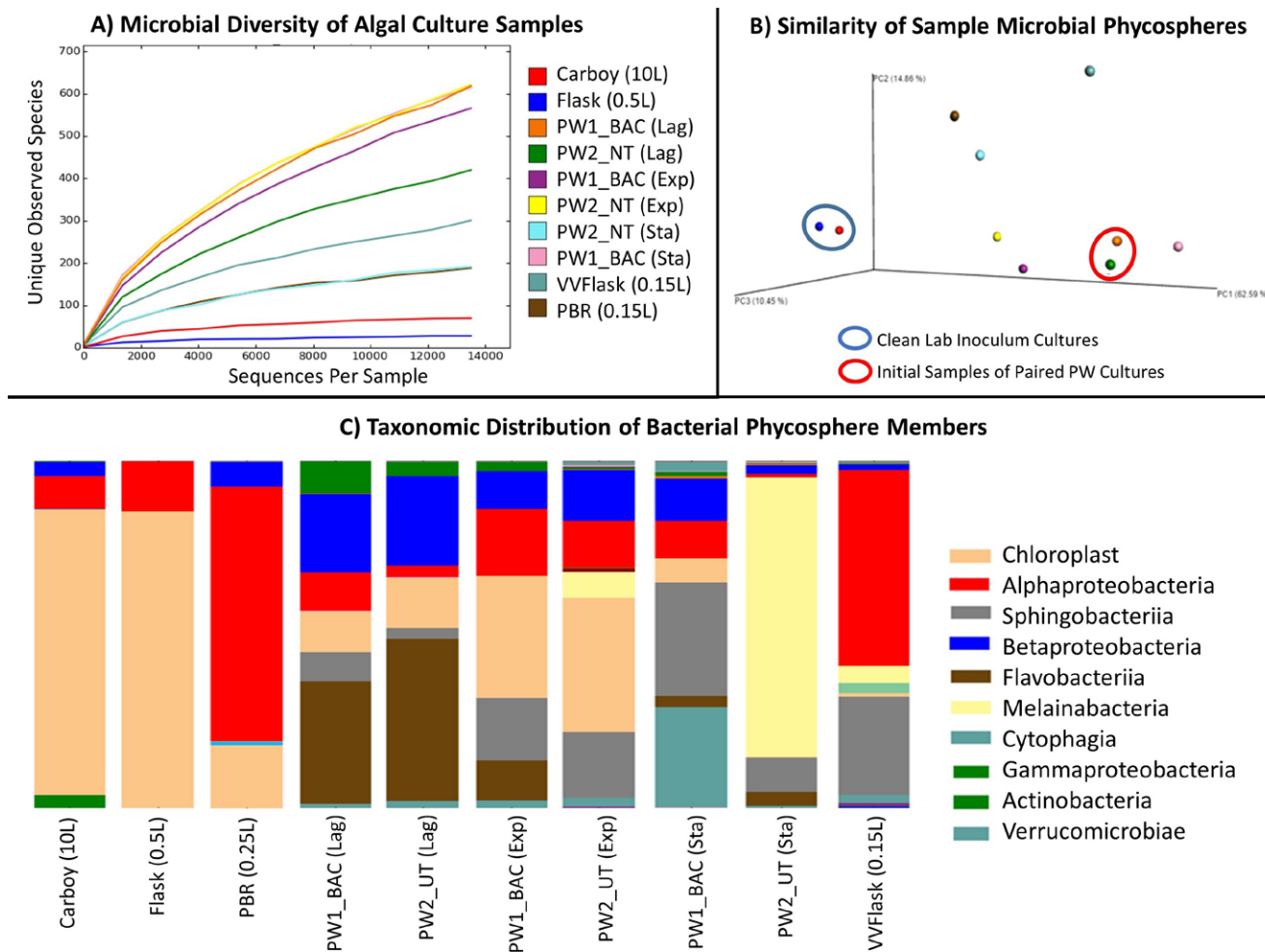
**Figure 12.** PCR analyses with *C. Sorokiniana* and *Scenedesmus* specific primers overlaid with *C. Sorokiniana* cell count and percent of *Scenedesmus* cells calculated from counts.

## 16S Methods for Identification and Quality Control of Algae Associated Bacterial Communities

In natural environments, bacterial cells are commonly found in greater abundance than their microalgal counterparts. Algal health within a given phycosphere has been shown to respond closely to different bacteria acting in mutualistic, parasitic and even symbiotic fashions (Cole, 1982). Several molecular diagnostic tools were developed to profile changing bacterial constituents of RAFT microalgae cultures based primarily on sequencing analysis of 16S small subunit ribosomal RNA genes (16S rDNA). The team used data resulting from these tools to

identify broad trends in microbial ecology and discover more specific problematic microbes.

Initially, the project employed Sanger sequencing methods to perform low throughput analysis of high-quality and full-length 16S rDNA. This allowed for confident assessment of the most prominent bacterial species and led to development of PCR-based assays to detect specific bacterial species of interest. Sanger sequencing-based methods were not capable of profiling complete bacterial communities and were augmented with a next-generation high-throughput sequencing approach. Using Illumina technology allowed for assessment of approximately 50,000 individual sequences for each culture sample, which revealed that project algal cultures contained as many as 600 unique species of bacteria (Figure 13).



**Figure 13.** Results of 16S community profiling data analysis on *C. sorokiniana* cultures grown in laboratory (Carboy/Flask) or paired outdoor paddlewheel units treated with benzalkonium chloride (PW1) or untreated (PW2) collected during lag, exponential and stationary growth phases. The data were assessed for A) sample diversity by rarefaction of unique observed OTUs (putative bacterial species); B) similarity of bacterial community composition by principal component analysis of weighted UniFrac distance; and C) taxonomic predictions displayed here at class-level resolution.

## Pathogen monitoring and control of *Vampirovibrio chlorellavorus*

16S rDNA profiling experiments described above led to the identification of pathogenic bacterium *V. chlorellavorus* that was consistently associated with declining *C. sorokiniana*, outdoor cultures. Previous studies indicated that the pathogen was capable of lysing cells of several *Chlorella sp.* (Coder and Goff, 1986) and observation during the RAFT project found the same for *C. Sorokiniana*. *V. chlorellavorus* cells reach only 0.6  $\mu\text{m}$  diameter, making them indistinguishable from other small

bacteria upon microscopic observation and necessitating development of another method for quantitation.

Quantitative PCR (qPCR) was chosen not only for its capability of highly accurate quantification of DNA targets, but also because it offers a more specific assay than the previous PCR techniques. The team designed a multiplexed assay to target both the pathogen and host DNA for normalization of the resulting data, and after optimization, detected minute concentrations of the organism (Table 5). The qPCR assay was used throughout lab and field experiments to accurately monitor the progression of disease.

**Table 5.** Statistical limits of qPCR assays

Target	Limit of Detection <sup>a</sup>	Limit of Blank <sup>a</sup>	Limit of Quantification <sup>a</sup>
<i>V. chlorellavorus</i>	12.67	9.87	18.67
<i>C. sorokiniana</i>	97.68	56.34	130.56

<sup>a</sup>All limit calculations are reported in units of target DNA copy number.

### Environmental Effects

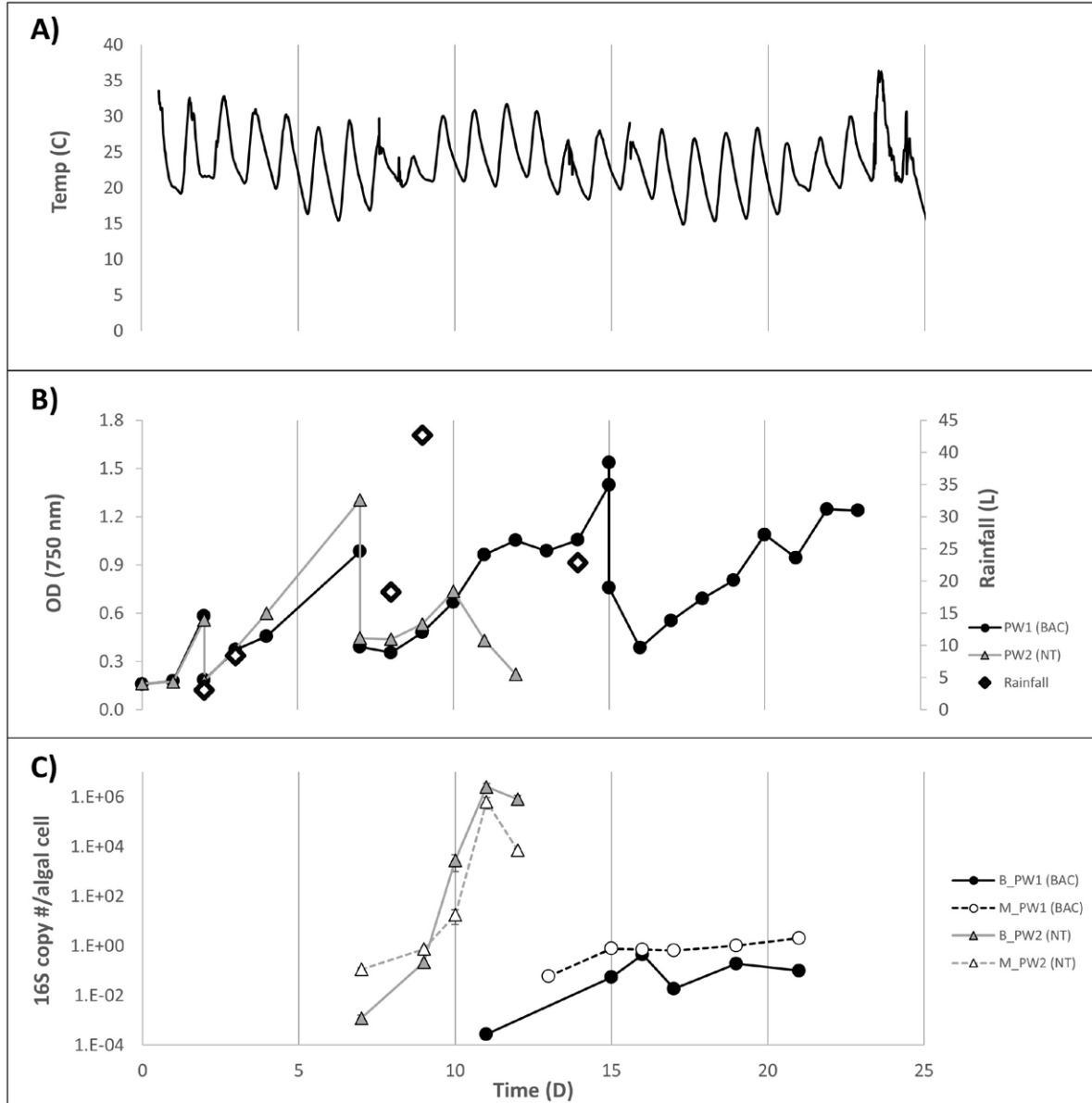
Experiments were undertaken to elucidate environmental effects on the progression of *V. chlorellavorus* through its pathogenic life cycle to provide a basis for predicting conditions for algal culture infection. These laboratory studies found that warmer temperatures, around 35 °C, induced more rapid and substantial *C. sorokiniana* cell death in the presence of *V. chlorellavorus*. The symptoms of pathogenesis were also observed to be most prominent in media held at neutral pH (Li, 2015).

### Chemical Treatment

Regular use of the detection methods described above revealed that *V. chlorellavorus* was present in nearly all *C. Sorokiniana* outdoor cultures. Quantitative monitoring indicated that the pathogen consistently accumulated in cultures with dying algae cells, particularly during the late spring and in the summer, when temperatures are

high. The team tested several methods for control of the bacterial pathogen prior to selecting the general biocide compound, benzalkonium chloride, for field-mitigation studies.

Experiments in both the laboratory and field informed the selection of a strategy for BAC at minimal effective concentrations that inhibited *V. chlorellavorus* activity while minimizing the direct effect on the cell wall to protect *C. sorokiniana*. Researchers at the RAFT field site evaluated the efficacy of treatments with the biocide at 1.5 ppm every fourth day and compared those results to algal growth in analogous, untreated control reactors. The treated *C. sorokiniana* cultures exhibited positive growth rates, over an average of 22 days, whereas the untreated cultures declined after only 12 days on average. The *V. chlorellavorus* qPCR assay confirmed the significantly higher number of cells of the pathogenic bacterium in the untreated cultures throughout growth cycles (Figure 14).



**Figure 14.** Benzalkonium chloride (BAC) effects on *C. Sorokiniana* growth and *V. chlorellavorus* accumulation in field reactors. The panels summarize data from an algal growth cycle between Aug. 30 and Sept. 22, 2016. A) Culture temperature is displayed as recorded by continuous data logger from PW1 reactor unit and compared across a time course of 25 days of growth against B) the optical density (absorbance at 750 nm) of *C. sorokiniana* culture in collocated PW reactor units either treated with BAC or a no treatment control (NT) as well as rainfall events quantified by the volume of water added to reactors. C) *V. chlorellavorus* accumulation is displayed in both biomass (B) and media (M) fractions by the ratio of 16S rDNA per algal cell 18S rDNA detected on a logarithmic scale, which excludes initial time point samples of undetected 16S rDNA.



Several publications, below, resulted from this work.

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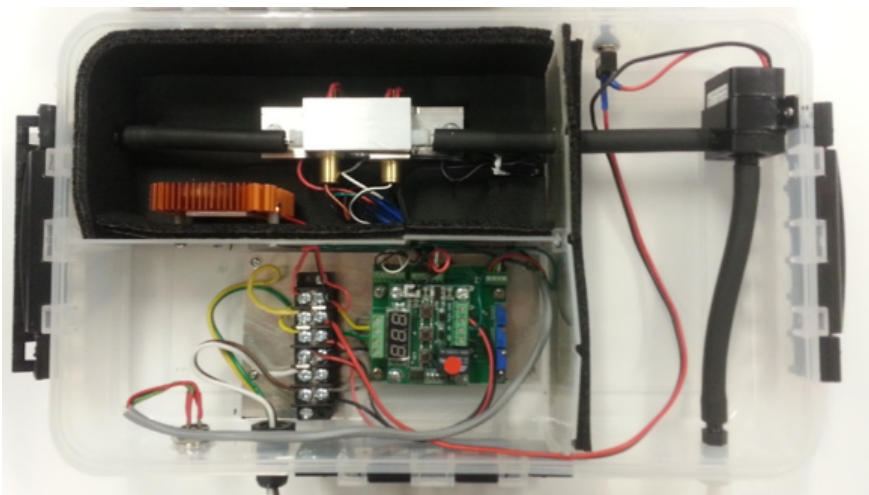
# CONTINUOUS MONITORING AND CONTROL

Optimizing resource inputs and maintaining high productivity are the key components of cost-effective algae production. Real-time monitoring and control provides the platform to acquire the environmental and physiological dynamics of a microalgae culture system. For large-scale microalgae production systems, effective decision making and overall production system management in terms of optimal resource use, harvesting and culture condition optimization (media composition, lighting, temperature, pH, dissolved oxygen levels, etc.) are crucial to achieving maximum profit and preventing or reducing economic losses in case of contamination.

Measurements of biological variables, including cell mass concentration, cell size, cell morphology, population composition (i.e., concerns with contamination), pigments and lipid content, are especially desirable because they directly indicate the dynamics of a microalgae culture system. Some

commercialized sensors monitor microalgae concentration, but most of them are designed to monitor microalgae concentration at an environmental level that is much lower than the cell concentration in microalgae production applications. Furthermore, these sensors are too expensive for low added value product applications. Therefore, they are not practical to integrate into outdoor raceways or photobioreactor-based algae production systems.

The team's objective was aimed at developing and implementing an optical sensor capable of measuring multiple biological parameters in real time both in an indoor PBR and outdoor raceway system at high cell concentrations without needing sample preparation (i.e., dilution, washing, filtration) prior to measurements (Figure 15).



**Figure 15.** Schematics of the components layout of the inline optical density sensor.

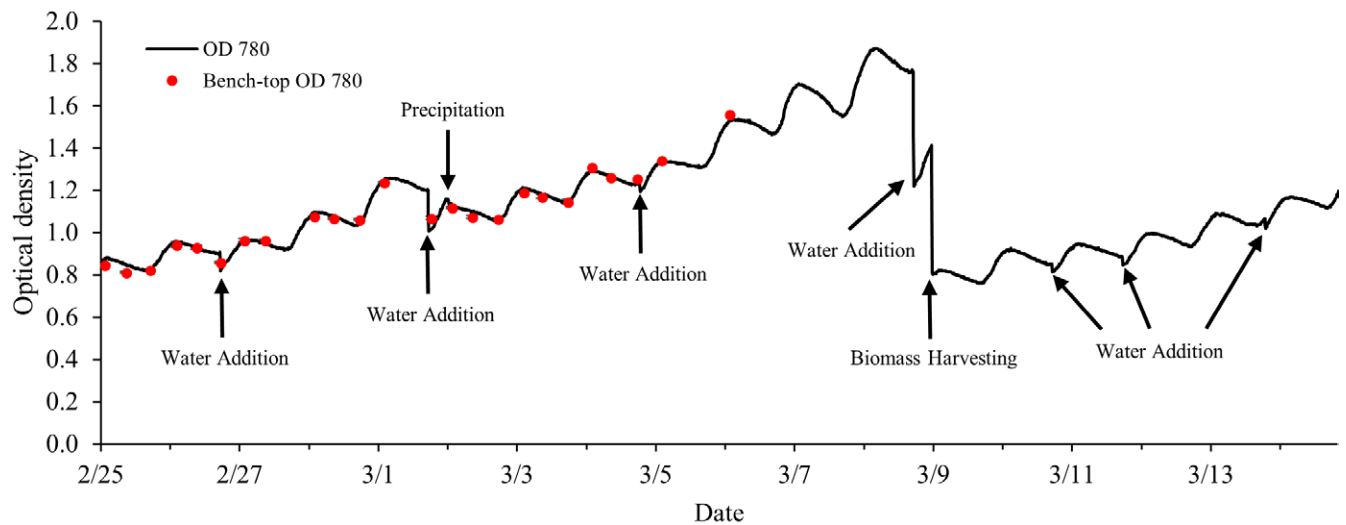
## Real-time monitoring and control for algae culture environment

As stated previously, the University of Arizona project site consisted of two types of raceway systems: the ARID and two small open pond raceways to conduct outdoor experiments. The key environmental variables in these raceways were monitored in real time using an autonomous data acquisition control system. Main variables monitored from both systems in the algae culture growing environment included electrical conductivity (HI3001, Hanna Instruments, USA), pH (HI1001, Hanna Instruments, USA), dissolved oxygen (DO1200/T, 101 Sensorex, USA), temperature (Type T and K, Omega Engineering Inc., USA) and, from the aerial environment, photosynthetically active radiation (PAR) (SQ-110, Apogee instruments, USA). The team monitored the environmental data using CR3000 and CR1000 (Campbell Scientific Inc., UT, USA) data loggers and also monitored carbon dioxide use in the two open pond raceway units. The researchers controlled the pH with carbon dioxide injection via data logger unit controls and developed a graphical user interface, enabling system operators to observe the

instantaneous and historical data from the UA-RAFT site for optimal systems management and operation. The AzMET weather station provided other climatic variables such as air temperature, humidity and total shortwave radiation for modeling studies.

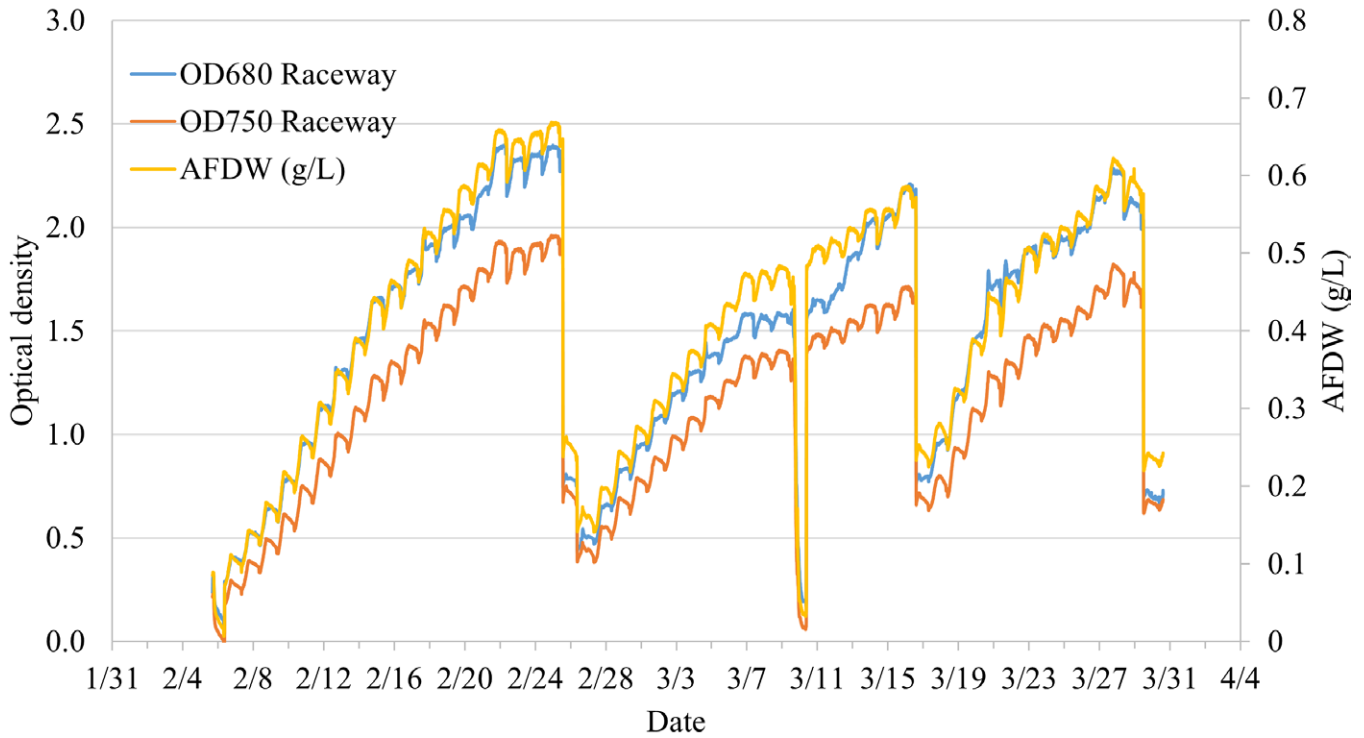
## Multi-wavelength based optical density sensor for microalgae growth and health monitoring

The optical sensor unit was integrated into the outdoor raceways at the University of Arizona RAFT project site. The optical density of the *S. obliquus* culture over 18 days was recorded by the optical sensor (Figure 16). The real-time optical density repeatedly showed an increase in OD reading, indicating the biomass increase during the day due to photosynthesis. The researchers observed a small decrease in optical density during the night, because photosynthetic microorganisms metabolize intracellular carbohydrate to sustain their metabolic activity during that time. Sudden decreases of optical density of the culture were due to water addition and rain (Figure 16).



**Figure 16.** Optical density change of *S. obliquus* in the open pond raceway over 18 days. Black arrows indicate events of water addition, precipitation and biomass harvesting.

Figure 17 illustrates the continuous monitoring of OD for microalgae *M. minutum* using the inline optical density sensor installed on a paddlewheel raceway at the UA-RAFT site for a 60-day experimental period. The calculated OD from the sensor outputs was calibrated to a benchtop spectrophotometer and the ash-free dry weight.



**Figure 17.** Continuous optical density and AFDW of microalgae culture *M. minutum* monitored by the OD sensor during a 60-day experiment.

The optical sensor unit prototype demonstrated the capability of estimating algal biomass concentration and changes of the physiological status of the microalgae culture in real time. For industrial microalgae production, the application of ultra-hydrophobic material (hydrophobic glass coating, Ultra Tech International, Inc., Jacksonville, FL, USA) on the surface of the flow chamber can further extend the maintenance interval without the need for cleaning biofilm build-up on the flow chamber. Biomass concentration was accurately estimated by optical density measurements at 650, 685 and 780 nm wavelengths. The sensor was capable of measuring maximum optical density of 5.41, 5.86 and 4.88 without sample preparation at 650 nm, 685 nm and 780 nm, respectively. A temperature control device for the sensor is necessary, especially for outdoor applications where air temperature can vary significantly, because the output power of laser diodes is temperature dependent. The cell concentration measurement range can be improved further by shortening the light path length of the flow chamber. With proper calibration, installation and operation, the optical sensor described in this study can be integrated into microalgae culture systems for monitoring and control purposes at a relatively low cost to ultimately help optimize product quality, quantity and resource use efficiency.

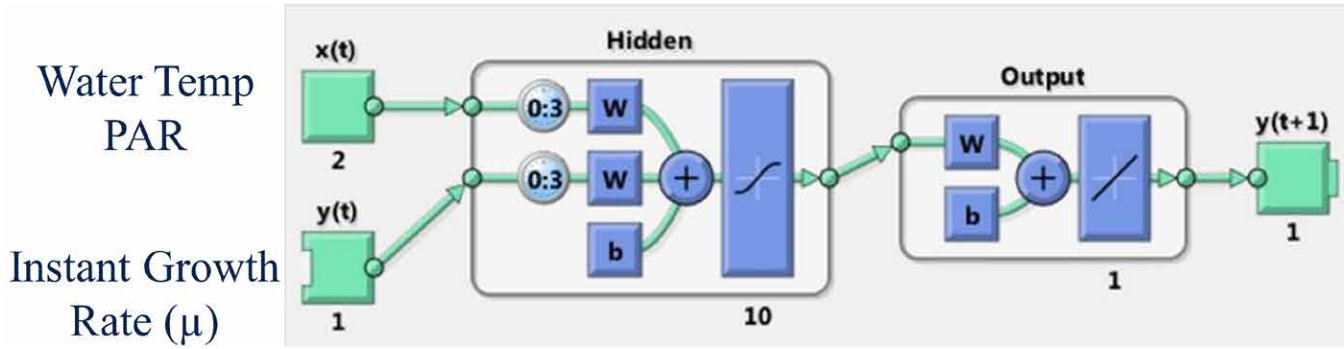
## Autonomous and timely detection of suboptimal microalgae growth

Due to the diurnal and seasonal changes of pond temperature and available photosynthetically active radiation, which greatly affect the growth of microalgae, the productivity of microalgae in open systems is rarely optimal. Although there are outdoor pond designs that aim to address this issue by reducing the heat loss of the pond when conditions are not favorable for microalgae growth, open pond systems are inherently susceptible to contamination, which can negatively affect the growth and eventually lead to a crash. To avoid the production

loss due to the suboptimal algae growth or crashing, a real-time microalgal growth prediction algorithm integrated with real-time sensor feedback can be advantageous for optimal monitoring and control of microalgae production systems. Artificial Neural Networks (ANN) have gained popularity for applications in modeling complex dynamic bioprocesses. ANN models, as a machine learning technique, were developed by training the ANN model with inputs and known outputs from a non-linear complex system of interest. The main advantage of ANN over parametric kinetic models is the possibility of modeling a system without knowing the theoretical knowledge of it.

The goal in this work was to predict the growth rate of healthy microalgae under a given set of environmental conditions using ANN and to compare it to the real-time growth rate measured by the real-time OD sensor. A substantial amount of discrepancy between the two serves as an indicator of abnormality in the microalgae culture and potentially signals early algae crashing or unhealthy growth.

The experiment was conducted in the paddlewheel raceway with *C. sorokiniana* cultivated in BG-11 media. The team used hourly average measurements of each parameter for ANN model training and used the neural network Time Series package within MATLAB for ANN predictive modeling. The researchers used a Nonlinear Auto Regressive Neural Networks with exogenous inputs (NARX) to predict future values of a time series from past values of the series and from that of external inputs (Figure 17). Rate change of daily growth ( $d_{\Delta OD/Day}/dt$ ), first derivative of the difference between current OD and OD at the same time from the previous day, can be used as an indicator of slowing growth or negative growth. Rate change of daily dissolved oxygen ( $\Delta DO/Day$ ) serves as a good indicator of microalgae growth. When growing under favorable conditions,  $d_{\Delta OD/Day}/dt$  and  $\Delta DO/Day$  should be close to zero. A statistically significant decrease of either parameter indicates “perturbed” microalgae growth.

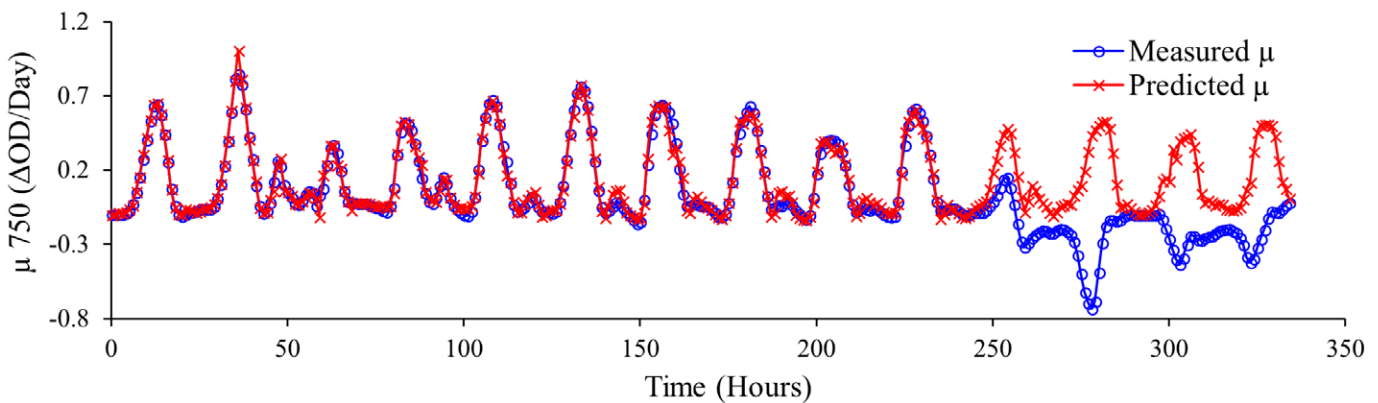


**Figure 18.** Schematic of an ANN structure with one hidden layer and output layer. In this experiment, two input parameters and 10 hidden neurons were used to estimate one output.

## Autonomous and timely detection of suboptimal microalgae growth

The NARX model was subsequently applied to the dataset collected from the entire outdoor culture. Figure 19 shows the time course overlay plot of model predicted  $\mu$  and measured  $\mu$ . The predicted and measured  $\mu$  showed good agreement during the first 248 hours of culture,

while none to a negligible amount of contaminants like *V. chlorellavorus* were detected in the culture media. The discrepancy in the two parameters, presented as prediction error in Figure 19, started to increase at hour 250 as a result of slowing growth of *C. sorokiniana*. The rapid decline of biomass led to a total crash of the culture. The ANN and NARX combined detected the potential for a crash at 250 hours, whereas raceway operators noted the beginning of a crash at hour 278.



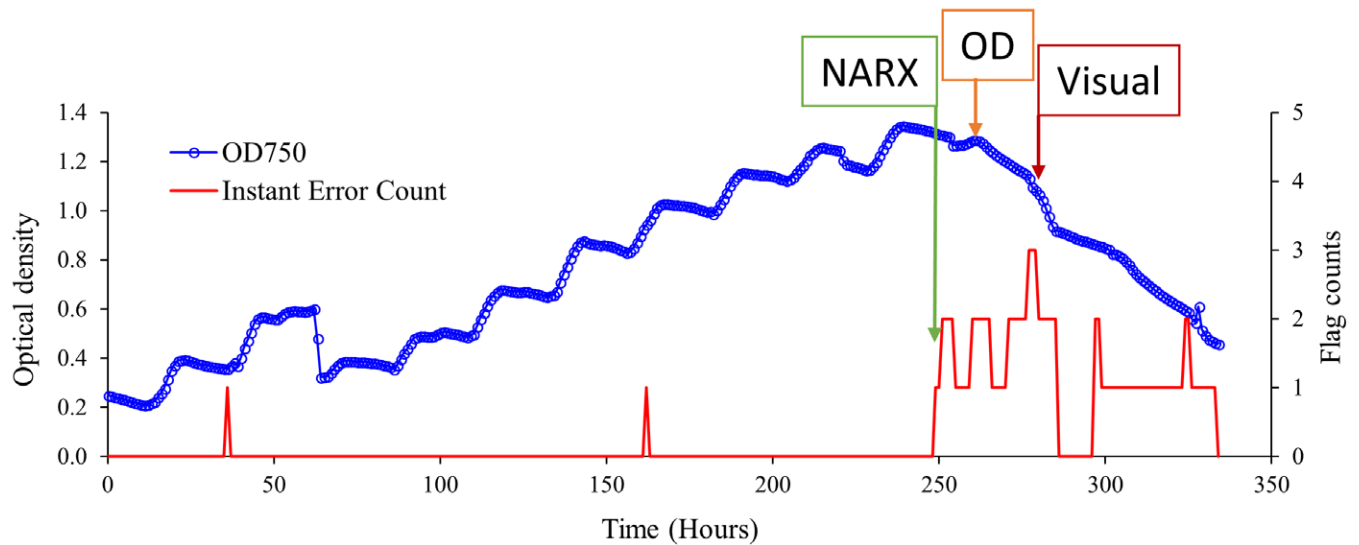
**Figure 19.** The time-course overlay plot of model predicted  $\mu$  and measured  $\mu$ .



The team developed an early warning algorithm for detecting suboptimal microalgal growth based on the NARX prediction model. The results of the simulation in terms of the number of flags triggered by the algorithm is shown in Figure 20. Model prediction error triggered the two flag counts at hours 38 and 164. However, both flags were isolated incidents, with no other flags triggered around the time points. These events also were single flag counts, meaning no flags were triggered by the other two variables. When an isolated single flag is prompted, operators ought to check the cultivation system to assure all instruments are in normal operating condition. In this case, the flags were triggered solely due to prediction errors. Starting at hour 250, flags were prompted continuously and the flag counts often were greater than 1, meaning that the model prediction error is in accordance with one or both of the  $\Delta\mu/\text{day}$  and  $\Delta\text{DO}/\text{day}$ . In case of continually multiple flag counts, operators should immediately take samples of the culture for further analysis to determine the cause of suboptimal

growth. Depending on the severity of the situation, countermeasures can be taken to avoid or minimize production losses (e.g., emergency biomass harvesting).

The team demonstrated that the NARX model and sensor feedback-based detection uncover suboptimal algae growth occurrences. Similar results were found when the instantaneous growth rate ( $\mu$ ) prediction model was trained by and applied to a dataset collected from a replicate experiment under similar environmental conditions. The increase of model prediction error started to show at hour 242, and the team visually confirmed the culture crash one day later (Figure 20). The results from the experiments demonstrated that the approach offers an early warning tool for operators of microalgal production systems to use to evaluate the growth conditions of the algal culture in real time and take the necessary preventive actions to avoid or minimize production losses (e.g., algal biomass). The machine training time is minimal.



**Figure 20.** Simulated results of early detection of suboptimal algae growth based on OD and DO sensor feedbacks and predictive ANN modeling.

A paper and patent resulted from this work.

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# RAFT MODELING

## PNNL Strain Parameterization for Biomass Growth Modeling

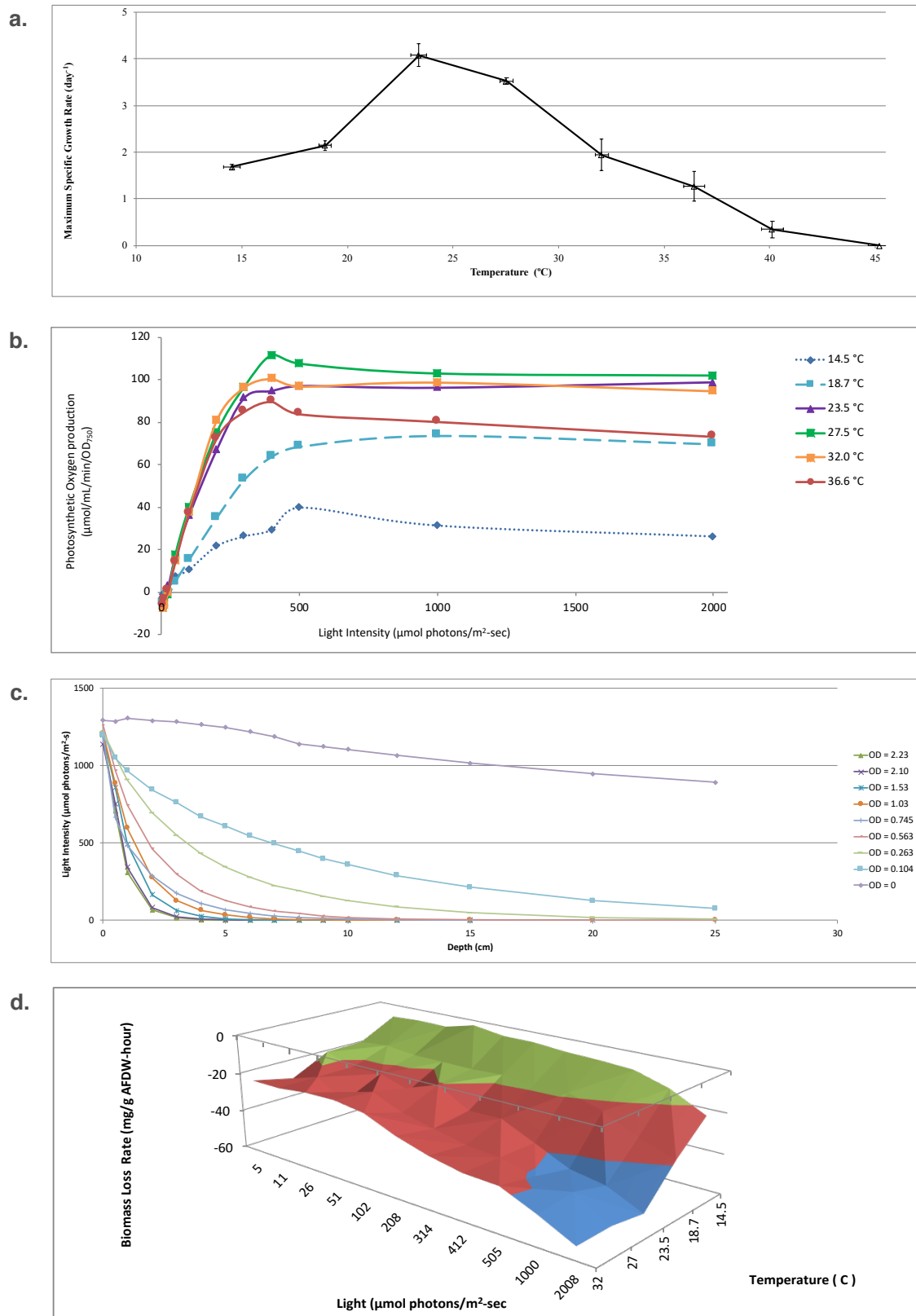
As described earlier in the Basic Strain Characterization section, the team further characterized a subset of the most promising strains to generate the required input parameters for the PNNL Biomass Growth model. This model enables the prediction of potentially achievable biomass productivities of these “parameterized” strains when cultured at different times of year in RAFT testbeds under different operating conditions. This more detailed characterization involved measuring the following key parameters for each strain: the maximum specific growth rate as a function of temperature and light intensity, the scatter-corrected biomass light absorption coefficient and (the biomass loss rate in the dark as a function of temperature and average light intensity during the preceding light period (see Figure 21 a-d).

The maximum specific growth rate as a function of temperature was measured in shake flask cultures on the PNNL Thermal Gradient Incubator (TGI), (Figure 21a). To determine the effect of light intensity on microalgae growth, the researchers took temperature-adapted samples from the thermal gradient incubator cultures and measured the rate of photosynthetic oxygen evolution, a proxy for growth rate, as a function of light intensity

(P–I curves) at each respective TGI temperature using a computer-controlled oxygraph Chlorolab system (Figure 21b). The scatter-corrected biomass light absorption coefficient was determined from underwater light intensity (PAR) versus culture depth data at different biomass densities (Figure 21c).

Biomass loss during the night can be a significant burden—as much as a 22% loss of the total AFDW biomass—on the net photosynthetic productivity of an organism. The team quantified biomass loss for several strains of microalgae and found that the value is highly variable. Biomass loss due to metabolic respiration is highly strain-specific and is affected by temperature as well as the light intensity of the preceding day (Figure 21d). In general, warmer nighttime temperatures increase metabolic rates and lead to higher night biomass losses. Similarly, higher daytime light intensities preceding the night also increase metabolic rates and lead to higher biomass loss rates.

These experimentally determined strain-specific parameters for *C. sorokiniana*, *S. obliquus* and *M. minutum* were used as inputs to the Biomass Assessment Tool Microalgae Growth Model, as described in the following section on the BAT Modeling Analysis and Experimental Design Support.



**Figure 21.** Parameterization of algal strains for predictive growth modeling: a) Maximum specific growth rate as a function of temperature; b) Photosynthetic oxygen production as a function of light and temperature; c) Light intensity as a function of depth at different biomass optical densities (OD); and d) Biomass loss rates as a function of both light and temperature. Data shown are for the marine strain *Tetraselmis striata*-LANL characterized during the RAFT project.

## PNNL Growth Model Validation

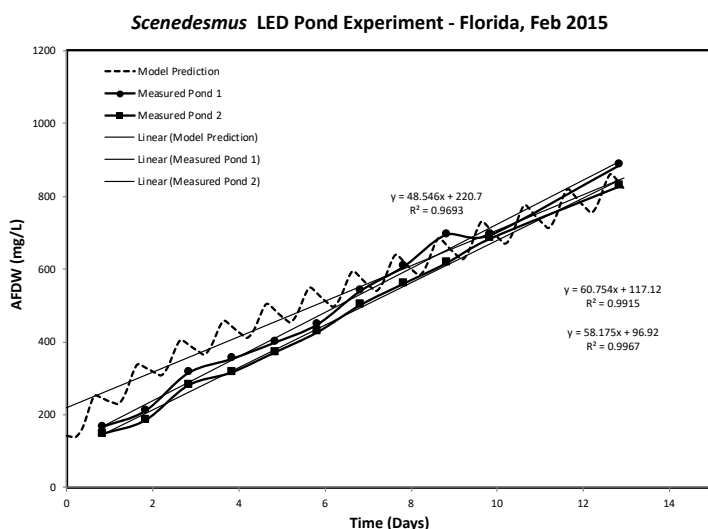
As mentioned in the previous section, the BAT modeling analysis was carried out with three strains: *C. sorokiniana*, *S. obliquus* DOE 0152.z and *M. minutum*. *C. sorokiniana* was parameterized during the NAABB project and *S. obliquus* and *M. minutum* were parameterized during the RAFT project as described in the section, PNNL Strain Parameterization for Biomass Growth Modeling. The researchers rigorously validated the PNNL Biomass Growth Model using raceway pond culture growth data before using this model as the basis for evaluating different BAT modeling scenarios to optimize annual biomass productivities via crop rotation, pond depth adjustment and harvesting strategy.

The researchers successfully validated the biomass growth model for *C. sorokiniana* using measured biomass versus time data from the following raceway pond experiments: a) outdoor batch and semi-continuous cultures that were performed during June and July 2012 as part of the NAABB consortium project at Rimrock, AZ;

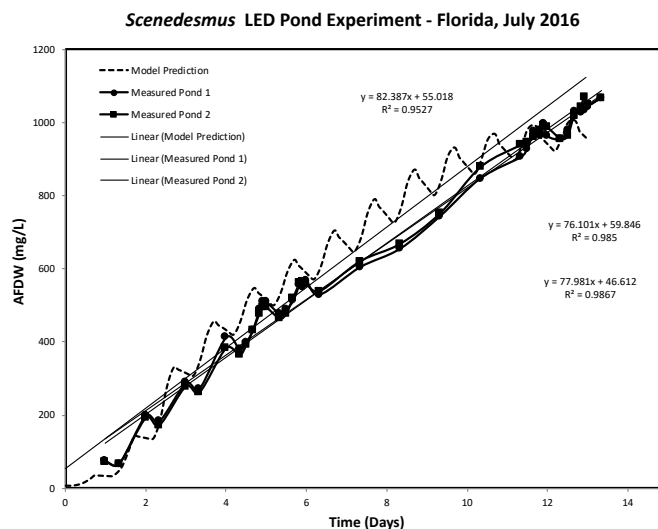
b) indoor PNNL raceway cultures simulating the months of January and July in Key West, FL, using BAT-generated light and temperature scripts; c) outdoor cultures performed in September 2015 at the RAFT testbed in Pecos, TX; and d) outdoor cultures carried out in July 2015 in Delhi, CA, as part of the BETO ABY1 project (Dr. Lundquist, PI).

The biomass growth model, after multiplication of the entire maximum specific growth rate matrix  $\mu(T,I)$  with a correction factor of 0.64, was successfully validated for *S. obliquus* using measured biomass versus time data from four different raceway pond cultures and one LEAPS culture, as shown in Figures 22 through 26.

Finally, the biomass growth model also was validated for *M. minutum* using the duplicate RAFT16 outdoor raceway experiment conducted at the University of Arizona in February and March 2016, and the triplicate raceway pond experiment conducted at the RAFT testbed in Pecos, TX, in January and February 2015 (data not shown).

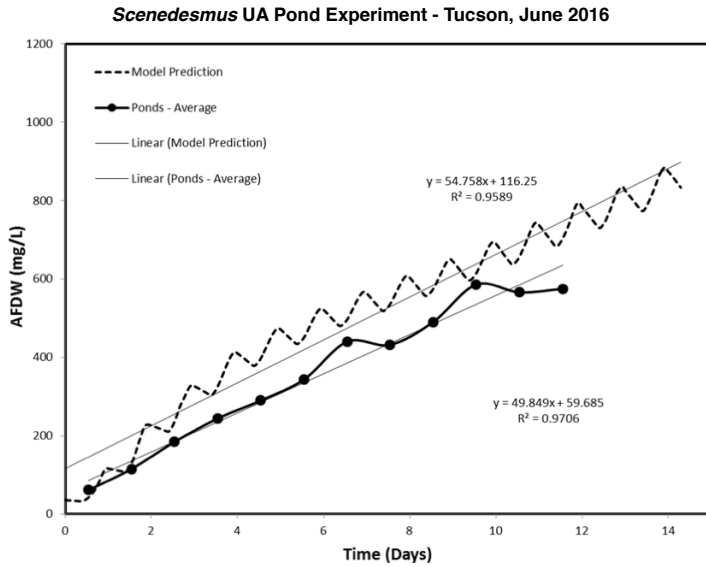


**Figure 22.** Measured and model predicted biomass (AFDW) concentration as a function of time for *S. obliquus* climate simulation pond culture experiments using BAT-generated light and temperature scripts for Key West, FL, for February 1.

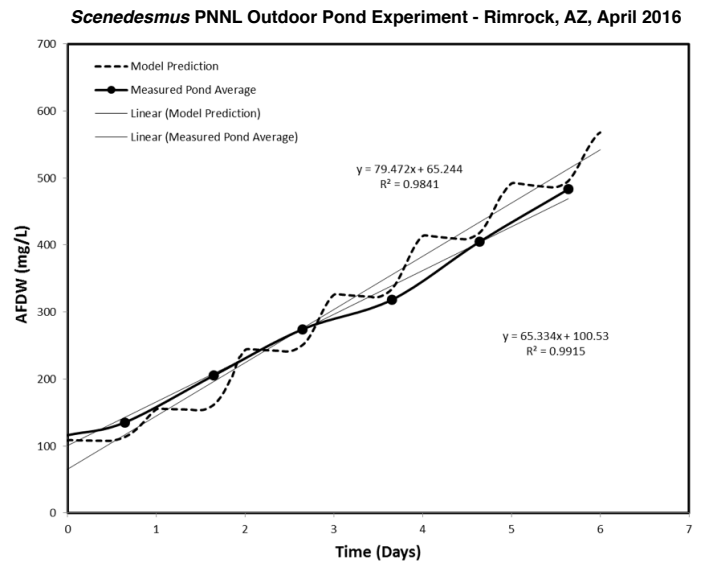


**Figure 23.** Measured and model predicted biomass (AFDW) concentration as a function of time for *S. obliquus* climate simulation pond culture experiments using BAT-generated light and temperature scripts for Key West, FL, for July 1.

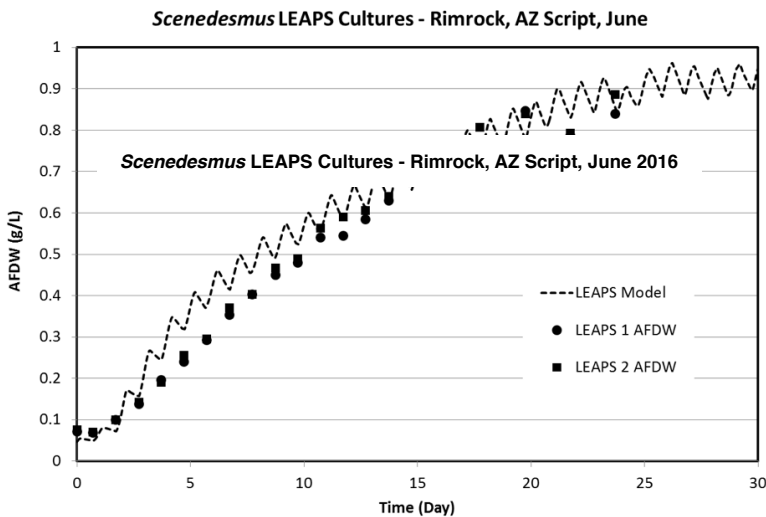




**Figure 24.** Measured and model predicted biomass (AFDW) concentration as a function of time for *S. obliquus* outdoor pond culture experiments conducted at the University of Arizona RAFT testbed site in Tucson, AZ, in June 2016.



**Figure 25.** Measured and model predicted biomass (AFDW) concentration as a function of time for *S. obliquus* outdoor pond culture experiments conducted by PNNL at the University of Arizona Agricultural Research Station in Rimrock, AZ, in April 2016.



**Figure 26.** Measured and model predicted biomass (AFDW) concentration as a function of time for *S. obliquus* LEAPS photobioreactor culture experiments using light and temperature scripts measured during a NAABB outdoor pond experiment conducted by PNNL at the University of Arizona Agricultural Research Station in Rimrock, AZ, in June 2012.

Several publications, below, resulted from this work.

### **Citations**

Edmundson, S., and M. Huesemann. 2015. The dark side of algae cultivation: A comparison of biomass loss at night in three photosynthetic algae, *Chlorella sorokiniana*, *Nannochloropsis salina* and *Picochlorum* sp. *Algal Research* **12**:470-476.

Edmundson, S.J., R. Kruk, and M.H. Huesemann. Winter algae crops: Characterization of algal strains with cold season production potential. *Environmental and Experimental Botany*. (In preparation, 2018).

Huesemann, M.H., T.S. Hausmann, R. Bartha, M. Aksoy, J.C. Weissman, and J.R. Benemann. 2009. Biomass productivities in wild type and a new pigment mutant of *Cyclotella* sp. (Diatom). *Applied Biochemistry and Biotechnology* **157(3)**:507-526.

Huesemann, M.H., J. Van Wageningen, T. Miller, A. Chavis, S. Hobbs, and B. Crowe. 2013. A screening model to predict microalgae biomass growth in photobioreactors and ponds. *Biotechnology and Bioengineering* **111(6)**:1583-1594.

Huesemann, M.H., B. Crowe, P. Waller, A. Chavis, S. Hobbs, S. Edmundson, and M. Wigmosta. 2016. A validated model to predict microalgae growth in outdoor pond cultures subjected to fluctuating light intensities and water temperatures. *Algal Research* **13**:195-206.

Huesemann, M.H., N. Sun, M. Wigmosta, R. Skaggs, and P. Waller. Enhancement of the Biomass Assessment Tool Microalgae Growth Model to Evaluate Alternative Strategies for Optimizing Biomass Productivities of *Chlorella sorokiniana* (DOE 1412). (In preparation, 2018).

Van Wageningen, J., T.W. Miller, S. Hobbs, P. Hook, B. Crowe, and M.H. Huesemann. 2012. Effects of light intensity and temperature on fatty acid composition in *Nannochloropsis salina*. *Energies*, **5**:731-740.

## **BAT Modeling Analysis and Experimental Design Support**

Given limitations on available RAFT testbed capacity and the time associated with each testbed experimental cycle (i.e., weeks to months), the team used the Biomass

Assessment Tool resource assessment modeling framework to assist in formulating cultivation experiment designs and operational practices and systematically assimilate experimental results for ongoing model validation and improvement. The team also needed resource assessment tools to extrapolate site-specific, short-duration growth experiments across multiple regions, multiple years of climate data, and numerous factors such as multiple algal strains, strain rotations and alternative operating strategies. Toward meeting these requirements, the objectives for this element of the RAFT program was to fully integrate the PNNL microalgae growth model into the BAT to enable support of RAFT experimental design, and evaluate the potential impact of RAFT advances in algal cultivation toward achieving BETO algal biomass and biofuel production goals. Specific model development and deployment activities are summarized below.

### **Incorporation of the PNNL Microalgae Growth Model into the BAT**

The BAT is a GIS-based analysis platform for high-resolution, national-scale resource and algal biofuels production assessments from open pond facilities (Wigmosta et al., 2011 ). Coupled models of the dominant biophysical processes affecting algal growth simulate the land and water resource requirements and potential biofuel production at high spatiotemporal scale. Basic data requirements for the BAT include highest available resolution elevation and remotely sensed earth systems data to identify locations suitable for algae cultivation, and high-resolution spatiotemporal meteorological data to drive the physics-based models. The analyses presented here utilize the North American Land Data Assimilation System (NLDAS) meteorological data at 5,832 stations from 1980 to 2009, including precipitation, air temperature, dew point, wind and incident solar radiation.

The original BAT algal growth model of Wigmosta et al. (2011) simulates the conversion of solar energy (photosynthetically active radiation) during photosynthesis to chemical energy storage in the form of oils and other biomass. The model accounts for reductions in photon absorption due to suboptimal light and water temperature. The light utilization efficiency, including light saturation and photoinhibition, is modeled using the Bush equation (Huesemann et al., 2009). While the above model

proved to be a useful tool to address a number of key questions related to algal biofuel resource assessment, it is not a true biomass growth model. Specifically, it cannot be parameterized to represent the growth characteristics of specific algal species (i.e., their unique response to varying climatic and sunlight conditions). Further, it ignores the impact of algal concentration and the associated attenuation of photosynthetically active radiation as a function of depth on biomass production. Consequently, the model cannot explicitly account for the impact of alternative pond operation characteristics such as the intensity and frequency of harvest on biomass growth. The original growth model, therefore, was replaced by the PNNL Microalgae Growth Model, a more complete formulation capable of representing concentration-dependent biomass growth as influenced by actual pond operations, including varying water depth and algal concentration associated with harvesting algal biomass.

The PNNL Microalgae Growth Model was developed to simulate biomass productivity in outdoor open ponds under naturally fluctuating light intensities and water temperatures. The model assumes that solar radiation and growth media water temperature determine microalgae growth and productivity, and that no other factors such as nutrients, carbon dioxide, and mixing (i.e., mass-transfer) are limiting. It also assumes a constant culture pH maintained by feedback-controlled carbon dioxide addition, and that there is no growth inhibition by photosynthetic oxygen or other compounds.

### **Deployment of the BAT to Evaluate Alternative Cultivation Design Parameters and Operational Strategies**

Previous studies (Wigmosta et al., 2011; Venteris et al., 2012, 2013, 2014a,b,c,d) have reported there are sufficient locations with suitable climate, land, water and nutrient resources to achieve the Department of Energy's 2017 production target of 1 million metric tons ash-free dry weight cultivated algal biomass (DOE, 2016). However, climate-driven seasonal and annual variability in biomass production present significant economic and lifecycle challenges. These challenges could be managed through careful design of operational cultivation strategies

based on an integrated approach to managing culture water depth and temperature, algal strain selection, timing and magnitude of harvest.

Key goals, then, of developing cultivation practices are, in part, to dampen seasonal and inter-annual variability of algal growth rate and ultimately maximize biomass production considering three operational design parameters: 1) intra-annual rotation of strains, 2) manipulation of pond water depth to optimize local seasonal light and temperature for algal growth, and 3) harvest strategy.

#### ***Intra-annual Strain Rotation***

The PNNL Microalgae Growth Model within the BAT was parameterized for three freshwater algal strains, including *C. sorokiniana* and *Monoraphidium* and *Scenedesmus*. Growth model parameters for these strains were determined experimentally and were observed to exhibit distinct and to some extent complementary responses to seasonal light and water temperature conditions.

Based on laboratory experiments, *C. sorokiniana* is a heat-loving, cold-intolerant strain with favorable water temperatures ranging from 30 to 40 °C with little to no growth potential when water temperature is below approximately 15 °C. In contrast, *Monoraphidium* is cold-hardy and heat-intolerant with favorable temperatures ranging from approximately 15 to 27 °C. Its growth rate rapidly declines when water temperature rises above approximately 27 °C. Although cold tolerant, *Monoraphidium* has a higher biomass loss rate at low light intensities (i.e., PAR < 50  $\mu\text{mol}/\text{m}^2\text{-sec}$ ), indicating potentially lower productivity with reduced light availability in winter. Compared to the strong seasonal responses of *Chlorella* and *Monoraphidium*, *Scenedesmus* has more consistent growth over a wide range of pond temperatures (20 to 40 °C) and PAR conditions (500 to 2000  $\mu\text{mol}/\text{m}^2\text{-sec}$ ). Generally, this suite of strains is well suited for strain rotation designed to reduce the impact of seasonal climate variability on algal production. In model application, the team implemented strain rotation on a monthly basis, selecting the algal strain with the highest biomass yield in each month for each location and climate.

### **Culture Water Depth**

Pond depth affects both exposure to photosynthetically active radiation and water temperature. Increasing pond depth enhances light attenuation (i.e., less light availability). In addition, available shortwave radiation (operating at 0.285–2.8  $\mu\text{m}$ ) has a primary control on pond water temperature. Increasing pond depth produces greater thermal inertia (i.e., varying degrees of dampening and latency to changing air temperature). Thus, the average daily pond temperature during warm seasons decreases as depth increases. In cool seasons, the reverse occurs.

### **Harvest Strategy**

Harvest involves the removal of algae-laden water and subsequent replacement with clean water. The harvest strategy employed in this analysis is designed to achieve and maintain a target algal concentration. The algae is permitted to grow in the pond until a target algal concentration is exceeded (e.g., 500 mg/L) at the end of an hourly time step. A volume of algae-laden water is then removed from the pond such that when replaced by clean water, the target concentration is reestablished. The target concentration can be varied seasonally, monthly or even weekly based on the expected growth rate in the pond. Ideally, harvest can be timed to maximize productivity by remaining in the steep part of the growth curve, balancing algal density and depth of light penetration.

## **Baseline Assessment of Cultivation Practices**

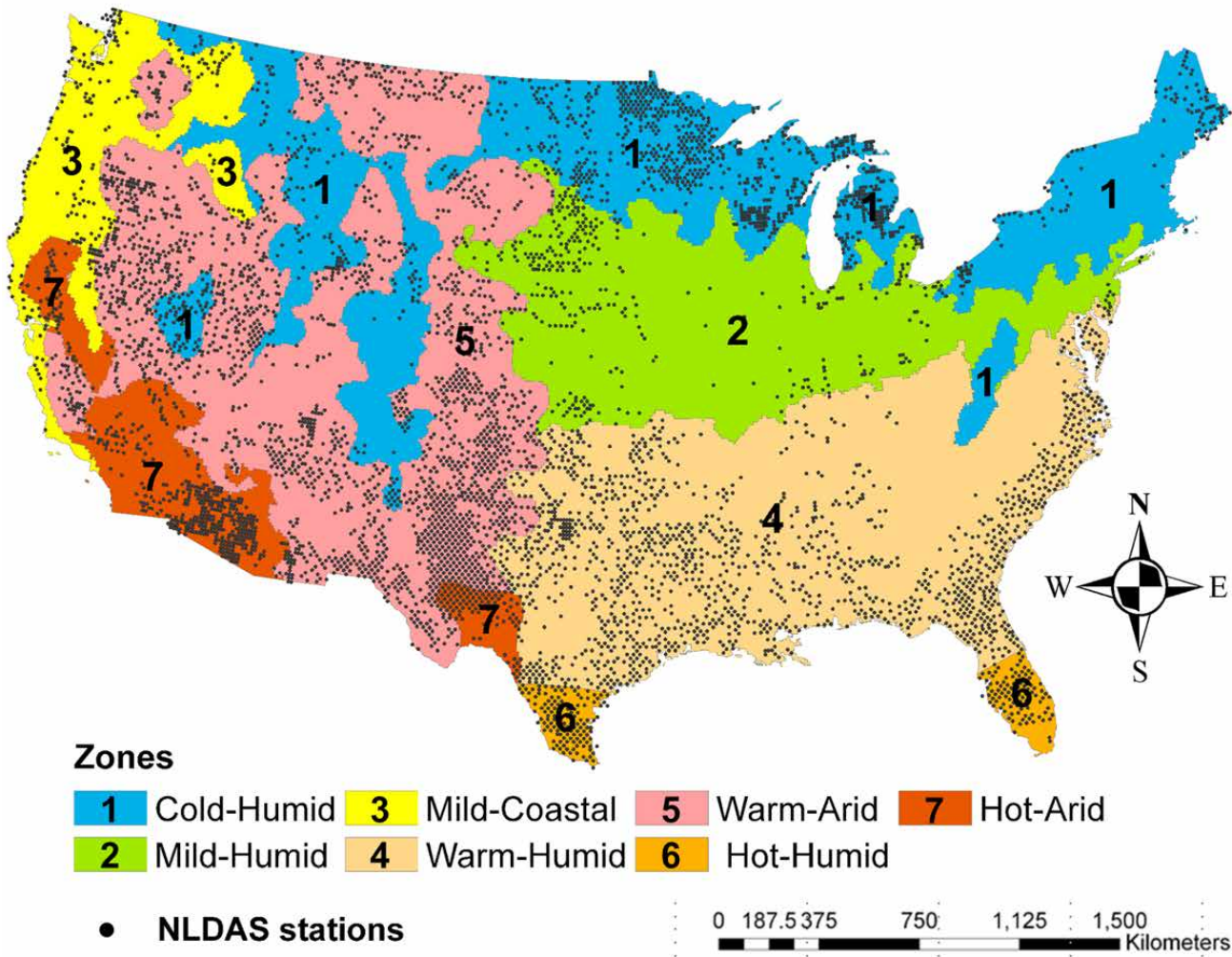
Recognizing that agricultural-related resource management decisions are generally made at scales beyond the individual site level, it was necessary to conduct the impact assessment of cultivation practices on a regional basis. To accomplish this, the team divided the 5,832 NLDAS stations into seven zones based on their representative climate characteristics, including temperature and humidity, which directly control algal growth variability and seasonality (Figure 26). The baseline cultivation practice was established in a manner

similar to Davis et al. (2014), such that the BAT was run for 30 years at each NLDAS station location for *C. sorokiniana* assuming, 25 cm pond depth with an hourly time step, and simulated harvest was triggered at 500 mg/L algae concentration. The cultivation parameters the team examined include: (1) harvest strategy, comparing triggering harvest concentrations of 300 mg/L and 700 mg/L to the baseline concentration 500 mg/L; (2) comparing open pond culture depths of 15 cm and 20 cm to the 25 cm baseline depth; and (3) strain selection, comparing *Monoraphidium* and *Scenedesmus* to the baseline strain *C. sorokiniana*. For each zone in Table 6, the median values (Q2) or the 50th percentile of the grouped data are reported to represent the zonal average. The median was selected rather than the mean because the latter tends to be overly sensitive to outliers. This applies hereafter unless otherwise stated.

Generally, results are as expected, with the baseline productivity being higher in the warmer zones than the colder zone (higher altitudes) due to more consistent and higher availability of radiation and associated higher water temperatures favored by *Chlorella*. The baseline productivity is also higher in the more humid than arid zones (e.g., Zone 4/Warm-Humid (e.g., the Southeast) vs. Zone 5/Warm-Arid (e.g., the Southwest)). The low relative humidity and limited cloud cover in the arid zones leads to greater thermal energy loss from open ponds at night, lowering morning water temperatures that inhibit *C. sorokiniana* growth.

In terms of the 30-year mean annual productivity (PYR), the baseline is highly variable across the zones in the continental U.S. (Figure 27a). As shown in Table 6, the baseline PYR is greater than 10.0 g/m<sup>2</sup>-day in only three of the zones: highest in Zone 6 (Hot-Humid), followed by Zone 4 (Warm-Humid) and then Zone 7 (Hot-Arid). The baseline PYR is lowest (3.7 g/m<sup>2</sup>-day) in Zone 1 (Cold-Humid), where algal growth often becomes limiting during cold-temperature months. At the station level, the baseline PYR ranges from 0.5 to 19.1 g/m<sup>2</sup>-day.





**Figure 27.** Map of the 5,832 NLDAS stations distributed over the continental U.S. To perform regional analyses, the team divided the stations into seven groups/zones based on their representative climate characteristics including temperature and humidity.



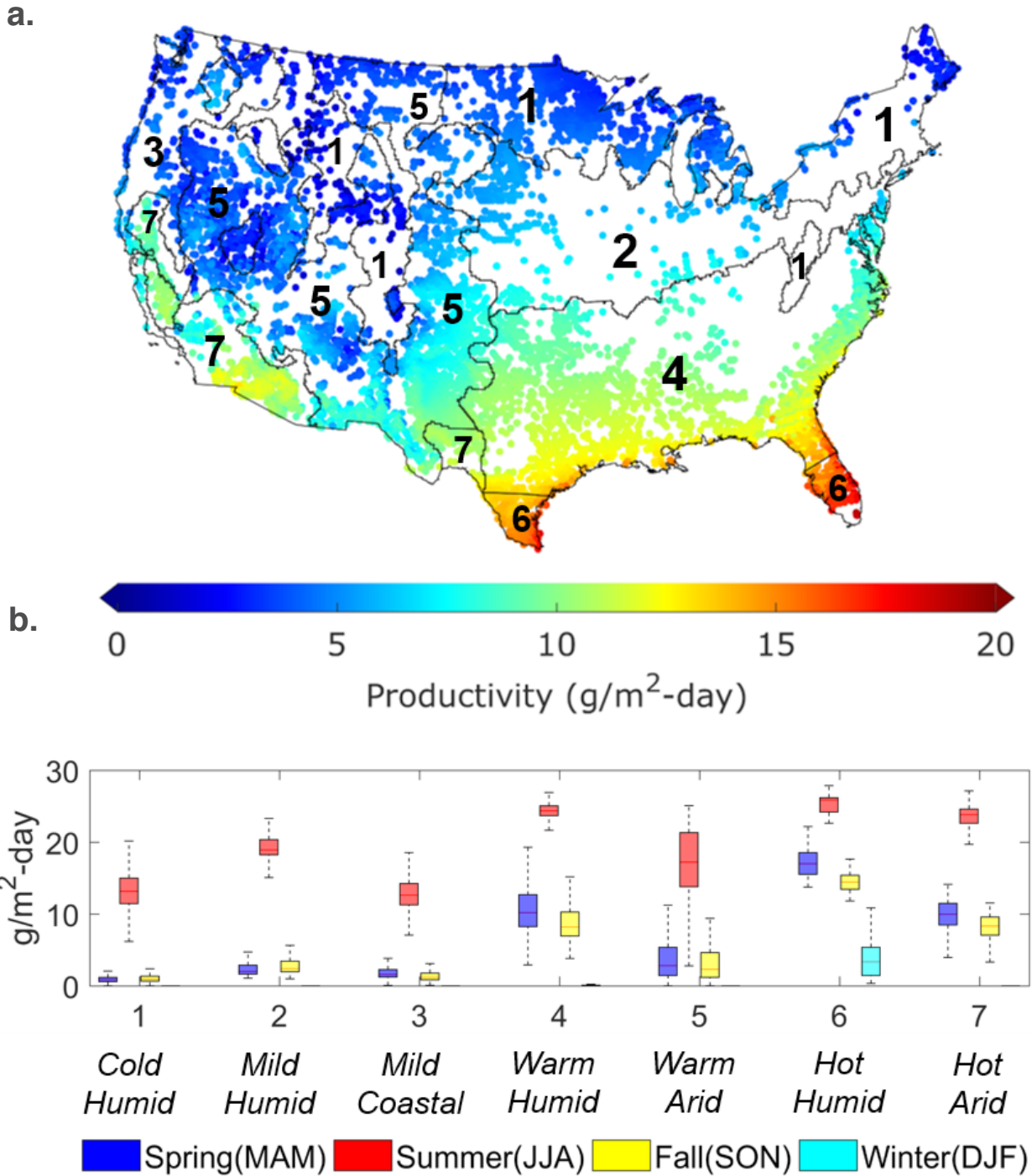
**Table 6.** Summary statistics of the mean annual productivity ( $P_{YR}$ , unit: g/m<sup>2</sup>-day) under the Baseline and Maximum cultivation strategy, and their differences  $\Delta$ .

Zone	Climate Type	Baseline $P_{YR}$	Maximum $P_{YR}$	$\Delta P_{YR}$
		Q2	Q2	Q2
1	Cold-Humid	3.7	17.5	13.8
2	Mild-Humid	5.8	18.7	12.9
3	Mild-Coastal	3.8	20.7	16.9
4	Warm-Humid	10.7	24.8	14.1
5	Warm-Arid	5.5	22.7	17.1
6	Hot-Humid	15.1	27.6	12.6
7	Hot-Arid	10.5	27.7	17.2

\*Note: Q2 is the 50<sup>th</sup> percentile of the data grouped by zone.  $\Delta P_{YR} = \text{Maximum } P_{YR} - \text{Baseline } P_{YR}$ .

Seasonally, the baseline 30-year mean seasonal productivity (PSEAS) exhibits strong variability in all seven zones, with peak productions occurring in summer (JJA), lowest productivity in winter (DJF) and comparable productivity in spring (MAM) and fall (SON) (Figure 27b). The baseline PSEAS is greater than 20 g/m<sup>2</sup>-day only during summer in three zones: Zone 6 (Hot-Humid), Zone

4 (Warm-Humid) and Zone 7 (Hot-Arid). The highest baseline PSEAS (=25.9 g/m<sup>2</sup>-day) is found in summer in Zone 6 (Hot-Humid). The winter-time productivity is zero in all zones but Zone 6 (Hot-Humid), which has warm winters with small diurnal variation (e.g., in southern Florida and southern coastal Texas).



**Figure 28.** (a) Map of mean annual biomass productivity under the baseline condition; (b) Mean seasonal biomass productivity over simulated 30 years for 7 climate zones. The Baseline production is simulated for *C. sorokiniana* in open pond operated at a 25 cm depth at an hourly time step for 30 years, and simulated harvest was triggered at 500 mg/L algae concentration. The boxes in the boxplot show 25%–75% quantiles, and the whiskers show the minimum and maximum.

## Evaluation of Alternative Cultivation Strategies

As a measure of relative importance of each of the cultivation strategies evaluated, the team independently examined on a zonal basis the sensitivity of biomass productivity to the three basic cultivation strategies. The level of sensitivity is expressed by the ratio of scenario-specific productivity to the baseline productivity.

### Impact on Mean Annual Productivity

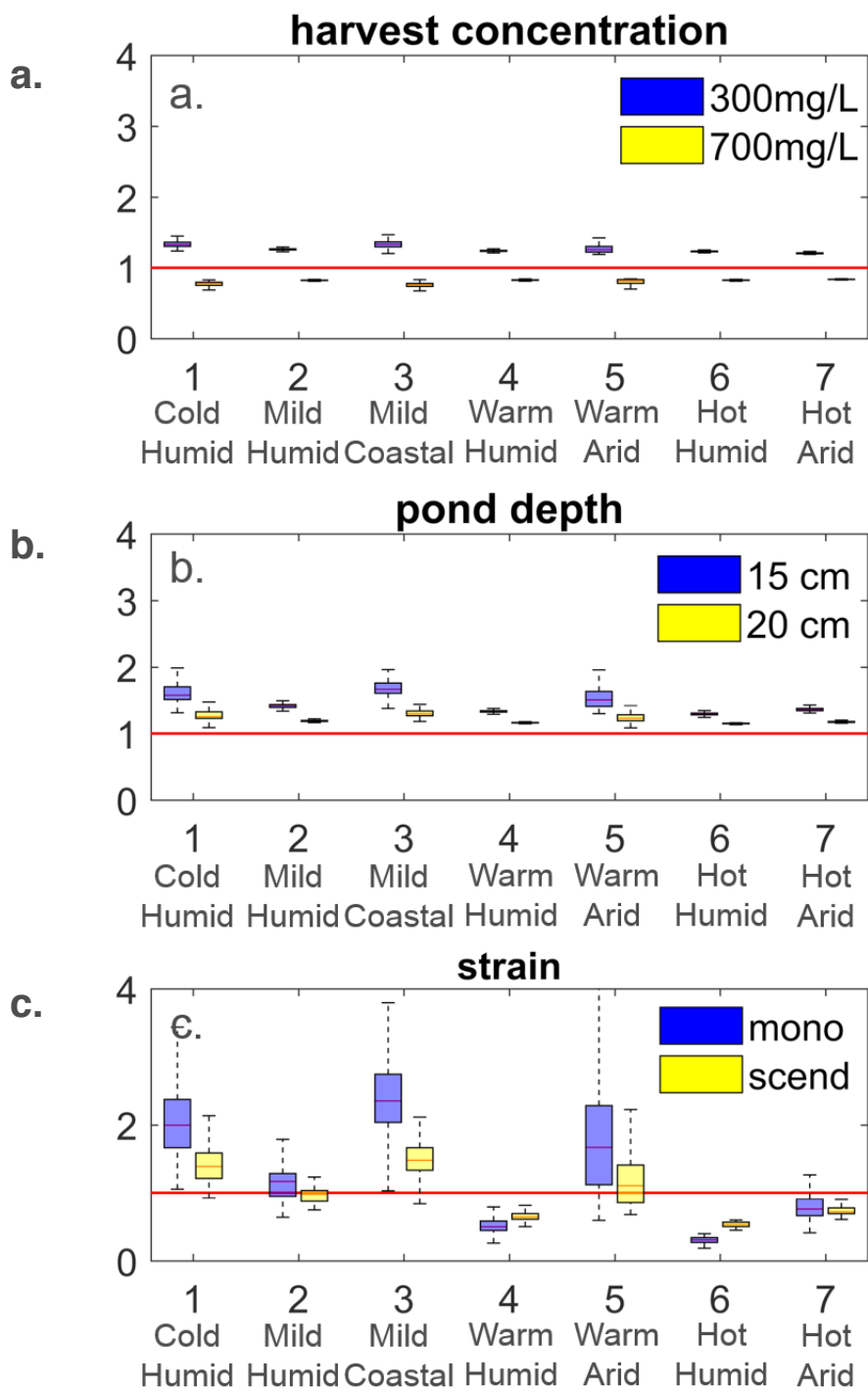
As shown in Figure 28, at the annual scale, the selection of strain (while the pond depth is prescribed at 25 cm and the harvest concentration is prescribed at 500 mg/L) has the most prominent influence on PYR in Zone 1 (Cold-Humid), Zone 3 (Mild-Coastal) and Zone 5 (Warm-Arid). In these zones, the PYR significantly increases when *C. sorokiniana* is replaced with cold-hardy *Monoraphidium*, with the (median) ratio ranging from 1.7 to 2.4. *Scenedesmus* also outperforms *C. sorokiniana* in these zones, with the ratio ranging from 1.1 to 1.5. On the other hand, the baseline strain *C. sorokiniana* produces highest yield in Zone 4 (Warm-Humid); Zone 6 (Hot-Humid), which has warm-mild winters with less diurnal temperature variations; and Zone 7 (Hot-Arid), which has warm winters but greater diurnal temperature variations in an arid environment.

Manipulating pond culture depth or harvest concentration alone (with the baseline strain *C. sorokiniana*) shows similar but lower magnitude impact on PYR. In Zone 1 (Cold-Humid), Zone 3 (Mild-Coastal) and Zone 5 (Warm-Arid), a shallower water depth produces a moderately higher increase in PYR (ratio = 1.5 ~ 1.7) and a similar degree of PYR increase in the other zones (ratio = 1.3 ~ 1.4). A smaller harvest concentration (300 mg/L) provides a similar degree of PYR increase in all seven zones (ratio = 1.2 ~ 1.3).

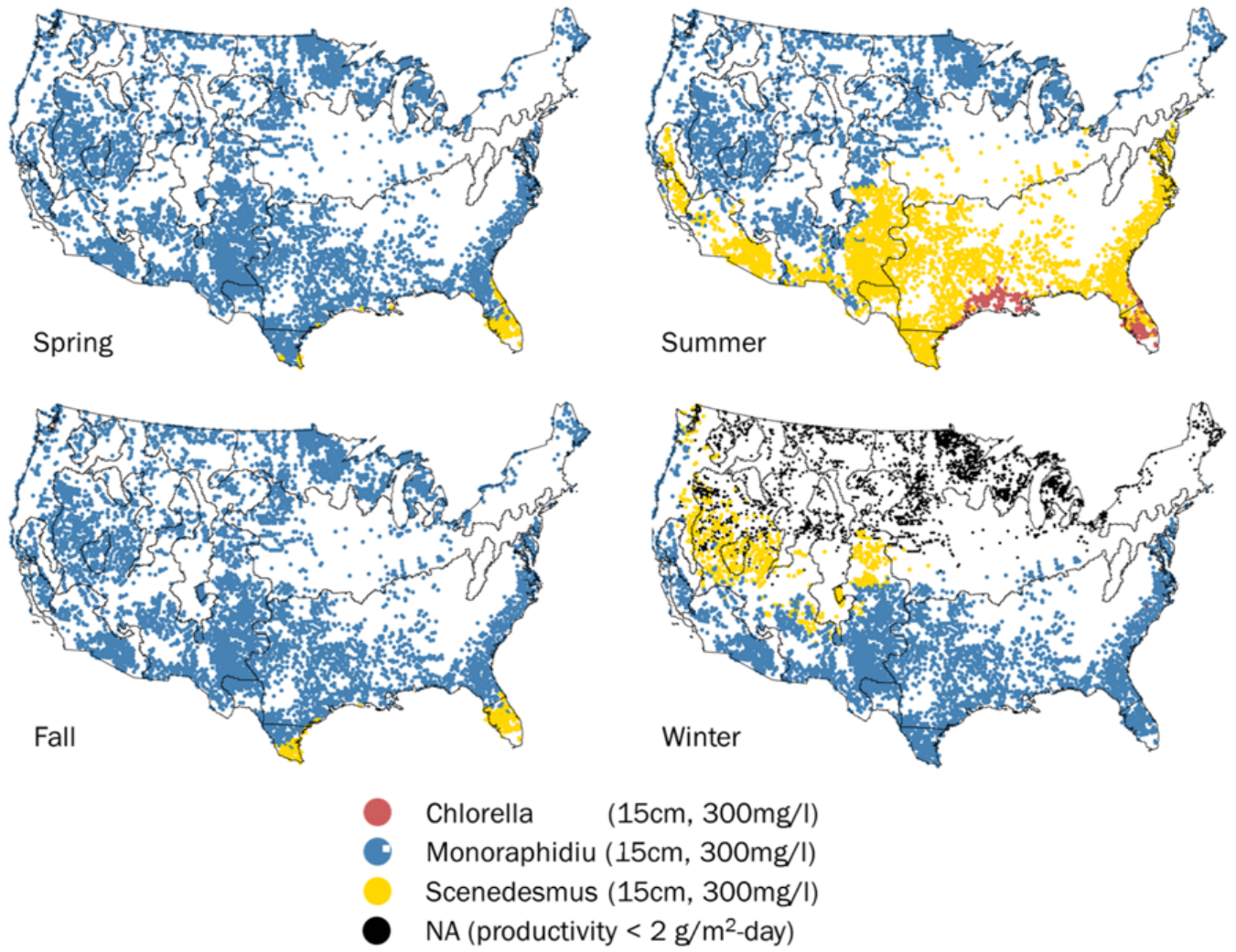
## Optimization of Cultivation Strategies

As a limited demonstration of using the BAT to screen alternative cultivation strategies, the team evaluated the maximum potential for biomass production from rotating three algae strains (*Chlorella*, *Scenedesmus* and *Monoraphidium*) in conjunction with manipulating three pond water depths (15 cm, 20 cm and 25 cm) and three harvest concentrations (300 mg/L, 500 mg/L, and 700 mg/L) at each NLDAS location for each month over 30 years. From the set of 27 alternatives, the cultivation strategy producing the highest biomass productivity was deemed to be the optimum.

The set of optimal strategies on a seasonal basis, across all sites, consists of only three variations. For all sites and all seasons, the optimal pond depth is 15 cm and the optimal harvest concentration is 300 mg/L. The optimal strain type changes with seasons and zones of the country (Figure 29). The team identified the combination of *Monoraphidium* at 15 cm pond depth and harvested at 300 mg/L to be the optimal practice for more than 90% of the stations in spring, more than 85% of the stations in fall and more than 84% of the stations in winter. In summer, *Monoraphidium* produces the highest seasonal yield in Zones 1 to 3 (cold-mild zone) at more than 90% of the stations. The team identified *Monoraphidium* and *Scenedesmus* to be the optimal strain for about the same percentage (approximately 50%) of the stations in Zones 4 to 7 (warm-hot zone). The stations that favor *Scenedesmus* over *Monoraphidium* generally feature hotter pond temperatures because *Scenedesmus* grows faster than *Monoraphidium* at temperatures over 32 °C and also has smaller night biomass loss (Figure 28). Although *Chlorella* has the highest growth rate in high-temperature conditions and produces the highest yield at the baseline depth (25 cm) and baseline harvest concentration (500 mg/L), it is likely subject to greater biomass loss in a shallower pond where water temperature falls more often below 15 °C at night.



**Figure 29.** Sensitivity of mean annual productivity to cultivation strategies: (a) target harvest concentration, (b) open-pond culture depth, and (c) strain selection. The level of sensitivity is expressed by the ratio of alternative scenario-driven mean annual production to the baseline mean annual production over simulated 30 years for seven climate zones. The boxes show 25%–75% quantiles, and the whiskers show the minimum and maximum.



**Figure 30.** Seasonal optimal cultivation strategy (strain type + pond depth + harvest concentration) for all sites.



From an operations standpoint for a specific cultivation site, it is likely that researchers would plan and execute implementation of strain rotation strategies on a monthly basis, guided by past experience plus short- and mid-term weather forecasts. As an illustration of how the BAT can help inform development of these strategies, Table 7 displays the optimal combination of strains, depths and harvest concentrations for a set of sites across the U.S. representing a wide range of climate conditions. As previously noted, the optimum depth (15 cm) and harvest concentration (300 mg/L) are the same for all sites and all months. Again, *Monoraphidium* is the dominant strain for all locations. *Scenedesmus* is the optimum strain for one or more summer months at all sites except Corvallis, OR, the most northern site listed. *Scenedesmus* is optimal for only one month of the year at two sites, Lancaster, PA, a northern site, and Tucson, AZ.

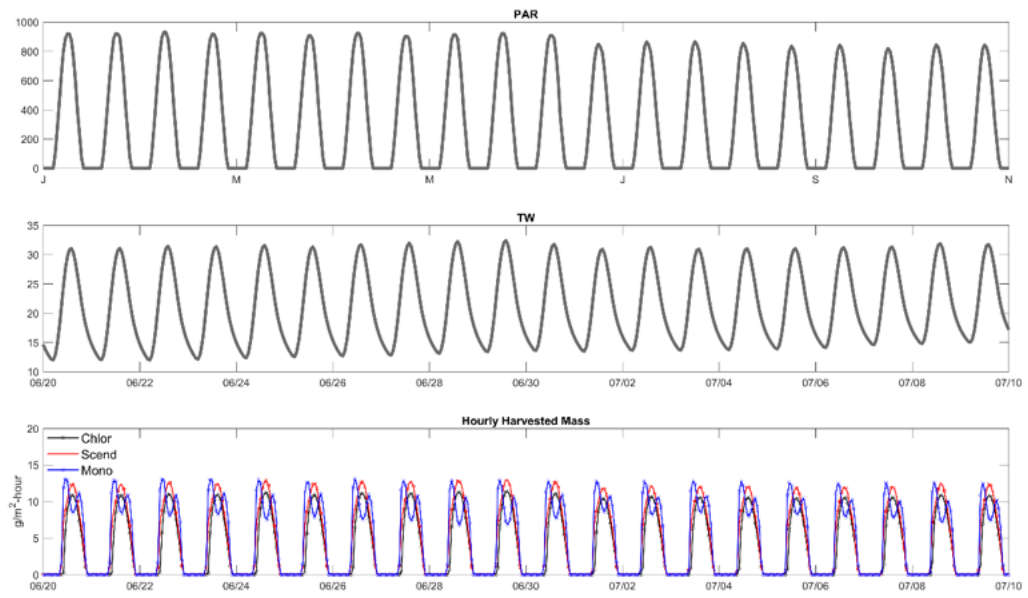
Overall, these findings are consistent with expected regional and seasonal performance of the three strains. However, a couple anomalies warrant further investigation and further illustrate the use of modeling to support

development of operational strategies. For example, the dominance of *Monoraphidium* in Tucson, AZ, was a bit unexpected. To help understand this, Figure 30 shows the 30-year average hourly time series from June 20 to July 10 of PAR, water temperature (°C) and harvested mass (g/m<sup>2</sup>-hr). During this time period, on average, PAR is less than 1,000 and water temperature ranges from about 15 to 30 °C but most often is less than 25 °C. Under these conditions, according to their respective growth curves and as confirmed by the harvested mass, *Monoraphidia* grows better than *Chlorella* and *Scenedesmus*. Regarding the three months where *Chlorella* is optimum for the two Florida sites, would there be a significant advantage to using a three-strain rotation instead of a two-strain rotation? To answer this question, Figure 31 shows the 30-year monthly average productivities for four sites including Ft Meyers and Vero Beach FL. The plots show that when *Chlorella* outperforms *Scenedesmus*, it is by only a slight margin. Therefore, it is reasonable that a two-strain strategy would be adopted to avoid the complexity of cultivating three strains.

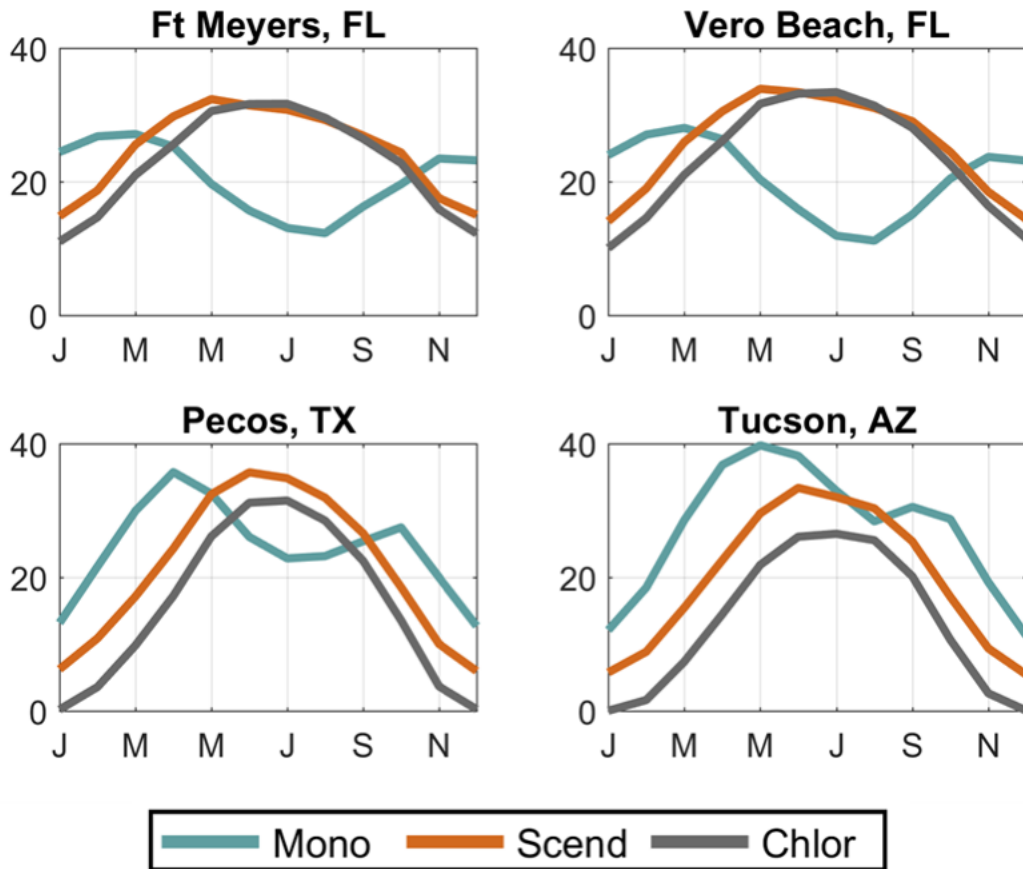
**Table 7.** Maximum potential for biomass production through strain rotation based on 30-year simulations at selected NLDAS sites considering three algae strains (*Chlorella*, *Scenedesmus* and *Monoraphidium*), three pond water depths (15 cm, 20 cm, and 25 cm) and three harvest concentrations (300 mg/L, 500 mg/L, and 700 mg/L).

Location	Lat	Lon	Month											
			1	2	3	4	5	6	7	8	9	10	11	12
Vero Beach, FL	27.94	-80.94	M	M	M	S	S	S	C	C	S	S	M	M
Ft Meyers, FL	26.56	-81.94	M	M	M	M	S	S	C	S	S	M	M	M
Mesa, AZ	33.31	111.69	M	M	M	M	M	S	S	S	S	M	M	M
Tucson, AZ	32.19	-110.94	M	M	M	M	M	M	M	S	M	M	M	M
Sacramento, CA	38.56	-121.56	M	M	M	M	M	S	S	S	M	M	M	M
Independence, KS	37.19	-95.69	M	M	M	M	M	S	S	S	M	M	M	M
Chapel Hill, NC	35.94	-79.06	M	M	M	M	M	S	S	S	M	M	M	M
Las Cruces, NM	32.31	-106.94	M	M	M	M	M	S	S	S	M	M	M	M
Corvallis, OR	44.56	-123.31	M	M	M	M	M	M	M	M	M	M	M	M
Lancaster, PA	40.06	-76.44	M	M	M	M	M	M	S	M	M	M	M	M
Pecos, TX	31.44	-103.56	M	M	M	M	M	S	S	S	S	M	M	M

\* Note: C = *Chlorella*, M = *Monoraphidium*, S = *Scenedesmus*.



**Figure 31.** The 30-year average hourly time series of PAR ( $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ), water temperature ( $T_w$ ,  $^{\circ}\text{C}$ ), harvest mass ( $\text{g/m}^2\text{-hour}$ ) for Tucson, AZ.



**Figure 32.** The 30-year monthly mean productivity ( $\text{g/m}^2\text{-day}$ ) with culture depth of 15 cm and 300 mg/L harvest concentration.

A number of publications, below, resulted from this work.

### Citations

Edmundson, S.J., R. Kruk, and M.H. Huesemann. Winter Algae Crops: Characterization of Algal Strains with Cold Season Production Potential. *Environmental and Experimental Botany*. (In preparation, 2018).

Huesemann, M.H., B. Crowe, P. Waller, A. Chavis, S. Hobbs, S. Edmundson, and M. Wigmosta. 2016. A validated model to predict microalgae growth in outdoor pond cultures subjected to fluctuating light intensities and water temperatures. *Algal Research* **13**:195-206.

Huesemann, M., T. Dale, A. Chavis, B. Crowe, S. Twary, A. Barry, D. Valentine, R. Yoshida, M. Wigmosta, and V. Cullinan. 2017a. Simulation of outdoor pond cultures using indoor LED-lighted and temperature-controlled raceway ponds and Phenometrics™ photobioreactor. *Algal Research* **21**:178-190.

Huesemann, M., A. Chavis, S. Edmundson, D. Rye, and M. Wigmosta. 2017b. Climate-simulated pond culturing: Quantifying the maximum achievable annual biomass productivity *Chlorella sorokiniana* in the United States. *Journal of Applied Phycology* to be submitted before Christmas.

Huesemann, M.H., N. Sun, M. Wigmosta, R. Skaggs, and P. Waller. Enhancement of the Biomass Assessment Tool Growth Model to Evaluate Alternative Strategies for Optimizing Biomass Productivities of *Chlorella sorokiniana* (DOE 1412). In preparation, 2018.

Perkins, W.A. and M.C. Richmond. MASS2, Modular Aquatic Simulation System in Two Dimensions: Theory and Numerical Methods. Report PNNL-14820-1. Pacific Northwest National Laboratory, Richland, Washington.

Wigmosta, M.S., A.M. Coleman, R.J. Skaggs, M.H. Huesemann, and L.J. Lane. 2011. National microalgae biofuels production potential and resource demand. *Water Resources Research* **47**.

## Results of UA Raceway Performance Modeling

Early UA RAFT experiments had observed productivities that were much lower than PNNL biomass growth model predictions. To account for actual conditions in the experimental paddlewheel raceway (1,000 L), ARID-HV raceway and paddlewheel ARID raceway, the team added nitrogen, salinity and shading algorithms to the PNNL biomass growth model and modeled temperature and evaporation in the experimental raceways. The original models were in Excel Visual Basic for Applications (VBA), but the models were converted to python and the team organized the entire set of RAFT experiments in Tucson in a database to efficiently model them.

The temperature and evaporation models initially included the temperature and evaporation algorithms from the PNNL open pond model. Recorded temperature and evaporation data from several years of UA RAFT experiments enabled calibration of the temperature and evaporation models to the experimental paddlewheel raceways. The best evaporation model was the Brady equation.

$$E = 6.9 + 0.49 W_2^2 (e_s - e_{air})$$

where

E = latent heat of vaporization

W = wind speed

$e_s$  = saturated vapor pressure

$e_{air}$  = actual vapor pressure

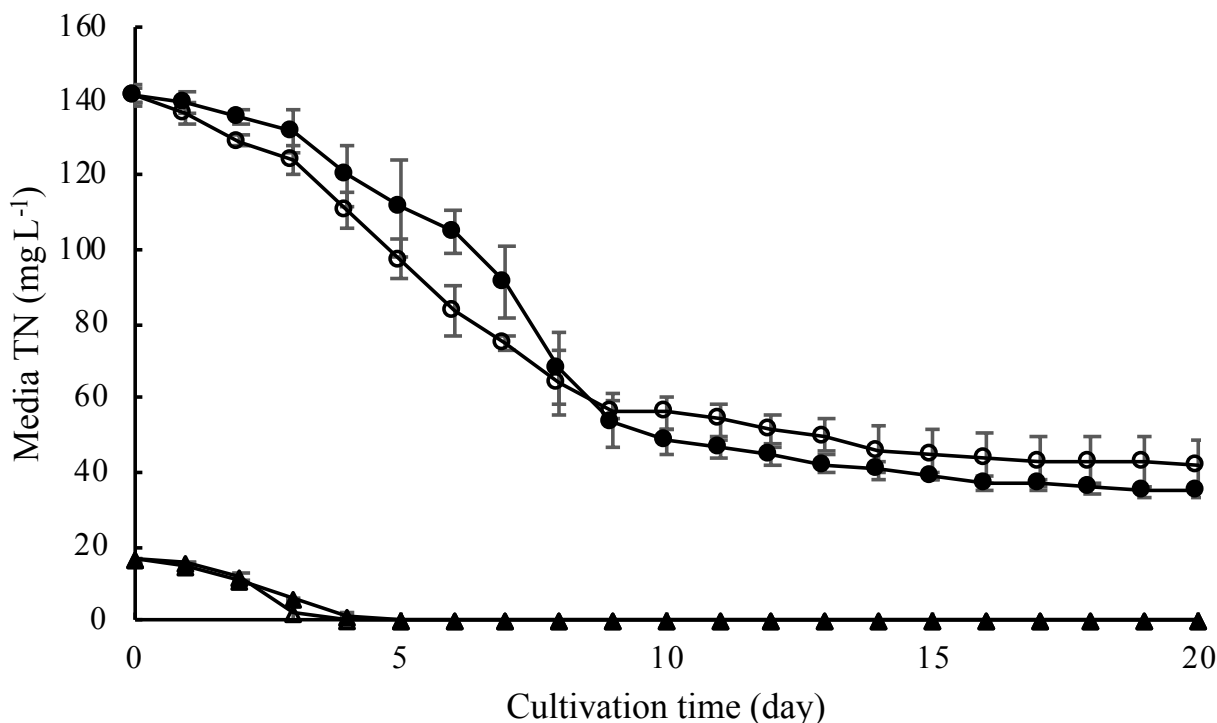
The energy balance model for calculating experimental raceway temperatures includes the latent heat of vaporization, solar radiation, long wave radiation and sensible heat flux. The team developed a shading model of the experimental paddlewheel raceways to calculate solar position and then define the shading line in the raceway. The model then uses various mathematical techniques to define the percent shaded area in the

raceway. Combined with albedo calculation, this model provides a more accurate estimate of solar energy input into the raceway than a model that ignores shading. This new shading model, combined with the Brady model of evapotranspiration (latent heat), the Bowen ratio model of sensible heat flux and the Brunt model of long wave radiation, provided the best fit to observed raceway temperature data. Two papers are in preparation that summarize the results of the evaporation, shading, and temperature simulations.

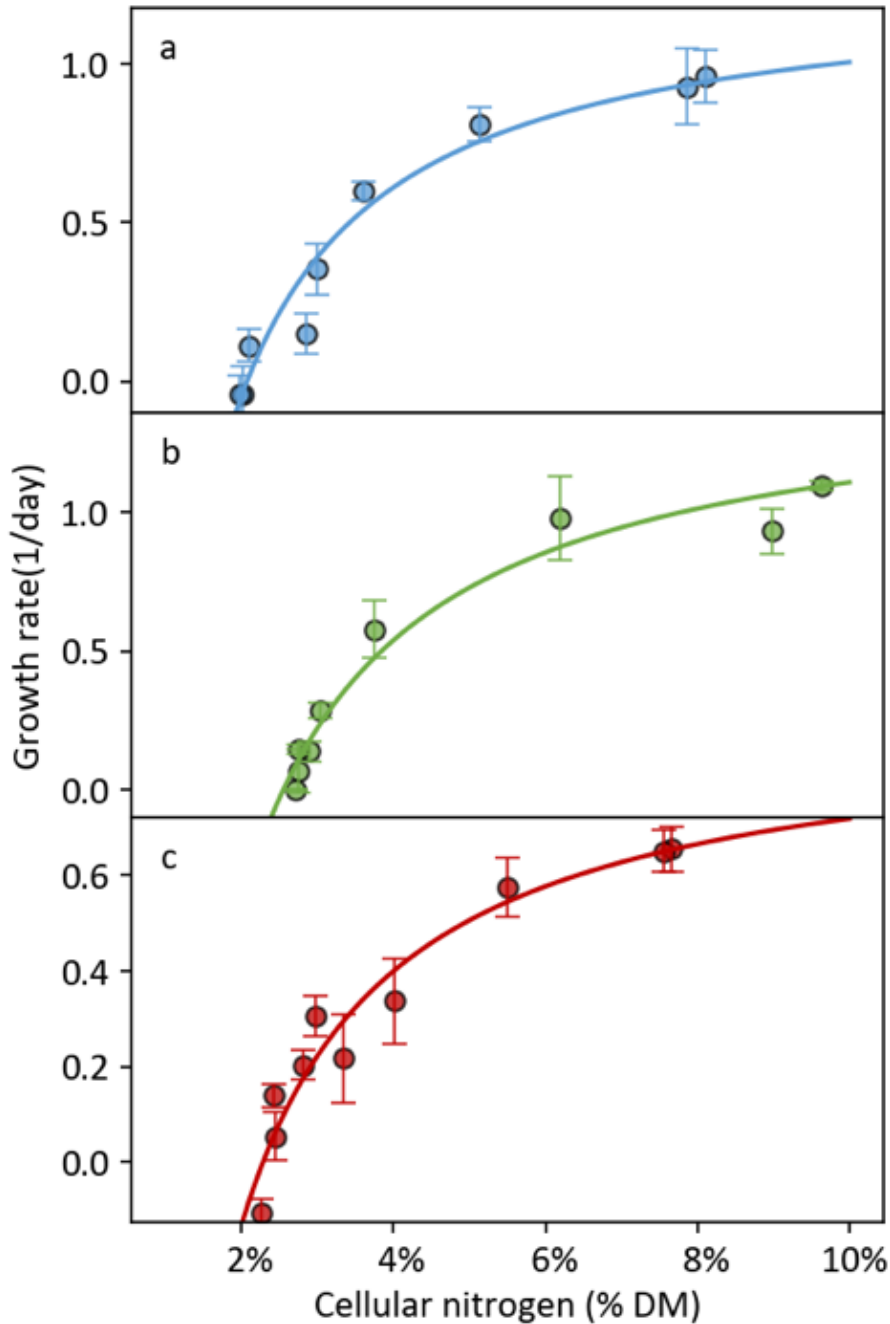
The RAFT project experiments had a pattern of reduced growth after the first batch. Experiments at PNNL-Sequim showed that nutrient depletion was the probable cause. The RAFT experiments also had an extremely wide range of nutrient productivities (biomass/applied nutrients), with dramatically low nutrient productivities in batches with excessive nutrient application and extremely high nutrient

productivities (but low overall productivity) in batches with nutrient stress. This variability demonstrated the need to develop models of growth rate vs. nutrient availability and guidelines for nutrient application rates.

The team also conducted nutrient experiments. The first set of experiments evaluated the required levels of nitrogen and phosphorous. The researchers evaluated four *S. obliquus* treatments with nitrogen concentrations of 15 (N-) and 45 mg/L (N+) and phosphorous concentrations of 2.5 (P-) and 11 mg/L (P+). Figure 32 shows nitrate concentration in the media vs. time for the four treatments and also demonstrates that intracellular nutrient storage was significant because intracellular storage supported growth after nutrient depletion. The maximum nitrogen yields and phosphorus yields were 49 g-dry biomass/g-N and 813 g-dry biomass/g-P, which were achieved simultaneously at an N/P ratio of 16.6.



**Figure 33.** Total nitrogen (TN) concentration in media under different culture conditions during the experiment: N-P- ( $\Delta$ ), N+P- ( $\circ$ ), N-P+ ( $\blacktriangle$ ), and N+P+ ( $\bullet$ ). Data are given as mean  $\pm$  standard deviation of triplicates.



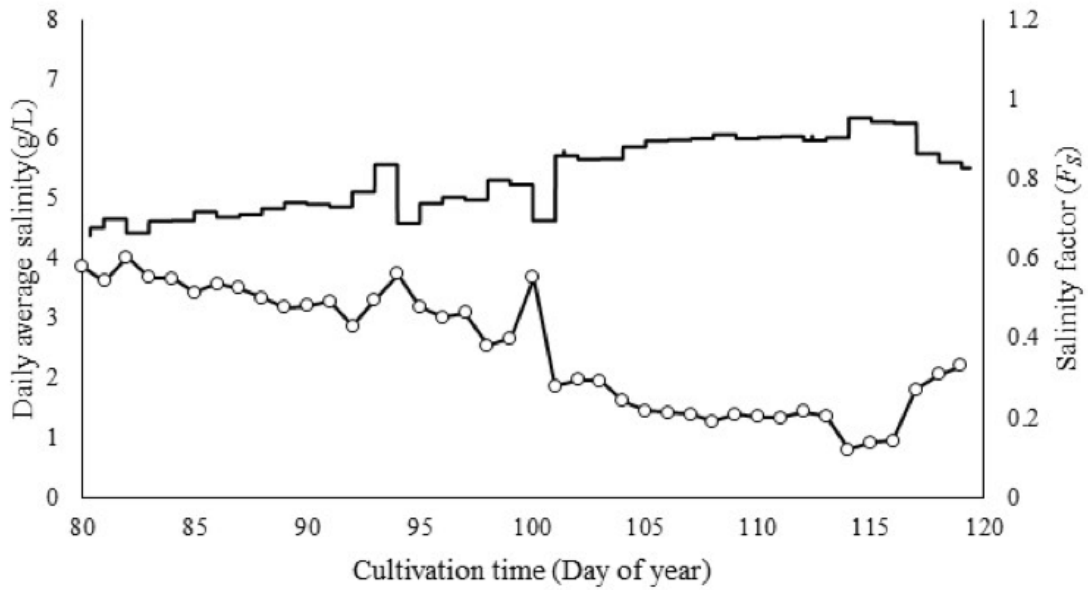
**Figure 34.** Plot of specific growth rates (dots) and the cell quota model (solid lines) as a function of cellular nitrogen for a) *S. obliquus*, b) *C. sorokiniana*, and c) *M. minutum*. Data are given as mean  $\pm$  standard deviation of triplicates.

The team then modified the nutrient depletion experiments to maintain constant light intensity by harvesting at the beginning of each day to have the same biomass concentration at the start of each day. In this experiment, three microalgae were cultured under nitrogen-deplete conditions, with a range of nitrogen stress levels. The team monitored growth rate, cellular nitrogen, chlorophyll content, lipid content and fatty acid composition as growth rate diminished due to nitrogen limitation. Growth rate was highly correlated with cellular nutrient concentration (Figure 34). Nutrient concentration also was highly correlated with optical density in the range sensitive to chlorophyll content, which can be used to evaluate nitrogen status in microalgae culture.

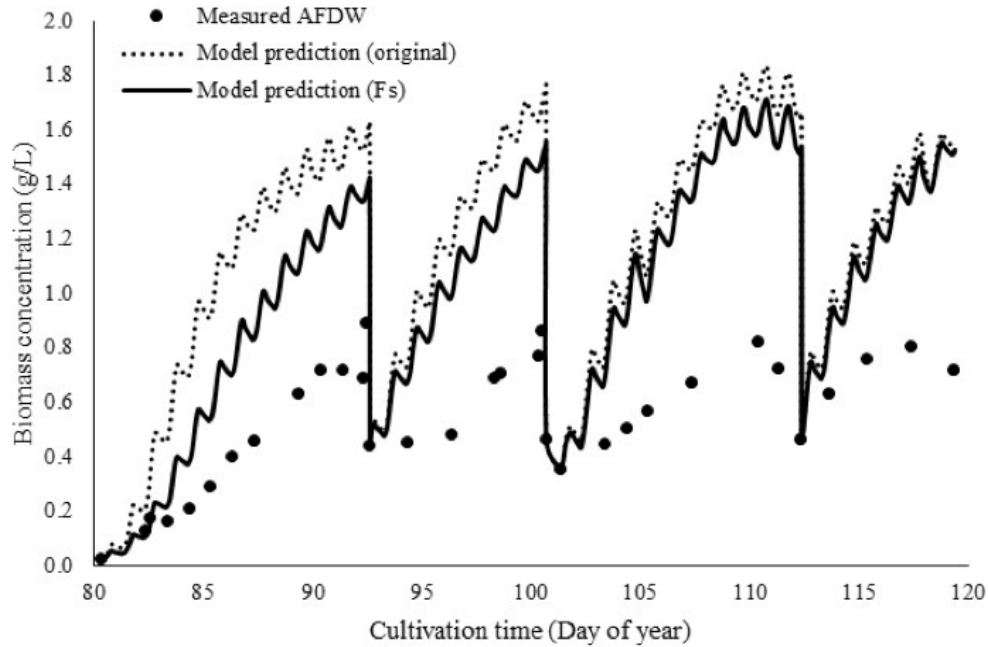


The team also evaluated salinity, nitrogen and shading in the HABG (Huesemann Algae Biomass Growth) model. A salinity stress algorithm was developed in the Huesemann laboratory, in which the relative growth rate was calculated as a function of salinity. Figure 35 shows the daily salinity and associated salinity growth factor in the RAFT 07 experiment with *S. obliquus* in the ARID-HV

raceway. Figure 35 shows the reduced calculated growth rate based on the product of the salinity factor and the unmodified growth rate from the HABG model. Note that the unmodified growth rate is extremely high compared to the observed growth rate and that including the salinity brings the simulated growth rate closer to, but still far higher than, the observed growth rate.

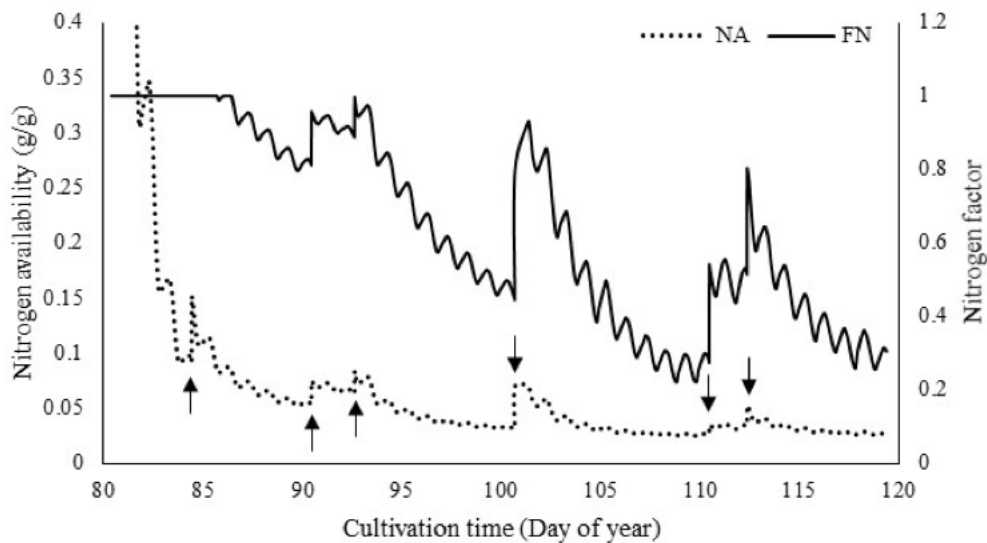


**Figure 35.** Daily average salinity (○) and corresponding salinity factor  $F_S$  (—) during RAFT07 ARID raceway experiment.



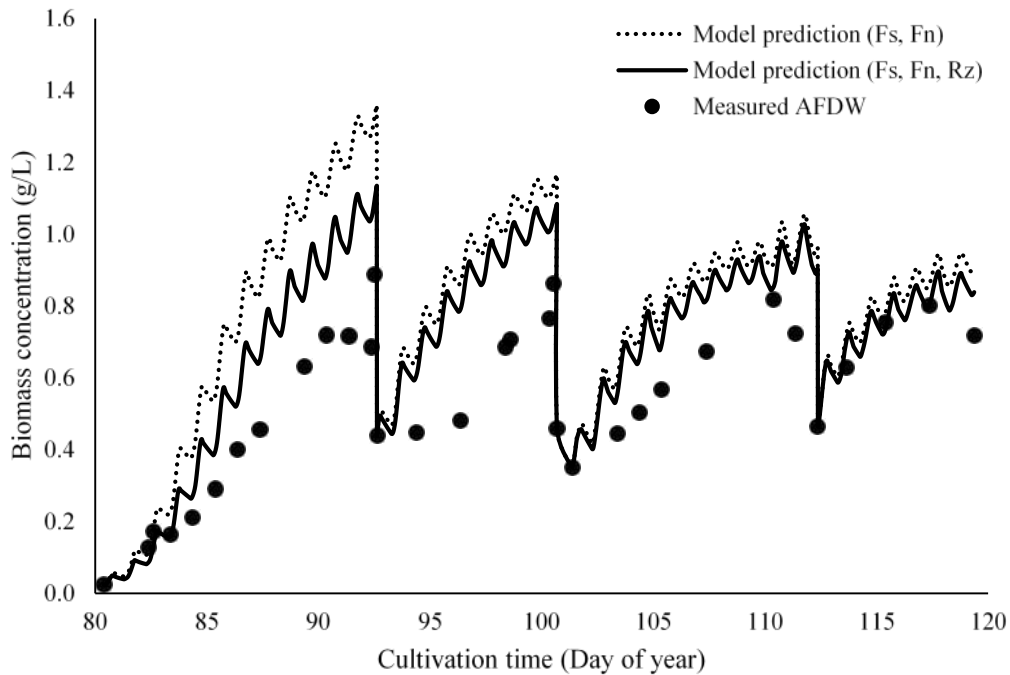
**Figure 36.** Measured biomass concentration, original growth model prediction and Fs - adjusted model prediction.

Based on results from this evaluation, the concept of nitrogen availability was derived (added nutrients divided by cell biomass) to develop a method to calculate growth reduction as a function of added nitrogen in fertilizer and biomass concentration. Figure 37 shows the nitrogen availability and the associated nitrogen growth factor (fraction of maximum) for the RAFT 07 experiment in the ARID-HV raceway.



**Figure 37.** Nitrogen availability (NA) and nitrogen factor (FN) as a function of time during RAFT07 experiment. NA increases due to nutrient addition (○).

Finally, the team added a shading algorithm to the HABG model to calculate the light intensity and fraction shaded at each layer based on raceway geometry, solar angle, albedo, angle of refraction and measured light intensity data (Figure 38). The estimated growth rate is much closer to the observed growth rate with all of these factors included in the HABG model.



**Figure 38.** Measured biomass concentration, FS FN - adjusted model prediction, and FS FN Rz - adjusted model prediction.

Comparison of the model with observed data indicated that ignoring the lag phase also resulted in overprediction. Delaying the start of the simulation until the end of the lag phase and inclusion of stress factors lowered model inaccuracy from 90% over-prediction to within  $\pm 10\%$  in six out of seven batches.

In addition, the team constructed and tested a new ARID raceway with paddle using data from the models to write two papers on temperature and growth in the new paddlewheel ARID raceway. A provisional patent was filed on this technology.

Several publications, below, resulted from this work.

### **Citations**

Attalah, S., P. Waller, G. Khawam, and R. Ryan. 2015. Energy productivity of High Velocity Algae Raceway Integrated Design (ARID-HV). Transactions of the ASABE 31(3):365-375.

Attalah, S., P. Waller, S. Steichen, J. Brown, and K. Ogden. Application of deoxygenation-aeration cycling to control the predatory bacterium *Vampirovibrio chlorellavorus* in *Chlorella sorokiniana* cultures. (Submitted, 2018).

Attalah, S., P. Waller, S. Steichen, S. Gao, C. Brown, J. Brown, and K. Ogden. Technoeconomic assessment of deoxygenation for control of *Vampirovibrio chlorellavorus* in *Chlorella sorokiniana* algae cultures. (In preparation, 2018).

Song, G., P. Waller, G. Khawan, S. Attalah, M. Huesemann, and K. Ogden. Incorporation of salinity stress, nitrogen stress, and shading into the HABG algae growth model. (Submitted, 2018).

Song, G., P. Waller, K. Ogden, and R. Qiu. Intracellular storage and the minimum amount of nitrogen and phosphorus required for growth of *Scenedesmus obliquus*. (In progress).

Song, G., R. Qiu, L. Toscano-Palomar, P. Waller, and K. Ogden. Nitrogen stress and health indicator in microalgae cultures.



# RAFT DATA

The primary objective of this project was to obtain long-term seasonal cultivation data in open systems across the southwestern United States. The public may access the data online at <https://raft.arizona.edu/cultivation-data/>. The data can be searched by strain, reactor type, location, depth, start date of the cultivation experiment, duration of experiment, maximum productivity, average productivity and maximum concentration. When a researcher double

clicks on the “experiment label,” an Excel spread sheet is downloaded. Within the spread sheet is discrete data, continuous data and some experimental comments, and there is either a link to weather station data or that data are included. The RAFT website (<https://raft.arizona.edu>) contains information regarding the strains, reactors and locations. If a researcher uses the data, please notify Kimberly Ogden via email ([Ogden@email.arizona.edu](mailto:Ogden@email.arizona.edu)).



# POND CRASHES

There is one part of cultivating algae that no one wants to discuss and of course is not published - how often ponds crash. Cultivating algae in open ponds is challenging, and a myriad of things can go wrong, so information regarding pond crashes from the University of Arizona testbed site is included here for completeness. A pond crash is defined as a drop in cell mass in the pond. Most of the experiments ended when the algae were no longer viable or when microscopic evaluation of the culture showed that there were many contaminant organisms in the pond in addition to algae. Recall that the goal of the RAFT project was to cultivate algae all year overall seasons, so the team kept the algae growing as long as possible, harvesting 75% of the biomass prior to the culture entering stationary phase.

Table xx provides a summary of why open pond experiments (RAFT 17 – 38) were terminated. These experiments were done in both paddlewheel systems (1000 L) and the ARID raceway using all 3 of the algal species (CS – *Chlorella sorokiniana*; MM – *Monoraphidium minutum*; SO – *Scenedesmus obliquus*). Many of the runs using *Chlorella sorokiniana* crashed

because *Vampirovibrio chlorellovorus* was present in the pond and caused the algae to die. However, the team discovered that addition of 1.5 to 2 ppm benzalkonium chloride (BKZ) every 4 days controls *Vampirovibrio chlorellovorus*. Many of the experiments shown in Table xx involved growing *C. sorokiniana* with various concentrations of BKZ that were added to the pond at varying intervals to develop a robust strategy for cultivation.

Another common problem was the presence of ciliates or protozoa that outcompete the algae. This was an issue regardless of the type of algae that was cultivated. Sometimes there were operational issues like leaks in the CO<sub>2</sub> supply or failed inoculation. Finally, sometimes the weather was just not conducive to algal cultivation. In Arizona, this is a few days during the monsoon season when up to 3 inches of rain can fall in an hour. The ponds tend to overflow. Every area has weather events that are out of our control like hurricanes or heavy rain fall in the Southern US. The good thing is that the ponds can be restarted quickly, and production continues.

Run	Reactor	Strain	Inoculation	Start of Crash	Crash Description	BZK Strategy	Days to crash
RAFT 17	ARID	CS	4/15/16 10:45	4/26/16 9:00	Crash. Concurrent VV spike. Ciliate count low ~6.	Cl- (0.5 ppm) during crash	11
RAFT 17	PW1	CS	4/14/16 15:00	5/11/16 9:00	Stagnant growth. Outcompeted by haematococcus.	BZK (2 ppm) during crash. pH 6 culture shock.	27
RAFT 17	PW2	CS	4/14/16 15:00	5/11/16 9:00	Crash. Slow growth prior to crash. OD drop 5/11. 5/10 - 5/11 small VV biomass spike. 5/11-5/12 large VV biomass spike.	Cl- (0.5 ppm). 3 doses.	27
RAFT 18	ARID	CS	5/5/16 14:00	5/29/16 9:00	Crash. 5/29 slight OD drop. 5/31 crash. VV media fraction and ciliate count increased at time of crash.	BZK (2 ppm), weekly.	24
RAFT 18	PW1	CS	5/19/16 17:00	6/2/16 9:00	Crash. VV spike 5/31 to 6/1. 6/1 harvest. Continued decline after harvest. Low ciliate count (~20)	No BZK control	14
RAFT 18	PW2	CS	5/19/16 17:00	6/9/16 9:00	Crash. VV spike concurrent with crash. Low ciliate count.	BZK (2 ppm), weekly.	21
RAFT 19	PW1	SO	6/9/16 9:00	6/23/16 9:00	Stagnant growth. Outcompeted by haematococcus.	N/A	14
RAFT 20	ARID	CS	6/14/16 15:30	6/27/16 9:00	Microburst caused flooding, electrical and structural damage. Compromised culture was terminated.	Post-harvest (2 ppm)	13
RAFT 20	PW2	CS	6/14/16 15:30	6/26/16 9:00	OD drop on 6/26. Ciliate count low, VV data unknown. Microburst on 6/27 led to heavy contamination and culture was terminated	Post-harvest (2 ppm)	12
RAFT 21	ARID	CS	7/11/16 19:30	7/23/16 9:00	Excessive leaking/ culture loss. Multiple leaking issues due to previous microburst. Early termination to repair damage.	1st @ OD=0.5 (2 ppm), post harvest (2 ppm)	12
RAFT 22	PW1	SO	8/12/16 14:40	8/24/16 8:00	Early termination/harvest.	N/A	
RAFT 22	PW2	SO	8/12/16 14:40	8/26/16 8:30		N/A	14
RAFT 23	ARID	CS	8/31/16 12:30	9/1/16 9:00	Failed inoculation. Immediate crash. No VV or invaders. Possibly residual chlorine in inoculation water		1
RAFT 23	PW1	CS	8/30/16 13:45	9/22/16 9:00	Early termination/harvest	Every 4 d (2 ppm)	
RAFT 23	PW2	CS	8/30/16 13:15	9/10/16 9:00	Crash. VV media and biomass spikes at time of crash. Low protozoan invader counts	No BZK control	11
RAFT 24	ARID	CS	9/15/16 10:45	11/16/16 9:00	Vc 16S biomass and media spike on 11/16. Ciliate count ~20. 6/17 protozoa infestation + damaged cells + yellow culture. 6/15 to 6/16 DO drop.	Inoculation (0.5 ppm), 1st @ full scale, every 4 days (1.3 ppm)	61
RAFT 25	PW1	CS	9/23/16 11:00	10/19/16 9:00	Early termination/harvest	Every 4 d (2 ppm)	
RAFT 25	PW2	CS	9/23/16 11:00	10/5/16 10:00	Crash. Infested with protozoan invaders. VV data unknown.	No BZK control	12
RAFT 26	PW1	CS	10/20/16 12:45	11/3/16 8:00	Early termination. Harvest. Healthy. OD750 = 1.24. Some ciliates	Every 4 d (2 ppm)	
RAFT 26	PW2	CS	10/20/16 12:45	10/28/16 9:00	Crash. OD dropped from 0.44 to 0.21. Flagellates increased from 100 to 1000/slide. VV data unknown.	No BZK control	8
RAFT 27	PW1	CS	11/9/16 15:00	11/30/16 9:30	Early termination. Healthy. OD750 = 0.898. Clean.	Every 4 d (2 ppm)	
RAFT 27	PW2	CS	11/9/16 15:00	11/19/16 9:00	Crashed. OD dropped from 0.7 to 0.6. VV biomass fraction increased steadily and then spiked between 11/18 and 1/20. Invader count was 20/slide at crash and increased to 2000/slide by termination (opportunists). Observation of vacuuous cells.	No BZK control	10
RAFT 28	ARID	MM	11/29/16 12:00	12/27/16 14:00	Crash. Very rainy season. 1670 L of rain prior to start of crash on 12/27. Additional 700 L of rain by 1/1. Overfilled reactor. Sump pump malfunction due to precipitation and settling algae combined with overcast days meant limited agitation. Replacement sump pump also failed. Invaders.	N/A	28

Run	Reactor	Strain	Inoculation	Start of Crash	Crash Description	BZK Strategy	Days to crash
RAFT 29	PW1	SO	12/2/16 12:15	1/23/17 12:30	Early termination. Relatively healthy, OD750 = 1.3. Ciliates	N/A	
RAFT 29	PW2	SO	12/2/16 12:15	1/23/17 12:30	Early termination. Harvest. Healthy. OD750 = 1.3. Some invaders	N/A	
RAFT 30	PW1	SO	1/23/17 17:00	2/23/17 10:30	Early termination. Healthy. (20 inv/slide)	N/A	
RAFT 30	PW2	SO	1/23/17 17:00	2/17/17 10:00	Crash. OD drop from 0.72 to 0.70. Many protozoan invaders. Few rain events, few issues with pH regulation and spikes to pH 9. 2/16, temp drop to 50C coincided with ciliate count reduction from 50 to 4.	N/A	25
RAFT 31	ARID	MM	1/24/17 12:45	2/23/17 10:00	Crashed. OD dropped from 0.41 to 0.38. Culture showed signs of physical stress. Abundant rain on 2/19 and 2/20 (~ 400L). pH spiked to 9 on 2/19. Invader counts ~10, increased to 1000 by 3/1, 3/1 experimental 1.5 ppm BZK dose reduced ciliate count to 12 on 3/2. Productivity continued to decline.	Attempted ciliate crash control (1.5 ppm)	29
RAFT 32	PW1	SO	2/24/17 14:00	3/14/17 10:00	Crash. OD drop 0.63 to 0.60. Cells became pale and damaged. Stressed scenedesmus, pairs and single cells, clumping. Ciliates. Notes suggest physical stress, but pH, temperature, and rain data appear normal	N/A	17
RAFT 32	PW2	SO	2/24/17 14:00	3/15/17 10:00	Crash. OD drop 0.61 to 0.53. Low nutrient stress, started to appear yellow. Yellow and clumping cells. Ciliate contamination.	N/A	17
RAFT 33	ARID	CS	3/27/17 11:30	4/11/17 10:45	Crash. Stressed culture due to operational issues. Delayed CO2 shipment. pH spiked to 9-10 for an extended time. At first (4/11) clumping cells and a few vacuous cells were observed, followed (on 4/12) by many damaged/clumping/vacuous/ lysed and few ciliates (#15), followed (on 4/13) by a brownish culture infested with ciliates (#1000).	Initial (0.5 ppm), every 4 d or post-harvest (1.3 ppm). Unknown [BZK] in inoculum. 4 doses	15
RAFT 34	ARID	CS	4/18/17 9:00	5/17/17 10:00	Crash. Following the 6th harvest, the OD continued to decline (0.5 to 0.3). Invaders count inc. from 50 to 300. Dusty rainy conditions may have played a role. VV data unknown.	Post-harvest (1.3 ppm). Initial (0.5 ppm). Additional dose during crash. 7 total	30
RAFT 35	PW1	SO	4/20/17 14:00	5/3/17 10:30	Crash. OD drop 1.1 to 0.9. Ciliate count 12 to 50 followed by gradual increase. Unintended snd/chl polyculture. Pale bubble like cells. On 5/4, Chl cell count 1 log reduction, 6 log drop to n.d. in one day. By termination on 5/5/2017, OD = 0.7. Ciliate # = 7000/slide.	N/A	13
RAFT 35	PW2	SO	4/20/17 14:00	5/2/17 10:00	Crash. OD drop from 0.9 to 0.7. Productivity decline on 5/30/2017 from ~12 to ~2 g/m <sup>2</sup> /day. Invaders = 400 (ciliates and flagellates). By termination on 5/3/2017, OD750 = 0.6. Inv # = 500	N/A	12
RAFT 36	PW1	CS	5/30/17 15:00	6/13/17 9:30	Early termination. Healthy. (45 inv/slide). VV detected on 6/10	Initial 1.3 ppm dose	
RAFT 36	PW1	CS	5/30/17 15:00	6/12/17 9:15	Crash. OD drop 1.6 to 1.1 + ciliate infestation (20000/slide). VV spike on 6/10 along with ciliate spike from n.d. to 24, followed by continued increase of both VV and ciliates.	Initial 1.3 ppm dose	12
RAFT 37	PWA	CS	5/31/17 12:00	6/25/17 8:30	Crash. Infested with ciliates. VV data unknown. Recycled media harvesting strategy, additional nutrients were not dosed at harvest prior to crash.	BZK + Recycled Media. Post-harvest (1.3 ppm). Initial (1.3 ppm). 8 total	25
RAFT 37	PW1	CS	6/13/17 13:00	6/19/17 7:30	Crash. Ciliate count spiked ~100 to ~1000. Night time DO dropped 6/18 to 6/19. Peak DO dropped 6/20 - 6/21	Initial (1.3ppm)	6
RAFT 38	PW2	CS	6/13/17 13:00	6/17/17 10:00	Crash. Inoculum contaminated with invaders and VV. Final VV data unknown.	No BZK control	4

# CULTIVATION BEST PRACTICES AND CONCLUSIONS

## Best Practices

A key result of the RAFT project is the development of best practices regarding outdoor cultivation of algal cultures to maximize productivity and minimize down time. This section will summarize suggestions for laboratory-scale characterization, strain maintenance, culture scale up, outdoor cultivation, data management and modeling.

### Characterization

Prior to running outdoor experiments, the team found that characterizing the strains in the laboratory aided in assuring outdoor success. More specifically, the team recommends determining the temperature, salinity and pH tolerance ranges prior to outdoor experimentation and then developing and implementing a crop rotation strategy. Ideally, the experiments are done in the LEAPS photobioreactors but can be done in other bioreactors that have temperature and pH control. When the researchers understand temperature tolerance, they propose a crop rotation strategy for a given location based on average seasonal weather data. Models can be used to determine the best strain rotation schedules, optimum culture depth and harvesting cycles for the characterized strains.

### Strain maintenance

Robust protocols for maintaining inoculum are another important aspect in the success of outdoor cultivation of microalgae. Culture maintenance is best done in a

growth room, growth chamber or, at a minimum, incubator/shakers fitted with fluorescent lamps that are on timers to allow for dark/light cycles. The team recommends aseptic techniques. It is advisable to have stock cultures on plates for long-term storage as well as in small liquid cultures of 50 to 500 mL. For plates, the light intensity is typically less than  $200 \mu\text{M}/\text{m}^2/\text{s}$  to avoid bleaching the culture. Antibiotics can be added to the plates to avoid bacterial contamination if the algal strain is resistant. For the liquid cultures, bicarbonate is typically supplied as the carbon source, cultures are agitated on an orbital shaker at approximately 200 rpm, and the cultures are visually inspected under a microscope at least weekly to assure the correct phenotype of the algae and to determine if the culture is contaminated. If needed, 1.5 ppm of benzalkonium chloride can be added to liquid cultures to limit bacterial contamination, but this can stress the cultures.

### Scale up

The success rate of scaling up cultures is higher when the inoculum is 5% or more on a volume basis. For example, a minimum of 500 mL of a dense culture (0.5 g/L) serves as the inoculum for 10 L. At this scale, the light intensity initially can be low, at  $200 \mu\text{M}/\text{m}^2/\text{s}$ , but can be increased to  $500 \mu\text{M}/\text{m}^2/\text{s}$  to avoid light limitation and to acclimate the cultures to the higher intensities observed outside. Agitation is accomplished by air sparging or ideally an air/carbon dioxide mixture (95/5). The growth of the scale-up

cultures is monitored every 48 to 72 hours; this can be done using a spectrophotometer. Periodically inspecting the culture with a microscope is a must.

The final step is scale up to outdoor systems such as paddlewheels. This step can be challenging because it usually involves moving from 10 to 20-liter carboys to 400 to 1,000-liter systems. Ideally, the 5% inoculum rule is followed but sometimes the researchers may not have all the necessary intermediate size reactors. Then, the best philosophy is to start at lower depth in the system and then scale up step-wise when the cell density is 0.05 g/L or higher. For example, a 20-liter carboy of algae serves as the inoculum for 400 liters of media in a 1,000-liter reactor initially. When the culture has doubled, add more media to bring the culture to the final total volume of 1,000 liters. Sometimes this is done by increasing the depth of the culture from 10 cm to 20 cm to 30 cm, etc.

### Outdoor cultivation

Once the culture is in an outdoor system, researchers continuously monitor many parameters, conduct discrete sample analysis daily and control the pH by carbon dioxide addition. Several data systems can be used. A common one used by the industry is the YSI 5200A system that monitors dissolved oxygen, temperature, conductivity, pH, ORP and salinity. The RAFT team used a variety of systems. The UA testbed choose to use a Campbell Scientific CR1000 measurement and control datalogger. This is a more generic datalogger that can be used for a wide range of measurement and control functions. The team recommends that, at a minimum, the dissolved oxygen, pH, electric conductivity and temperature are measured continuously. For example, actual measurements can be taken every 10 to 30 seconds, averaged, and stored every 10 minutes. The pH is controlled by carbon dioxide addition at a value of 8.0. The higher value limits contamination. Calibrating the flow meter to monitor the amount of carbon dioxide added is useful. Typically, the carbon dioxide source is disconnected or turned off during the night automatically since it is not used. Weather station data, including wind speed, wind direction, precipitation, solar radiance and air temperature are linked to the cultivation data.

Discrete sample analysis is also important. In addition to monitoring the amount of water and nutrients added, researchers take daily samples to determine the ash-free dry weight of the culture. Although this is tedious, it is important during research and development to understand how much biomass is present in an open system compared to how much dust or other non-organic material is present. Optical density measurements provide a quick measurement of algal growth and are straight forward. Evaluating the culture under the microscope is also extremely important for monitoring contaminants such as ciliates or rotifers as well as contaminant algae that have distinct phenotypes. Nutrient analysis is another useful tool during research and development. Algal cells have specific requirements in terms of nitrogen, phosphorous and some trace elements. At this point, unless determinantal to growth, all trace elements are supplied in excess. Nitrogen and phosphorous monitoring, however, helps researchers understand the requirements to optimize growth and cell composition in terms of percentage lipid, protein and carbohydrate. Hence, the team recommends monitoring the amount of these nutrients every 48 to 72 hours and relating to cell growth and composition information. Researchers can use ion chromatography or a total nitrogen analyzer for analysis.

Substantial discussion has focused on harvest strategies for outdoor systems. Some researchers have harvested 10% to 30% of every other day for example. This team chose to harvest 75% of the biomass near the end of linear growth phase. The philosophy is to maximize the use of nutrients and harvest before growth slows due to severe light limitation. This requires advance knowledge of the growth of the particular algal strain. This strategy also allows for consistency in the protocol regardless of season. For example, when the days are shorter and temperatures are cooler, the harvest may occur every 7 to 10 days, whereas in the summer the harvest may occur every other day. When the researchers harvest the reactors, they recycle the spent media to the reactors and add more nitrogen, phosphorous and trace elements. The team observed no adverse growth effects when media was recycled and hence recommends this procedure to conserve water.



As molecular diagnostic techniques continue to advance, the tools allow for characterizing the microalgae strains as well as the bacterial cells that are naturally found with the microalgae. The team recommends the routine use of molecular assays to identify both unknown microalgae contaminants and assure the presence of the specific project strains based on the sequences of their 18S small subunit ribosomal RNA genes (18S rDNA). To further understand the community of microorganisms or the phycosphere, the team recommends the use of 16s rDNA with the long-term goal of identifying both the helpful bacterial and the harmful ones as a measure of

culture health in open systems. A better understanding of these interactions will lead to more productive cultivation systems.

The final best practice is to continue implementing online monitoring of the microalgae culture environment and biomass concentration to significantly improve system process control. Most industries use process control strategies and the algal cultivation industry is behind. Further research, development and deployment of real-time sensors and control algorithms are key to increased productivity and less down time.

## Lessons Learned

### Strain Selection and Characterization

- Invasive strains may make excellent crop candidates (e.g., *M. minutum*, a NAABB isolate from the AgriLife Research Testbed); researchers should pay careful attention to isolating, identifying, evaluating and maintaining these strains. The RAFT project collected important outdoor cultivation data on this strain. (Note: *M. minutum* turned out to be the best (highest productivity) winter season strain among nine tested in the DISCOVER project under winter-season climate-simulated conditions in the LEAPS. It also performed best among three winter season strains tested outdoors in the DISCOVER project (no contamination, no crash). Most likely, this strain will be the winter season Statement of Technology (SOT) benchmark strain for many years to come for BETO, unless a better winter season strain can be found. NREL found this strain to have very favorable biomass composition.
- Crop rotation improves annualized productivity (i.e., using appropriate summer and winter strains). Finding strains optimized for different seasons is essential to effective crop rotation.
- To address crop rotation challenges, the team developed a more streamlined strain screening procedure (compared to NAABB) to quickly identify fast growing strains and identify their temperature tolerance range to match to the correct growing season.
- The team used the newly developed PNNL LEAPS climate-simulation photobioreactors to evaluate strains in terms of biomass productivity prior to deployment in outdoor RAFT testbeds. This down-select procedure is reducing the risk of outdoor strain deployment.
- An algal polyculture derived from locally occurring natural algal species had good long-term growth and stability. This could be a viable strategy for sustained algal cultivation.
- Water management is more important and encompassing than previously thought.
- Site specific bio-prospecting is important. Sometimes the best strains available are not found in the lab.

### Molecular Diagnostics

- Microscope-aided morphology with molecular diagnostics are extremely important for quality control at all levels of cultivation, from primary cultures to small and large scale ups.
- Algal taxonomic tools need huge improvement, curated databases; type collections standardization. Still unable to identify many algae to genus level by sequence comparisons; many unidentifiable even at higher level without experts.
- The U.S. has a serious shortage of phycologists and there is a need to provide support/incentives to train the next generation of phycologists.

- Metagenomic analysis of the phycosphere community has moved from a dream to a reality in five years. The best so far uses the V4 conserved region of ribosomal subunit (16S) and Silva database.
- A serious need exists for research on algal biology, physiology, metabolomes and biochemistry, as has been done for higher plants – knowledge essential to modulating conditions to optimize growth and productivity. Research with *Chlamydomonas* does not answer many questions relevant to cultivatable microalgae (Chlorophyta).
- Algal viruses and obligate pathogens are nearly entirely unstudied for fresh water algae. For cultivation to move forward successfully, a systems biology approach together with engineering advancements are essential.

### **Algal Cultivation**

- Growth media should be tested in ponds or pond simulators (such as LEAPS) prior to outdoor use to determine maximum achievable biomass yields to avoid growth limitations in outdoor ponds, and thus sub-optimal productivities.
- Growth media and cultivation practices should be aligned to provide either maximum biomass yield or maximum biomass productivity by continuous cell harvest at low cell densities (i.e., different media for different pond operations).
- Knowledge of strain-specific biomass losses over the dark period can influence the operational strategy chosen for managing a pond (e.g., optimal harvest time).
- Growing algae for long periods of time at a high rate of production is very challenging. There can be large unexplained differences in growth rates even between the same cultures grown at the same time.
- Integrating monitoring with management decisions – basis for, machine learning may offer lucrative approaches.
- Flexibility is key; large-scale cultivation is tough and uncertain. Being able to adapt to any situation is important.

### **Modeling**

- Begin modeling efforts with standard models from the literature that are designed for the system in question.
- Organize experimental and modeling data in database format that researchers can access through standard modeling languages such as python or C.
- Collaborate with other modeling and scientific groups.

# PUBLICATIONS, PRESENTATIONS, PATENTS

## Publications

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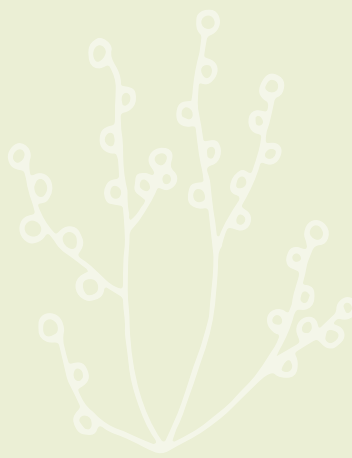
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