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DE-STRESSING BIOFUEL MICROALGAE: LEVERAGING BENEFICIAL MICROBIOMES TO ALLEVIATE HARMFUL CONDITIONS THAT REDUCE ALGAL GROWTH

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DE-STRESSING BIOFUEL MICROALGAE: LEVERAGING BENEFICIAL MICROBIOMES TO ALLEVIATE HARMFUL CONDITIONS THAT REDUCE ALGAL GROWTH

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ABSTRACT

Industrial-scale production of algal biofuels is hampered by two major stressors: environmental and biological. Specifically, high light intensities and temperatures combined with microbial predators can wipe out entire crops of microalgae, which increases prices (Shurin et al., 2013). We are examining interactions between *Desmodesmus* sp. CO46, a species of microalgae used in biofuel production, and its microbiomes (also known as bacterial communities) that attach to its cell periphery. This work seeks to establish and monitor bacterial species that beneficially interact with *Desmodesmus* to enhance its growth by mitigating the negative effects of high light, high temperature, and pathogen attack. Algal cells were cultivated with samples of diluted microbiome bacteria in sterile artificial seawater (F/2 media), and algal growth was measured via chlorophyll-a fluorometry. By monitoring samples of *Desmodesmus* cultured with simplified microbiomes under high light cycled incubation, we find an array of algal growth responses ranging from highly beneficial to severely negative. Utilizing this library of helpful microbiomes will assist with the reliable, larger scale cultivation of *Desmodesmus* for biofuels and bioproducts and provide cheaper renewable energy. Future experimentation is intended to compare bulk algal dry weight between the microbiomes when grown in largescale, to see whether combining beneficial simplified microbiomes would result in an additive effect on algal growth enhancement, and to investigate more closely the specific metabolic exchanges between mutualistic bacteria and *Desmodesmus*. Eventually, the beneficial bacteria will also be identified through DNA sequencing.

INTRODUCTION

Attention has been given to microalgae as a potential source of green energy due to its high concentration of lipids and renewability. In fact, biofuels produced from microalgae can be carbon neutral or even carbon negative. Through photosynthesis, they intake CO_2 produced from fuels and recycle it into algal biomass, which can then be stored, sequestered, or converted into other products. However, the production cost of algae-based biofuel is high, and the amount produced is low in comparison to those of current fuel sources (Wijffels and Barbosa, 2010). Due to the lack of knowledge on the factors influencing algal productivity, there is still much to be understood in terms of the ecology of microalgae in their environment. The scientific work described here has focused on efforts to find ways to increase their production efficiency.

The phycosphere of microalgae cells provides a key environment for nutrient exchange between microalgae and their "microbiome", also known as bacteria or bacterial communities. It is the environment immediately at the surface of algal cells where bacteria have direct access to attach to. Both the phycosphere and microbiomes are critical because they control the algal-bacterial metabolic interactions happen at the microenvironment - in other words, the "single-cell level". It is therefore crucial to study this intimate environment to fully understand the role of these microbes in the natural ocean ecosystem (Azam and Smith, 1991) and production-scale engineered ecosystems (). However, the type of interaction varies depending on the species of both the algae and bacteria. Bacteria can ingest organic products exuded by microalgae and then produce remineralized nitrogen as well as vitamins, growth promoters, and antibiotics important for microalgae growth. Additionally, bacteria can help shade algal cells from intense light. Despite this, bacteria can also compete with microalgae for inorganic nutrients, and some can even lyse dying algal cells to access nutrient stores (Seymour et al., 2017).

In their recent study at Lawrence Livermore National Laboratory (LLNL), Samo et al. experimented on two microalgae species used in biofuel production: *Nannochloropsis salina* and *Phaeodactylum tricornutum.* Each algae species was observed under exudate conditions, where the surrounding bacteria community was naturally occurring, and under phycosphere enriched conditions, where only the phycosphere involved bacteria were present. Algal growth was monitored by chlorophyll autofluorescence measurements as well as the release and uptake of carbon and nitrogen between the microalgae and bacteria using Nanoscale Secondary Ion Mass Spectrometry technology (NanoSIMS). The study showed that each algae species responded differently: *N. salina* did not seem to increase carbon fixation with attached bacteria while *P. tricornutum* did show significant growth improvement with attached bacteria (Samo et al., 2018).

In the current project, experiments are conducted on *Desmodesmus sp. CO46*, another strain of microalgae used in biofuel production. Now that specificity of the bacterial species is understood to be necessary for enhanced algal growth, samples of *Desmodesmus* phycospheres from six different locations in Hawaii and the San Francisco Bay Area are being studied in attempt to find ideal mutualistic bacterial environments that not only support growth but also can mitigate growth reduction in stressful conditions. High light intensity and temperature can stress algal cells during cultivation, while pathogenic bacterial species can kill the algae; therefore, a microbiome that contributes to decreasing the effects of these stressors will greatly improve algal production.

The technical objectives of this current project focus on using the available technology to monitor *Desmodesmus* microalgae growth and find how different bacterial species interact with the microalga. The goal is to eventually identify microbiomes that benefit *Desmodesmus* as well

as those that are harmful in order to formulate an engineered microbiome to support the microalga in stressful environments.

PROGRESS

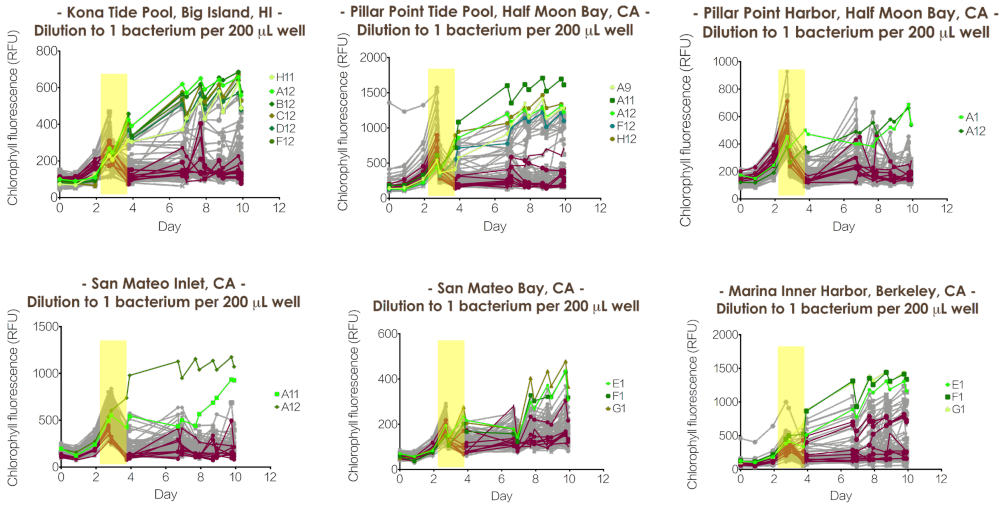
The technical approach of this project was to grow samples of *Desmodesmus* microalgae in the presence of different phycosphere-associated bacteria and monitor their growth effects on the algae under stress. Phycosphere samples from six different locations were experimented with: Kona Tide Pool, Big Island, HI; Pillar Point Tide Pool, Half Moon Bay, CA; Pillar Point Harbor, Half Moon Bay, CA; San Mateo Inlet, CA; San Mateo Bay, CA; and Marina Inner Harbor, Berkeley, CA.

Two 96-well plates were prepared for each of the six microbiome samples and the microalgae chlorophyll autofluorescence was monitored over almost two weeks. One well-plate was for the microbiomes diluted to ~1 bacterium per well, while the other was for the microbiomes diluted to ~0.01 bacterium per well to ensure bacterial isolation. This way, the effects of small groups of bacterial species on the algae can be distinct from one another, as there can be numerous types of bacteria in each microbiome. The growth media in each well consisted of F/2 ESAW media filtered through 0.22 µm pore size filters to mimic sterilized seawater. One column was left axenic (no bacteria added) on each plate to act as a control.

For the first three days, the plates were placed under low light incubation, with a light intensity of about 123 $\mu Em^{-2}s^{-1}$, so that the few algal cells would not be damaged by too much light. Once the cells multiplied, the plates were placed under high light incubation where growth is possible due to self-shading behavior, where cells take turns shading each other so that they do not experience high light intensity constantly. Under this high light, the shading that bacteria also offer can be monitored by how well the algae grow as well. The high light incubator was set to mimic the 24-hour sunlight cycle in Arizona state where microalgae plants are located, therefore the light intensity ranged from darkness for 10 hours then increased up to 1000 $\mu Em^{-2}s^{-1}$ by afternoon before decreasing back to darkness to begin the cycle again. Algal growth was measured by chlorophyll autofluorescence with the Cytation 5 plate fluorometer for about two weeks. The well plates were stored in the low light incubator for an additional week without measurements.

Choosing at least one well per phycosphere sample, 34 of the wells with the highest and most consistent measured growth rates were transferred to new sterile seawater media in tubes. Most of the high growers came from the lower dilution of one bacterium per well. This is probably because transferring from the lower dilution has a higher chance of putting one or even multiple bacterial cells into each well. In contrast, the higher dilution of 0.01 bacterium per well reduces the probability of even transferring one bacterium, and the lack of bacteria may have contributed to poor algal growth (see Fig. 1).

A)



B)

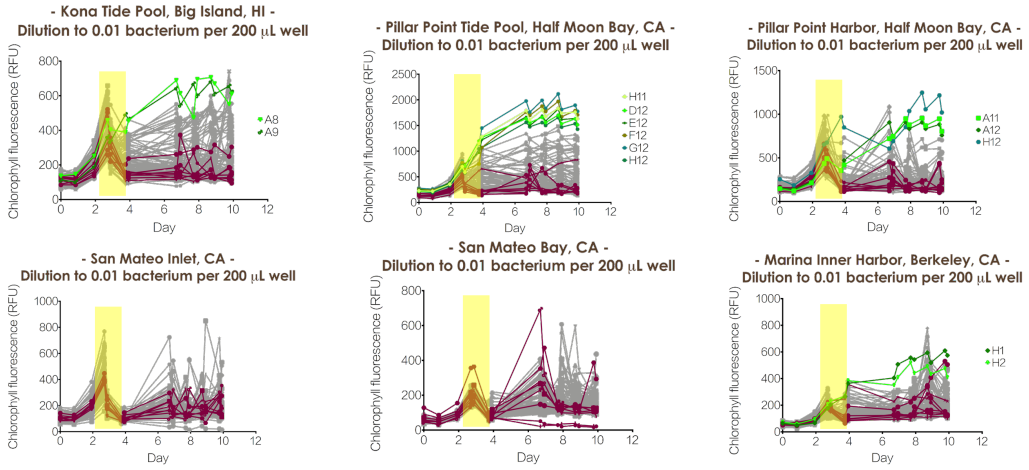


Figure 1. Chlorophyll a fluorescence measurements of 96-well plate samples over course of two weeks of A) dilution to one bacterium per well and B) dilution to 0.01 bacterium per well. The 34 wells chosen for further growth are shown in green hues, lower/no growth wells are in gray, and axenic wells in magenta. The transition from low to high light incubation is denoted in yellow.

For the chosen 34 wells, each transfer tube contained 3mL of 0.22 µm filtered F/2 ESAW media, 15 µL of additional axenic *Desmodesmus,* and 75 µL of sample from the well. Duplicate tubes were made for each chosen well. Axenic suspensions were also prepared for comparison. Chlorophyll autofluorescence measurements were resumed as the tubes were kept in the high light incubator (see Fig. 2). The Trilogy Laboratory Fluorometer was used to read the tube chlorophyll fluorescence. After the first measurement during cultivation, a significant fluorescence reduction occurred due to the transition to high light intensity and the presence of too few algal cells. As a result, a shading screen was put in place until the algae was robust enough to survive under the full light intensity. Because the fluorescence still showed drops the next few days, an additional 50 µL of supplemental axenic algae was added to each tube.

Interestingly, most of the tubes continued to show a decreasing fluorescence trend, and only about five showed significant increase. The screen was removed after two weeks, when the significant growers showed strong fluorescence in the thousands of relative fluorescence units (RFUs).

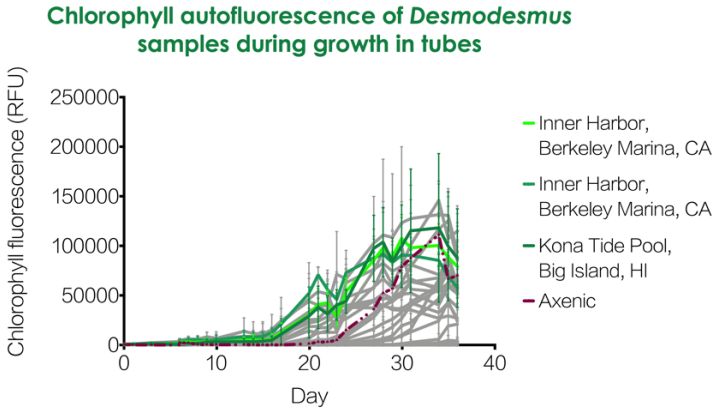


Figure 2. Chlorophyll a fluorescence analysis of high grower growth in test tubes. The statistically significant high growers are shown in green hues, the non-significant growers are in gray, and the axenic sample in magenta. Vertical bars display variation as standard errors of the mean.

After four to five weeks of additional growth, the high growing individual tube samples were imaged using fluorescence microscopy as well as SEM microscopy (see Fig. 3). The images displayed both attached and unattached bacterial-algal relationships. Additionally, it was clear that multiple bacteria morphologies were present in the high grower environments, which supports that multiple bacterial species can be beneficial for algal growth. An interesting find was that bacteria were seen attached to other bacteria in contact with algal cells, showing possible associations within associations that can be researched further.

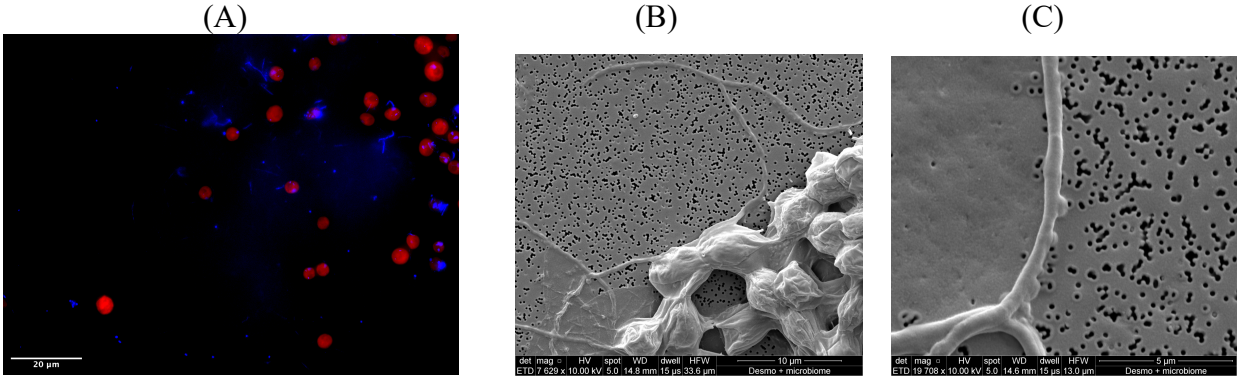


Figure 3. Microscopy images of algal-bacterial associations via (A) fluorescence microscopy and (B) (C) scanning electron microscopy (SEM). (A) Fluorescence microscopy displayed the algal cells in red and the bacterial cells in blue. A variety of bacteria were found in the high growing samples including attached and unattached bacteria as well as filamentous and round morphologies. (B) SEM allowed visualization of the close association between algal cells and

bacteria. (C) Bacteria-bacteria associations were also found and could be a source of future investigation.

Using the extra sum-of-squares F test, $P < 0.05$, three statistically significant high growing samples were found compared to the axenic rate. These samples were transferred to large flasks in triplicate for larger-scale cultivation. Separate triplicate flasks were also prepared for an axenic sample and a xenic sample, where the microbiome is naturally occurring. It is planned for these samples to be grown, and then the bulk algal dry weights will be measured as a way to quantify algal biomass productivity.

FUTURE WORK

Future activities planned for the progress of this project include: growing the samples up in largescale so that dry biomass can be measured and compared, monitoring the growth of *Desmodesmus* in the presence of more than one beneficial microbiome, and studying the details of the metabolic processes that occur between the microalgae and mutualistic bacteria versus those with antagonistic bacteria. Furthermore, identification of the helpful bacterial species, through DNA sequencing, would eventually need to be done. Once more experimentation is performed on these topics to further identify the bacterial species involved, the project deliverables can finally include a more direct answer to the original question on finding the ideal microbiome for *Desmodesmus sp. CO46* in the face of environmental and biological stresses.

IMPACT ON LABORATORY MISSIONS

The DOE laboratory mission to ensure national security through finding energy resources that are efficient and environmentally friendly is especially supported by this project. Microalgae, being a potential source of biofuel due to its high lipid content, is additionally fast growing and does not take up as much land as many present-day crops. Furthermore, it is predicted to release much less pollution than the current use of fossil fuels does, and it is capable of absorbing excess CO_2. Microalgae have also been considered as a new form of livestock feed. However, there is still much to be researched to lower production costs before microalgae can be used on a larger scale and begin replacing nonrenewable sources of energy (Wijffels and Barbosa, 2010).

To align with the mission statement of the laboratory, the projects of the “Algae Microbiome” group at Lawrence Livermore National Laboratory (LLNL) are aimed to ultimately discover methods to increase the efficiency of microalgae production. Through many experiments, the group has been studying how bacteria plays an important role in the survival of microalgae species. The current project discussed in this report only covers a small part in finding helpful bacterial mutualists that enhance the growth of one biofuel-related algal strain, *Desmodesmus sp. CO46*. Specifically, the experimental goal was to find which bacterial species would help the microalgae grow the most, beyond the amount it could grow in its natural microbiome, in the presence of stress. An enormous amount of biomass is necessary for the potential largescale use of microalgae in biofuel and feed production; thus, production efficiency is crucial to allow high growth at lower energy and economic costs.

Within this current project, there is still experimentation to be done. The samples are still being monitored with chlorophyll fluorescence until their values stop increasing, signifying they have reached stationary growth phase. At that point, high growing samples would be grown up in much larger quantities so that bulk algal dry weights can be measured as a means of quantifying algal productivity. Also, the discovered beneficial microbiomes can be combined to test if multiple microbiomes contribute to even more enhanced algal growth. As seen in the well plate growth curves of the *Desmodesmus* samples, shown in Figure 1, there were a large number of wells showing intermediate growth that were not chosen for further growth monitoring due to time constraints. These algal-bacterial samples have the potential to demonstrate mutualistic behavior and should be observed more if this project is furthered. Outside of this work, many other algal strains can be experimented with in a similar fashion to find even better algae and bacteria mutualistic relationships.

This work was supported in part by the U.S. Department of Energy, Office of Science, Office of Workforce Development for Teachers and Scientists (WDTS) under the Community College Internship (CCI) program.

CONCLUSIONS

So far, the results of this research project supported the concept of simplifying phycosphere-associated bacterial consortia to confer microalgal tolerance to stress. Multiple microbiome samples allowed algal growth to surpass the rate of the axenic algae samples (no bacteria present). Furthermore, microscope images revealed that samples which displayed high growth contained some sort of bacterial consortia, indicating that more than one species of bacteria can work together in boosting algal growth. As for the samples that grew poorly compared to the axenic samples, it is possible that these microbiomes consisted of pathogenic bacteria that harmed the algae or bacteria that could not help the algae enough to grow under stress. The project is intended to be extended to further study interactions between the *Desmodesmus sp. CO46* strain and simplified microbiomes in order to find and identify the ideal microbiome for efficient growth of the microalga.

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APPENDIX

Participants

Name	Institution	Role
Adrienne Ly	CCI Program Intern; Nuclear and Chemical Sciences Division; Lawrence Livermore National Laboratory, Livermore, CA, USA	Summer student intern; Learned about the project and lab procedures from mentor, collected and analyzed data, produced poster and report to document the project at end of internship
Kristina A. Rolison	Biosciences and Biotechnology Division; Lawrence Livermore National Laboratory, Livermore, CA, USA	Assisted student intern during lab procedures
Xavier Mayali	Nuclear and Chemical Sciences Division; Lawrence Livermore National Laboratory, Livermore, CA, USA	Worked with mentor in overseeing student intern work
Ty J. Samo	Nuclear and Chemical Sciences Division; Lawrence Livermore National Laboratory, Livermore, CA, USA	Mentor; Guided student intern on project scope and goals, instructed and assisted student intern during lab procedures, main advisor for student throughout duration of project

Scientific Facilities

- For this project, the scientific facilities used were the Algae Cultivation Facilities at Lawrence Livermore National Laboratory, Livermore, CA, USA.

Notable Outcomes

- Poster Designed for LLNL Poster Symposium (under same title)

Ly, A., Rolison, K.A., Mayali, X., and Samo, T.J. (2019). Destressing biofuel microalgae: Leveraging beneficial microbiomes to alleviate harmful conditions that reduce algal growth [Poster]. Lawrence Livermore National Laboratory, Livermore, CA, USA.