Broad Spectrum Antifungal Pond Protection
WBS# 1.3.2.044

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Advanced Algal Systems Technology Area
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Sandia National Laboratories

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Quad Chart Overview

Timeline

Project start date 10/01/2021

Project end date 9/30/2024

	FY22 Costed	Total Award
DOE Funding	$333K	*$1 M*

TRL at Project Start: 2

TRL at Project End: 5-6

*Only fill out if applicable.

Project Goals

Create a low to zero cost method of prophylaxis against pond crashes caused by a wide range of fungal agents.

These protective strains or consortia will be stable in co-cultivation with algae and will not require separate cultivation or multiple additions to the production culture.

Demonstrate the ability of a microbial consortia to reduce the pond crashes from fungal infection

End of Project Milestone

SNL and outdoor cultivation partner (TBD) will demonstrate in 1000L outdoor cultures a 100% increase in the MTTF in the summer seasonal cultivation run in algae co-cultures with protective consortia and or strains

Funding Mechanism

Laboratory Call L-0C000009 (Technical Area 1), 2021

Project Overview: *Context and Project History*

Peer-reviewed analyses indicated that at least 30% of annual algal production is lost through biocontaminant-mediated crashes.

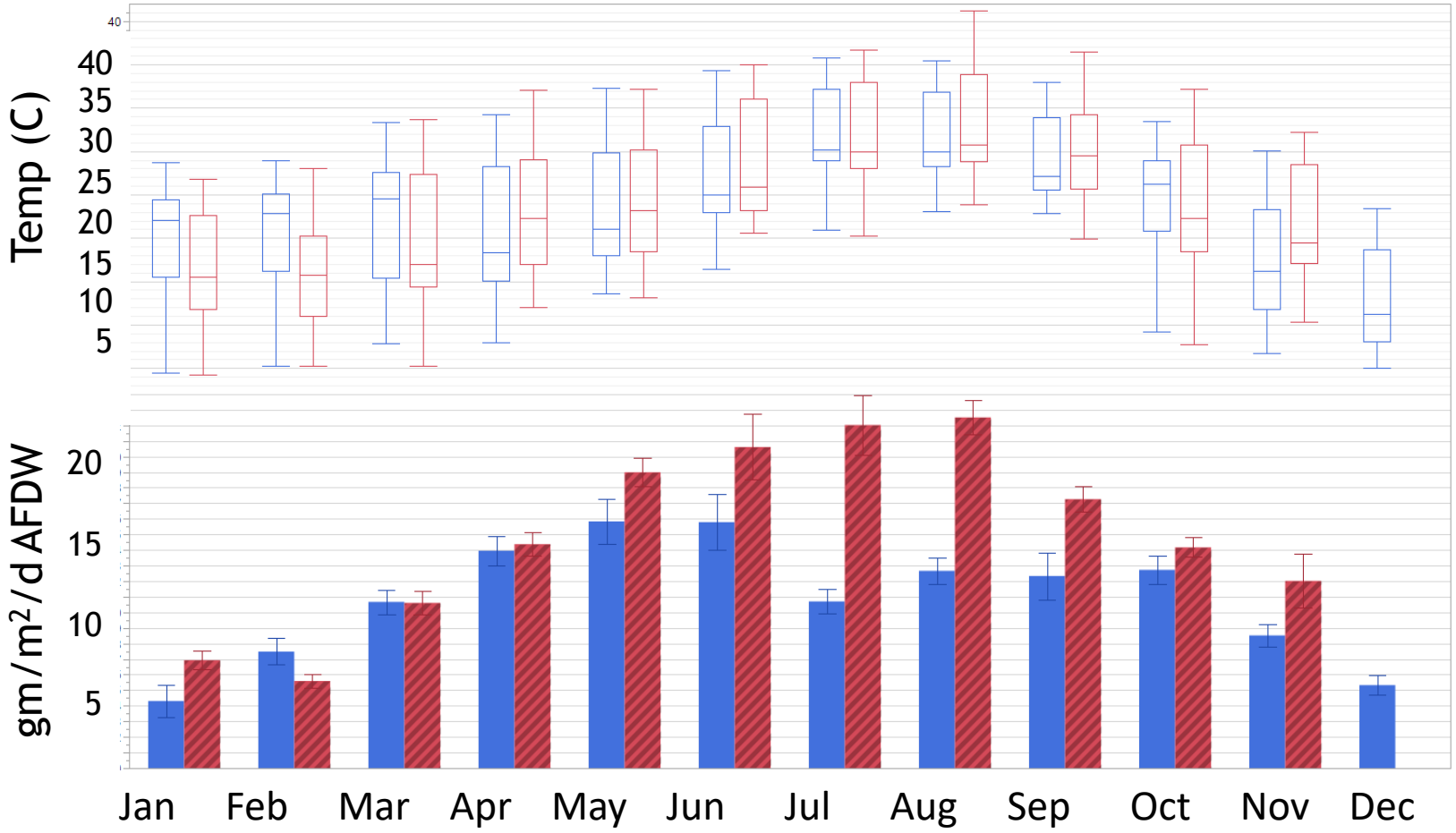
The goal of this project is to provide a low to zero cost prophylactic treatment to prevent pond crashes due to fungal infection.

The driver for this project is the potential loss of productivity due to fungal infection.

The environmental and economic costs associated with treatment with biocides.

Not all algal strains tolerate biocides.

Fungi develop resistance to biocides



The history of fungal infection during the SOT runs for DISCOVR at AzCATI. Blue: 2018 cultivation runs with uncontrolled an uncontrolled fungal infection. Red: 2019 cultivation runs with fungal control. McGowen et al 2019

This project leverages previous results from DISCOVR

Koch's postulates for pond crashes: The set of criteria that establish whether a particular organism is the cause of a particular disease (pond crash).

SNL carried out Microbiome analysis of crashed ponds from AzCATI

Identified *Amoeboaphelidium sp* as putative agent

Carried out PCR analysis of pond crash samples with *Amoeboaphelidium* specific probes.

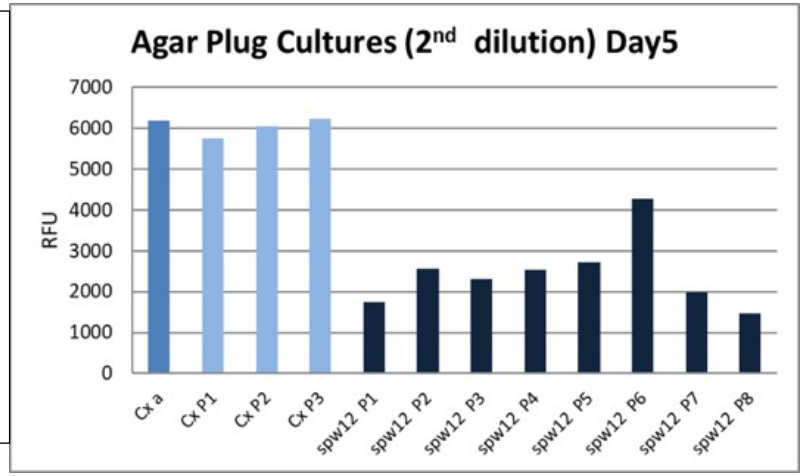
Individual isolates were used to recapitulate crashes.

Isolates that were sufficient to crash cultures we identified by PCR and sequencing

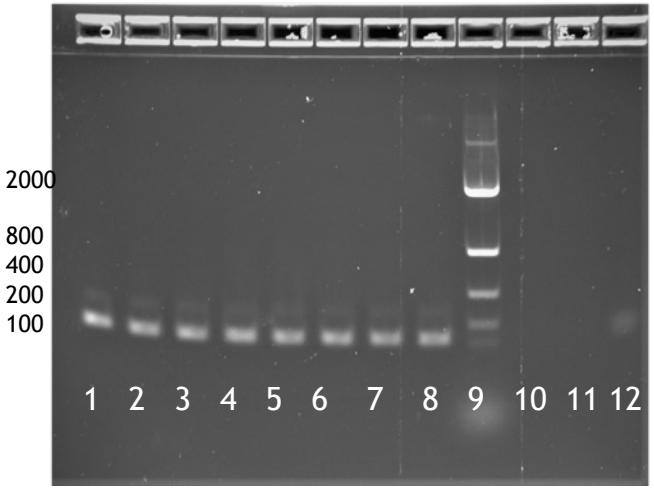
MiSeq FD01 Hits

		SPW15 6/4/18	SPW10 7/23/18	SPW12 7/23/18	SPW14 7/23/18	SPW10 10/23/18	SPW12 10/23/18	SPW13 4/24/19	SPW14 4/24/19	SPW16 4/1/19
OTU 11	Amoeboaphelidium sp. PML-2014 isolate FD01					29274	42642	280	2519	15301

Right: Agar plug isolation of *Amoeboaphelidium*. Cored plaques from agar overlay plates of algae + *Amoeboaphelidium* were added to cultures of algae and fluorescence of the cultures we measured on day 5. Light bars: control cultures, dark bars: cultures infected with plugs from plaques



Agar plugs from spw12 recapitulated crash



1. spw12 agar plug 1
2. spw12 agar plug 2
3. spw12 agar plug 3
4. spw12 agar plug 4
5. spw12 agar plug 5
6. spw12 agar plug 6
7. spw12 agar plug 7
8. spw12 agar plug 8
9. DNA Ladder
10. No template
11. 26BAM agar plug 2
12. 26BAM agar plug 3

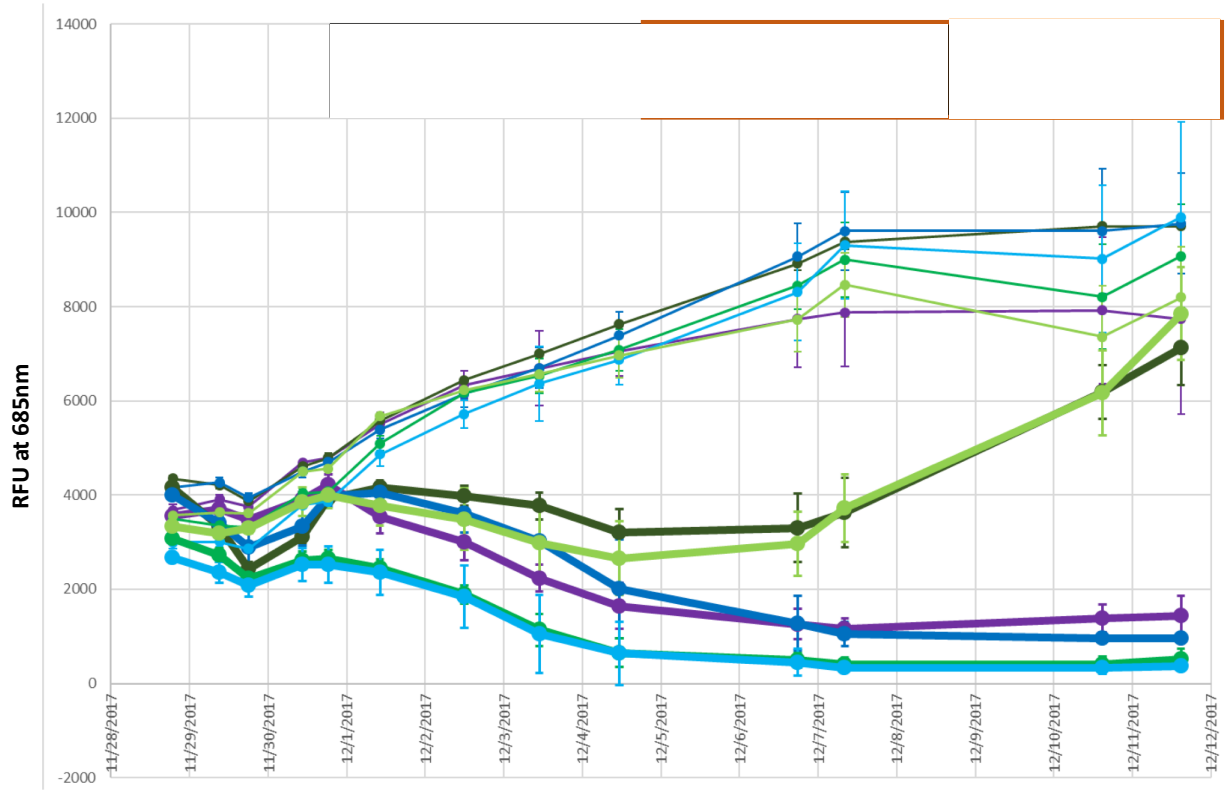
Left: PRC analysis of isolates capable of producing laboratory crashes with *Amoeboaphelidium* FD01 specific primers

This current AOP based on successful strategy of “Continuous Biological Protection and Control of Algal Pond Productivity”

The previous project resulted in development of consortia that protected *Microchloropsis salina* from grazing by the rotifer *Brachionus plicatilis.*

These protective consortia persisted in outdoor cultivation for at least 40 days and still provide protection against rotifer grazing.

Providing a potential low cost solution for the protection of production cultures from grazing by rotifers.

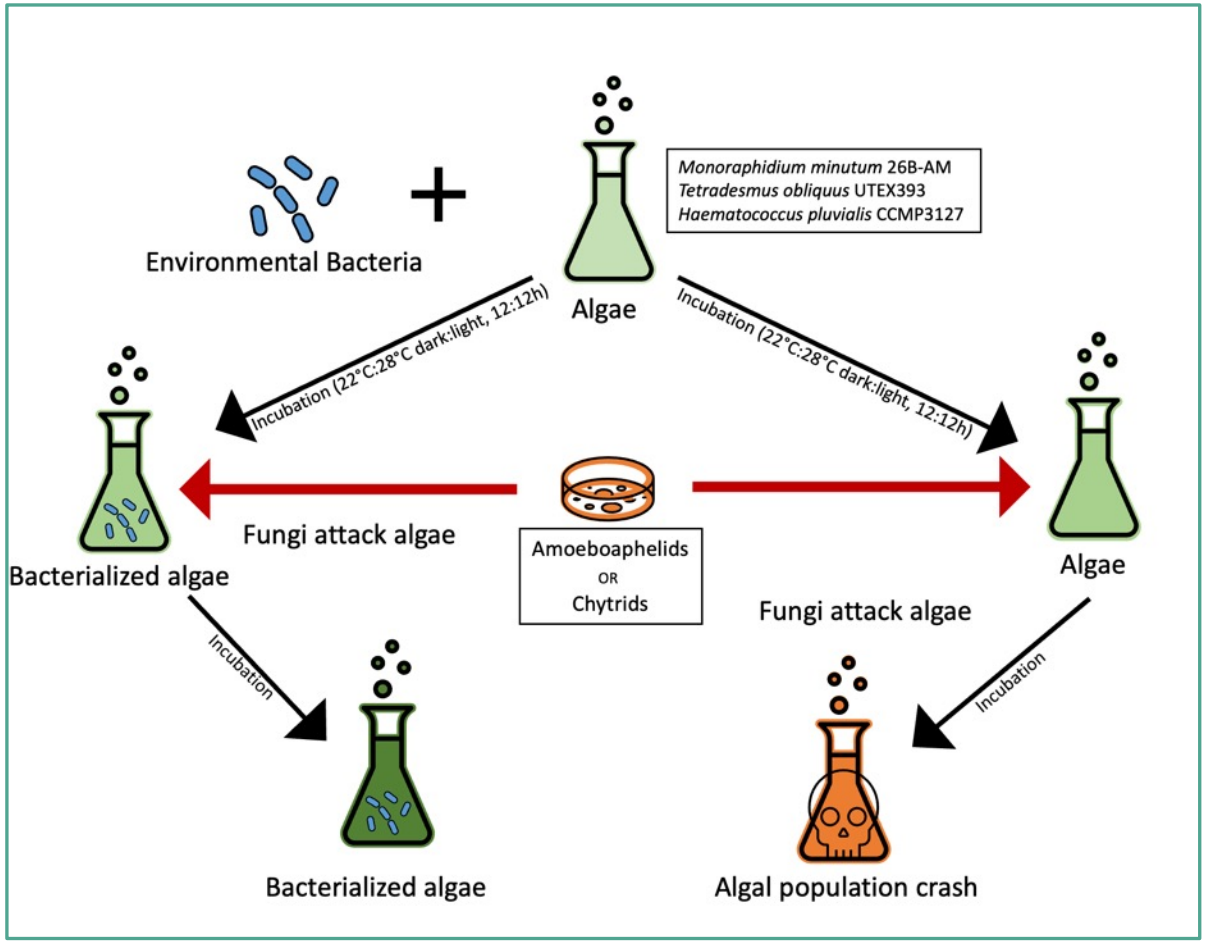


Rotifer challenge experiments of algae + protective consortia co-cultures grown outdoors for 40 days. After 40 days of outdoor cultivation algal cultures without protective consortia (A1,A2,A3) and those with protective Consortia (C1, C2,C3) were challenged with 10 rotifers/mL (thick lines) Rotifers were added on 11/29/2017. Consortia C1 and C3 were still protective against rotifers after 40 days. Thin lines = uninfected controls. Fisher et al 2019

AOP Workplan: *Development of Protective Consortia*

Similar to TABB project strategy used for developing anti-rotifer consortia

- 6+ different host parasite pairs
- Multiple xenic algal samples will be obtained from a variety of sources
  - Bacterial fraction harvested 0.8 micron filtrate -> 0.2 micron retentate
- Multiple Algae+consortia maintained in co-culture for at least 30 days
- Algae+consortia used as “food” for maintenance of fungi
- Once fungal cultures crash (algal cultures become resistant)
  - Bacterial fractions harvested as before
  - Susceptible algal cultures are bacterialized
  - Transfer of resistance to new algal cultures
- Consortia are tested for stability
- Consortia are dissected to potentially identify responsible bacterial species.
- Consortia are tested for range of specificity
- Combined consortia are created for broad range protection



1 – Approach: *Metrics and assumptions*

Technical metrics

TTF: Time To failure in standardized laboratory or pilot scale crash assays

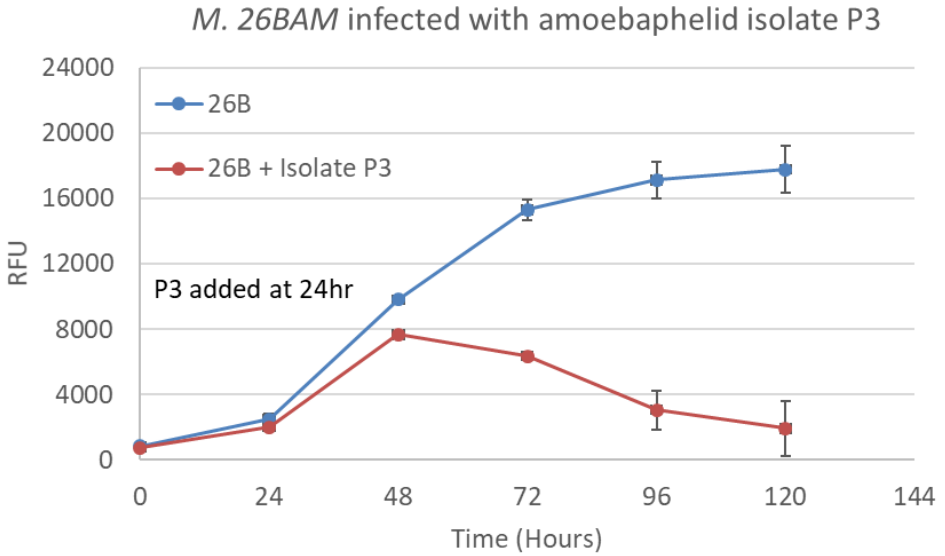
Protective co-culture stability: Number of days of laboratory cultivation is protective capacity maintained

Outdoor cultivation stability: Number of days of outdoor cultivation is protective capacity maintained

MTBF: Mean Time Between Failure for outdoor cultivation systems prone to fungal infection or loss of pond productivity.

Economic assumptions

It is assumed that cultivation of a stable coculture does not pose significant production costs beyond that of standard inoculum based algal cultivation.



Left: Standard crash assay with *Monoraphidium minutum* 26B-AM and *Amoeboaphelidium* isolate P3.

1 – Approach: *Communication and DEI plans*

Communication and collaboration with related projects and relevant stakeholders.

The PI is also the task lead for crop protection for the DISCOVR. The approaches and goals of this AOP has been briefed to the DISCOVR team and both the PI and the DISCOVR team are in weekly communication.

The interim and final results from this project will be communicated through presentations at national and international conferences and publication in relevant journals.

Diversity, equity, and inclusion.

Our primary DEI goal is to enrich the hiring pipeline with under represented candidates. As a part of our recruitment strategy for a postdoc and undergraduate interns on this project, we have directly contacted faculty at MSIs to make them aware of position so they can advertise it to their students. We have successfully recruited a postdoc and a summer intern from MSIs, and actively recruiting a year round intern from a MSIs.

I – Approach: *Risks and Mitigation Strategy*

Inability to isolate protective consortia. We believe that this risk is low based on previous success with isolating consortia that protect against rotifers and the fact that other protective bacteria and consortia against fungal pathogens have be isolated in a range of other systems	Extend the number and range of source culture material for the enrichment and selection cultures. The greater the diversity of source material and the larger the number of selection cultures the higher the likelihood of success. This risk is the subject of our go/no go decision point.
In house fungus collection supplemented with samples from partners and culture collections may not represent sufficient diversity. Individual isolates and consortia may prove to have a narrow range of protective effect in terms of fungal species. In simulated and non-simulated outdoor production conditions at pilot pond scale, protective consortia may not be stable over the length of the cultivation cycle.	We will seek additional collaborators and sources for the acquisition of additional fungal strains. Individual strains and consortia will be combined to create expanded consortia with broader range of protection. Genetic probes for monitoring consortia composition will be created. Growth conditions for selecting for and enhancing consortia levels in algal inoculum and stabilizing consortia in ponds will be developed. By increasing the initial consortia concentrations we will extend the life of the consortia in the production pond.

Milestones

Project Milestones:

Type	Description and Criteria	End Date
Quarterly Progress Measure (Regular)	**Establish fungal pathogen panel** Description: SNL will obtain a panel of at least 6 distinct fungal pathogens of algae.	**12/31/2021 Complete**
Quarterly Progress Measure (Regular)	**Establish selection cultures.** Description: SNL will establish cultures of "bacterialized" algae. Criteria: SNL will set up and maintain for at least 30 days laboratory scale algal cultures "bacterialized" with at least 6 different source bacteria for each of 6 fungal pathogens for a total of at least 36 selection cultures.	**3/31/2022 Complete**
Annual Milestone (Regular)	**Establish protective consortia** Description: SNL will demonstrate at l at laboratory scale multiple protective strains or consortia that each protect against at least one fungal pathogen in standard crash assays.	**9/30/2022 Complete**
Annual Milestone (Regular)	**Algal test bed demonstration of antifungal protection** Description: SNL will demonstrate in 100L and 1000L climate simulation ponds the persistence and stability of protective consortium in 30, 60, and 90 day growth trials under simulated outdoor conditions. Criteria: At the end of each trial each culture shall be challenged by experimental fungal infection and ***culture protection will be measured by both a 100% increase in the time to failure over control crashes and an 50% increase in algal growth rate in protected cultures versus non protected.***	**9/30/2023**
Annual Milestone (Regular)	**Field demonstration of antifungal protection.** Description: Sandia in collaboration with the DISCOVR consortium and the AzCATI algae test bed will demonstrate the effectiveness of antifungal consortia. Alternative test sites include Cal Poly SLO University of AZ and 100L ponds that can be operated outdoors at SNL.	***project end date 9/30/2024**

Gantt chart: *Task duration and milestones*

		2021			2022								
Task #	Task or Milestone Title	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep
Task 1	Fungal isolation and Antifungal enrichments.												
Milestone 1.1	Establish fungal pathogen panel												
Milestone 1.2	Establish selection cultures.												
Milestone 1.3	Establish protective consortia												

		2022			2023								
Task #	Task or Milestone Title	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep
Task 2	Lab scale characterization and cultivation trials												
Milestone 2.1	Algal test bed demonstration of antifungal protection												
DP1	Go/No-Go Decision Point 1												

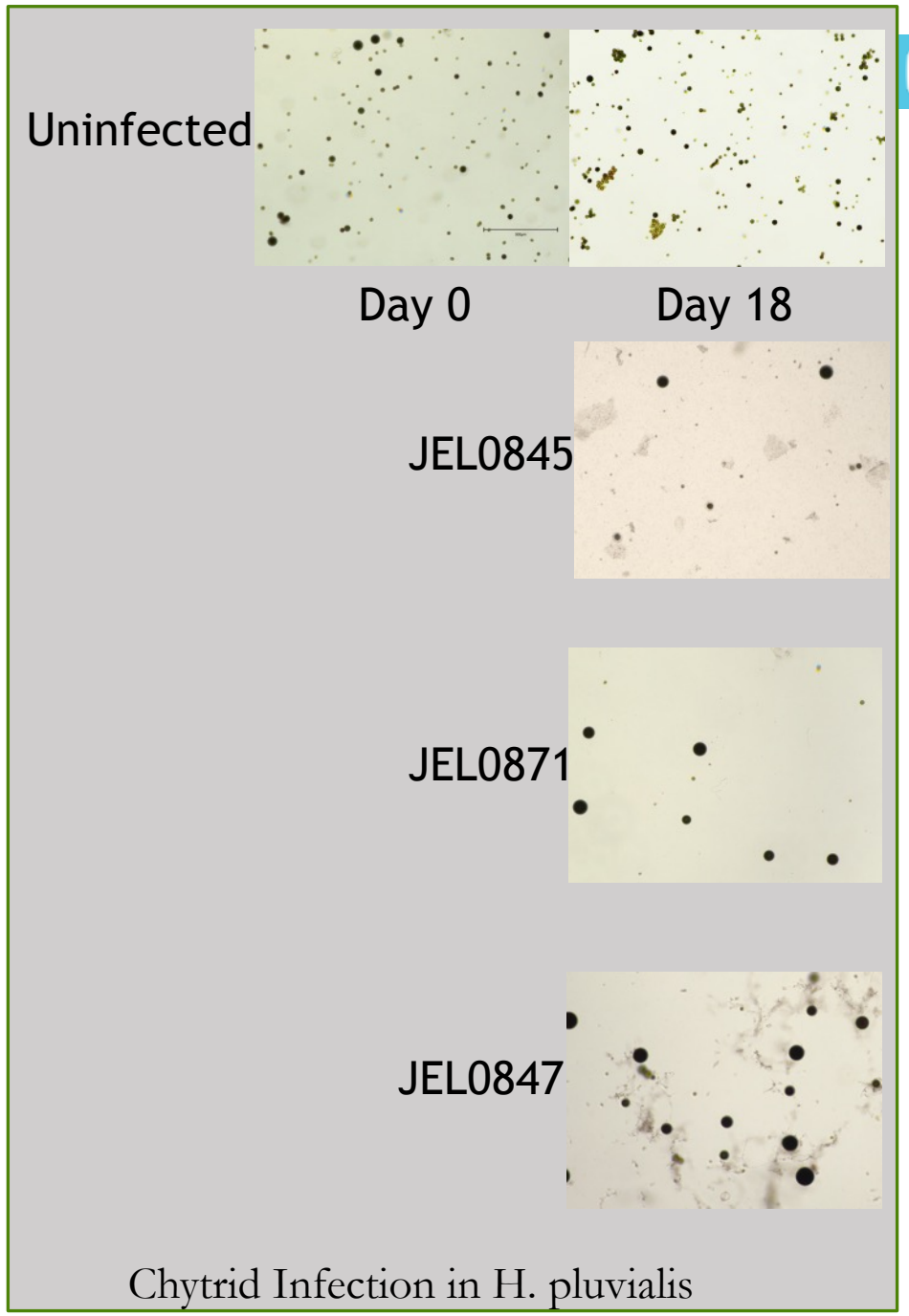
		2023			2024								
Task #	Task or Milestone Title	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep
Task 3	Field Testing and Validation												
Milestone 3.1	Field demonstration of antifungal protection.												

Progress and Outcomes: *Establishing Fungal Species Panel (12/21/21 milestone)*

Identifier	Organism	Classification	Host
JEL0821	*Paraphysoderma sedebokerense*	Chytrid	*Haematococcus pluvialis*
JEL0845	*Angulomyces* sp.	Chytrid	*Haematococcus pluvialis*
JEL0847	*Boothiomyces* sp.	Chytrid	*Haematococcus pluvialis*
JEL0871	*Catenaria* sp.	Chytrid	*Haematococcus pluvialis*
JEL0873	*Gaertneriomyces* sp.	Chytrid	*Haematococcus pluvialis*
JEL0915	*Aquamyces* sp.	Chytrid	*Haematococcus pluvialis*
PL3	*Amoeboaphelidium* sp.	Amoeboaphelid	*Monoraphidium minutum, Scenedesmus obliquus*
HG101	*Amoeboaphelidium* sp.	Amoeboaphelid	*Monoraphidium minutum*

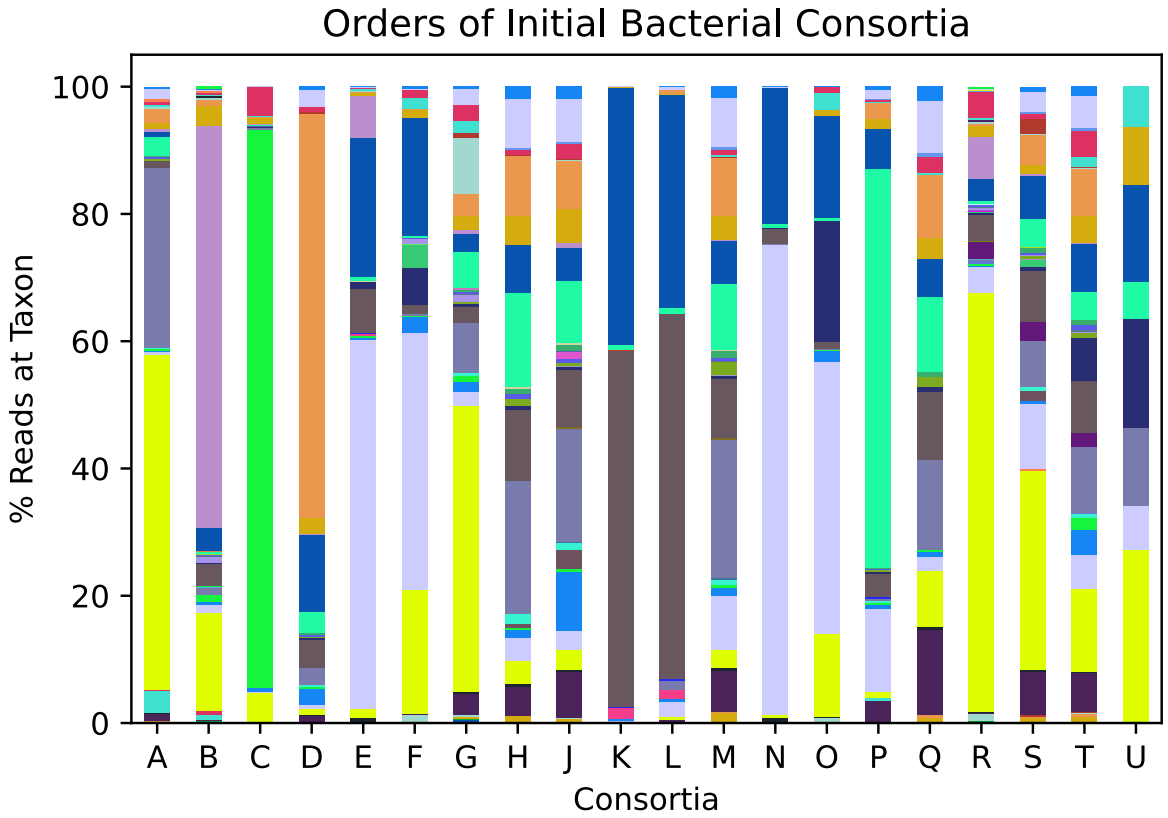
We have established a panel of fungal parasite species for the testing and development of protective consortia.

We have developed standard crash assays for each.



Chytrid Infection in H. pluvialis

Progress and Outcomes: *Algal Bacterialization (3/31/22 Milestone)*



16s Amplicon sequencing analysis of 20 independent starting consortia prior to bacterialization of algal host strains.

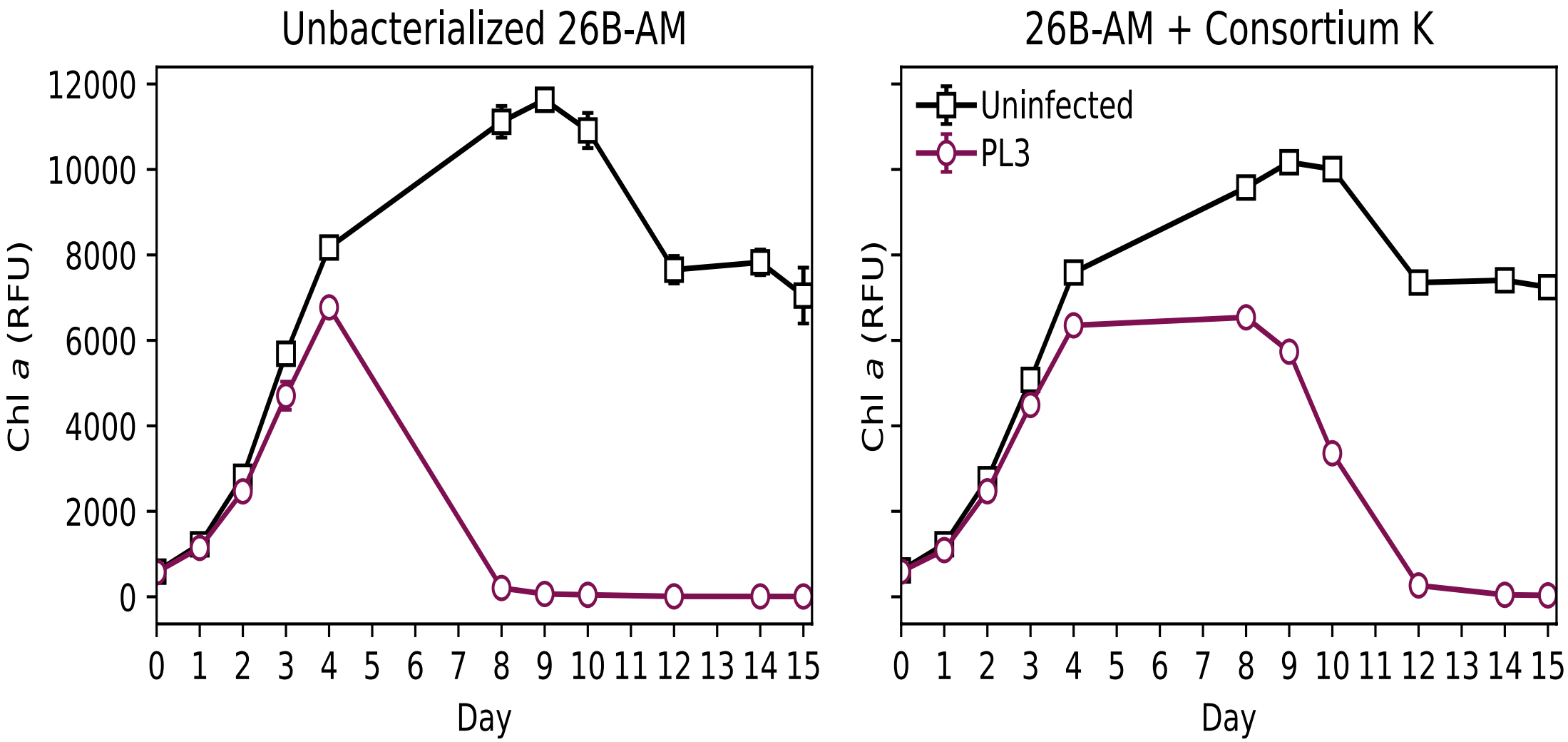
Bacterial community structure is routinely monitored by amplicon sequencing

Three species of algae (*Monoraphidium minutum* 26B-AM, *Scenedesmus obliquus* UTEX393 and *Haematococcus pluvialis* CCMP3127) were co-cultured with bacterial consortia and were considered established if the algae showed signs of growth after one week

Samples were collected of the bacterial consortia immediately prior to addition to the algal cultures and at 30-day, 90-day, 120-day, and 200-day timepoints for sequencing

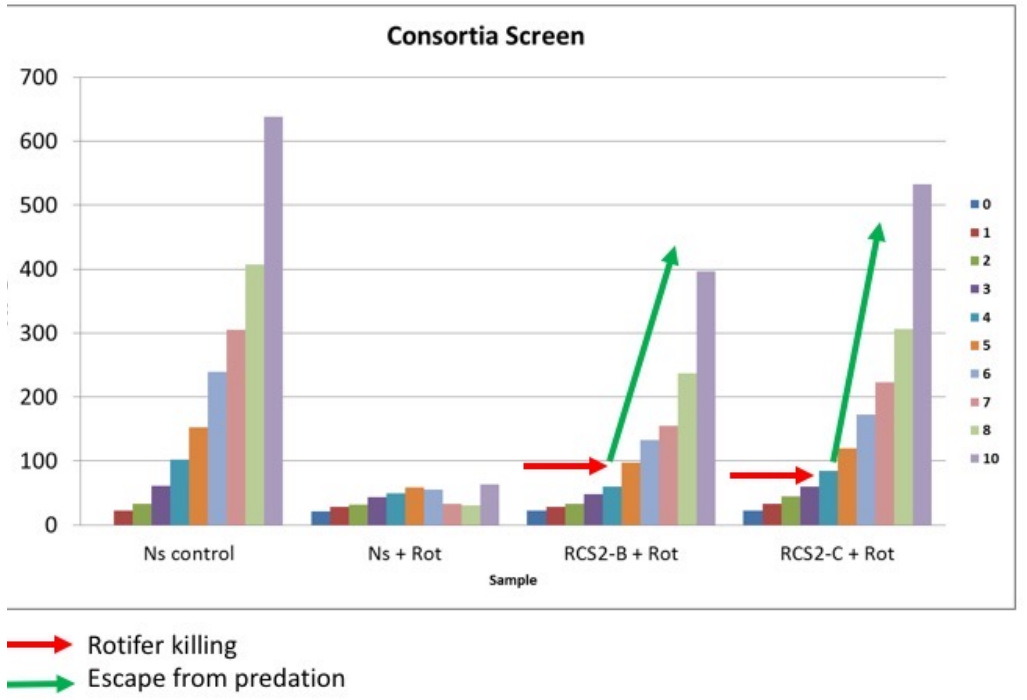
Sequencing was completed for initial and 30-day timepoints at SNL using Illumina MiSeq for DNA sequencing and Kraken2/Bracken for classification

Progress and Outcomes: *Consortia Increase TTF in Aphelid Infection (9/30/22 milestone)*



- Bacterial consortia were chosen based on initial tests to test against PL3 at the 60-day timepoint
- Consortium K showed approx. 70% increase in time to infection and time to crash

Consortia Increase TTF in Haematococcus/chytrid Infections (9/30/22 Milestone)



- (Right) Growth of uninfected *Haematococcus* cultures (green) and culture infected with the chytrid JEL0845 (*Angulomyces* sp.).
- CCMP3127 were infected at 1:1000 volume on day 0 **and reinfected** at the same concentration on day 20
- Bacterial consortia showed up to 100% protection against culture reinfection.
- (Left) Dynamics similar to those observed with anti rotifer consortia: a period of continued grazing while the consortia was taking effect followed by an “escape from grazing once the rotifers were killed.

Go/No Go milestone

Project Go/No Go Decision Point:

Decision	Description	Criteria	Date
Go/No Go DP 1	**Establish consortia/isolates suitable for pond protection**	SNL will culture algae with and without protective consortia and or strains in 500mL EPBRs under simulated outdoor production conditions for no less than 30 days. At the end of the growth period, the cultures shall be challenged with one or more fungal pathogens under previously determined standard crash assay conditions. After the cultivation trial the consortia and or strains shall continue to provide protection as evidenced by 100% increase in the MTBF in standard crash assays.	6/30/2023

For Go/No Go we want to demonstrate the feasibility of the overall approach

Demonstrate the algal/bacterial co-cultures are stable for at least 30 days under simulated production conditions

Demonstrate that the consortia retain the ability to protect algae from infection by parasites as evidenced by 100% increase in TTF

Upon completion of Go/No Go we will move to 1000L simulated outdoor cultivation in order to develop protocols for outdoor deployment and cultivation trials.

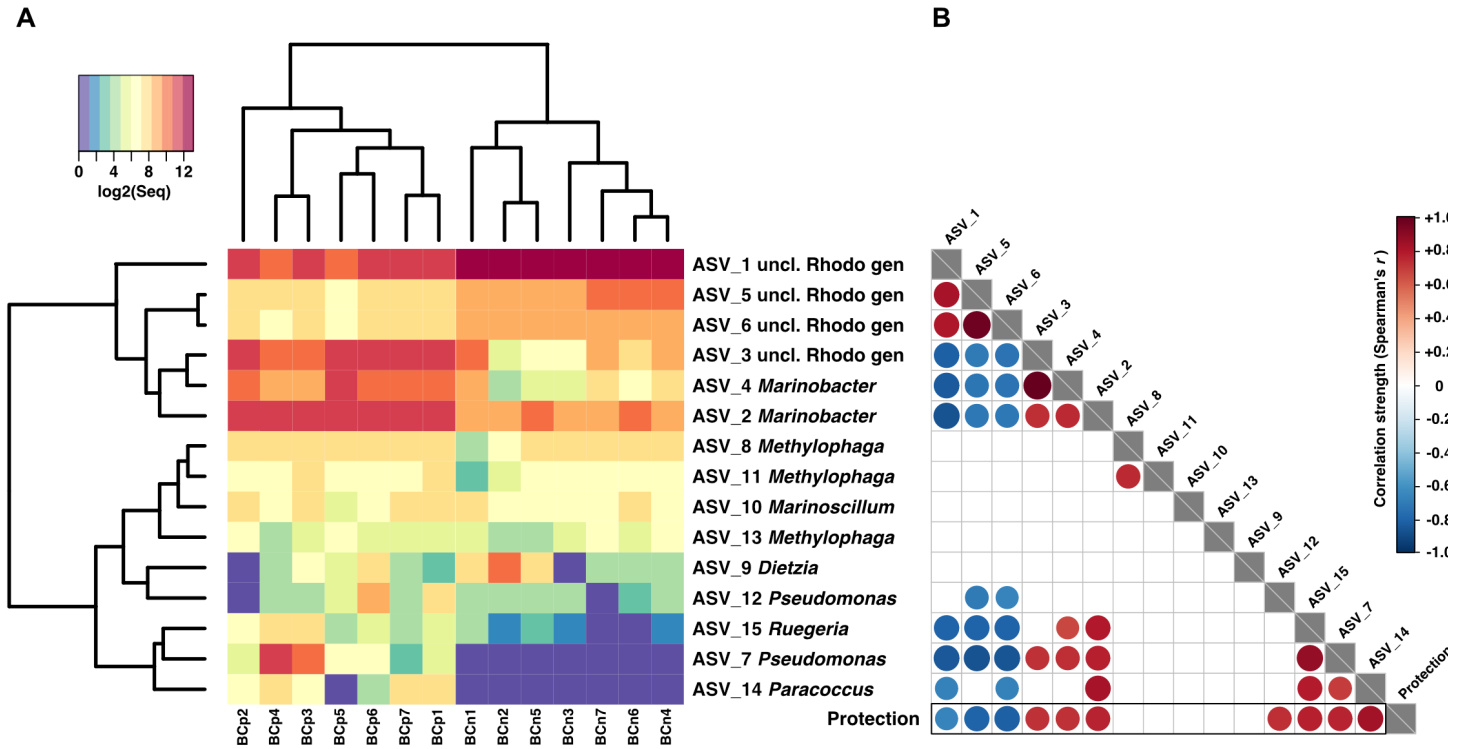
Next steps: *Deconvolution and Recombination*

We are currently carrying out amplicon sequencing of samples taken from timecourses of consortia evolution.

Similar to the previous rotifer resistance study (left),we will identify bacterial strains that are most highly correlated with protection.

We will identify differences in consortia based on algal host and fun)gal parasite species.

This information will be used to recombine consortia for broader resistance or to assist in the possible isolation of individual bacterial strains that are responsible for fungal resistance.



Abundances of top 15 ASVs and correlations with protection. (A) Heatmap displaying sequence abundances of the 15 most abundant ASVs across all bacterial consortia. sequences. (B) Correlations between ASVs and growth rates of bacterial consortia algal cultures in the presence of rotifers from the grazing assays (‘Protection’). Fisher et al 2019

Next steps: *Testing at scale*

We are currently carrying cultivation and infection trials under simulated outdoor conditions at 500mL scale in phenometrics EPBRs.

When successful we will test effectiveness at 1000L under simulated outdoor conditions at SNL Crash Lab.

This allows for crashes on demand with any agent of choice.

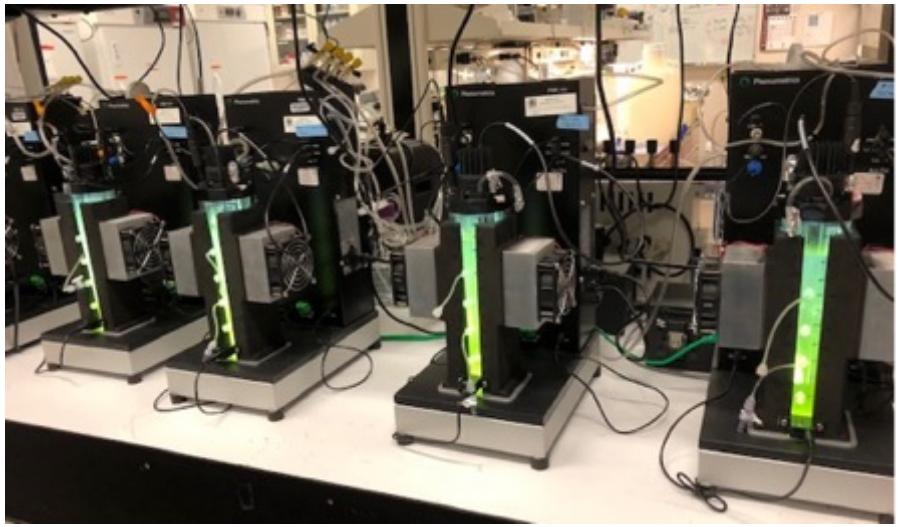
Our USDA permits do not allow for uncontained cultivation of the fungal agents.

After successful testing at 1000L scale we will either:

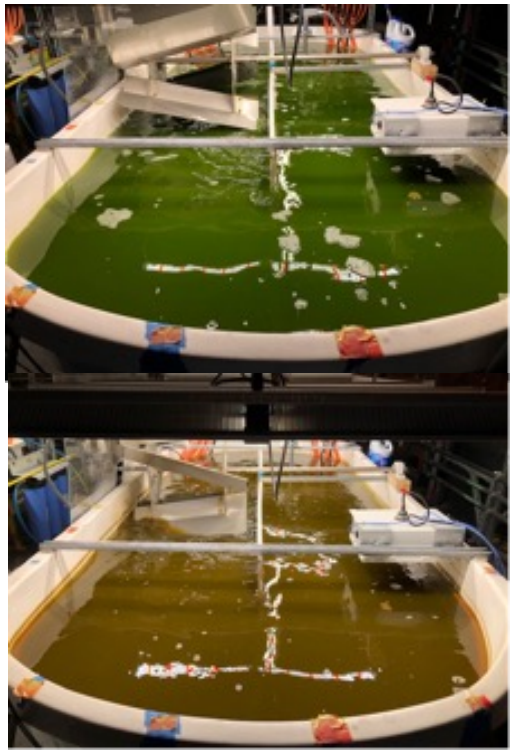
- Contract with an organization such as AzCATI to carry out outdoor trials.
- Or carryout outdoor cultivation trials at SNL combined with indoor testing.

The relevant criterium for making this choice is whether a potential contractor is experiencing relevant crashes at a reasonable frequency (untreated)

This decision will be reached in FY24Q1. We could carry out a combination of both types of outdoor trials.



Phenometrics EPBRs.



1000 L crashes on demand at SNL testbed:
Top: control algal culture.
Bottom: amoeboaphelid infected algal culture

3 Impact: *Broad spectrum, zero-cost prophylaxis against fungal infection*

Goal: Select microbial consortia to protect algal production systems from multiple fungal species

- Persist in algal co-cultivation with algae and not require separate cultivation
- No negative effect on algal production, final biomass concentration, or composition.
- Broadly applicable to a variety of production algae and deleterious species.

Stakeholders and end users of this technology: Algal production companies

- Will use the protective consortia that we develop and provide
- Will use the general strategy and protocol for the development of their own protective consortia.
- Will reduce biomass losses due to pond crash or “subacute” fungal infections
- Will reduce the costs (economic and environmental) associated with interdiction technologies such as biocides.

Contribution to BETO and EERE Goals: Overcome barriers associated with algal biomass availability and cost.

- Increase annualized yields and thus reduce the overall cost of algal biomass production.
- Enable the reliable and consistent supply of biomass in order to reduce financial technical and operational risk to biorefineries.

Summary: *Broad Spectrum Antifungal Pond Protection*

Project Objectives

- Create a low to zero cost method of prophylaxis against pond crashes caused by a wide range of fungal agents.
- These protective strains or consortia will be stable in co-cultivation with algae and will not require separate cultivation or multiple additions to the production culture.

Technical Approach

- Expand a previously developed protective strain/consortium selection process to include multiple fungal species in a parallel process.
- Evaluate the antifungal consortia at laboratory and pilot pond scale
- Deploy isolates or consortia to outdoor cultivation trials
- Demonstrate the ability of a microbial consortia to reduce the pond crashes from fungal infection

Current progress

- Developed consortia that extend MTTF by 70% in standard *Amoeboaphelidium* induced crashes.
- Developed consortia that extend MTTF by 100% in standard chytrid induce crashes.
- Consortia retain activity after 30-90 days of co-cultivation with host algae.

Acknowledgements

SNL Team

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