DOE Bioenergy Technologies Office (BETO) 2023 Project Peer Review

Ecological monitoring technologies to enhance large-scale microalgae cultivation, stability, and productivity

April 4, 2023
Advanced Algal Systems

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SCRIPPS INSTITUTION OF OCEANOGRAPHY
UC San Diego

GLOBAL ALGAE

Project Overview

- ***Project Goal***: Develop and employ real-time monitoring using 3rd generation long-read sequencing on algal cultures. Toolkit and *shared learning deliverables* will include a combination of a curated algal microbiome database, analysis workflows, and a collection of mitigation strategies.
- Increasing algal productivity in outdoors cultivation ponds through ecological monitoring and manipulation of algae and their microbiome will have a direct impact on the economic viability and sustainability of algal biofuel production.

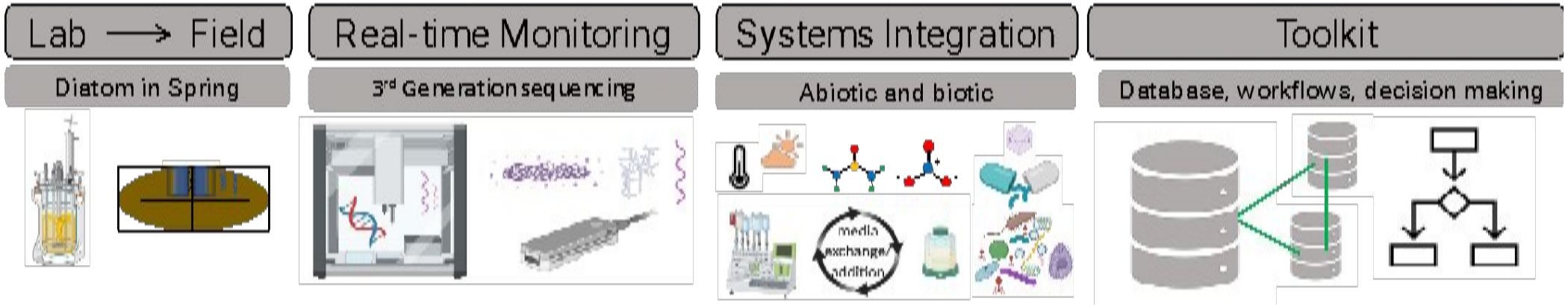
- ***High Feasibility***: Based on our Team's previous successes during DOE PEAK project
  1. *Development of Standard Operating Procedures (SOPs) at field site*
  2. *Microbiomes of high-performance alga cultivated within field-research scale*
  3. *Isolation of relevant microbiome constituents.*

Project Overview

Team Member Organization	Area of Expertise
Scripps Institution of Oceanography, University of California, San Diego	Development of rapid, real-time field monitoring of algae pond microbiomes and experimental validation and interpretation.
	Computation infrastructure development and validation and interpretation.
Global Algae Innovations Inc.	Laboratory to outdoor microalgae cultivation & harvesting implementation of experimental workflow.

Area	Key Personnel
Experimental Validation and Interpretation	Lisa Zeigler, Ph.D. PI, SIO, UCSD
Computation and Interpretation	Eric Allen, Ph.D. Co-PI, SIO, UCSD
Phycology and Farm Cultivation	Aga Pinowska, Ph.D. Co-PI, GAI
Phycology and Project Management	Jesse Traller, Ph.D. Co-PI, GAI

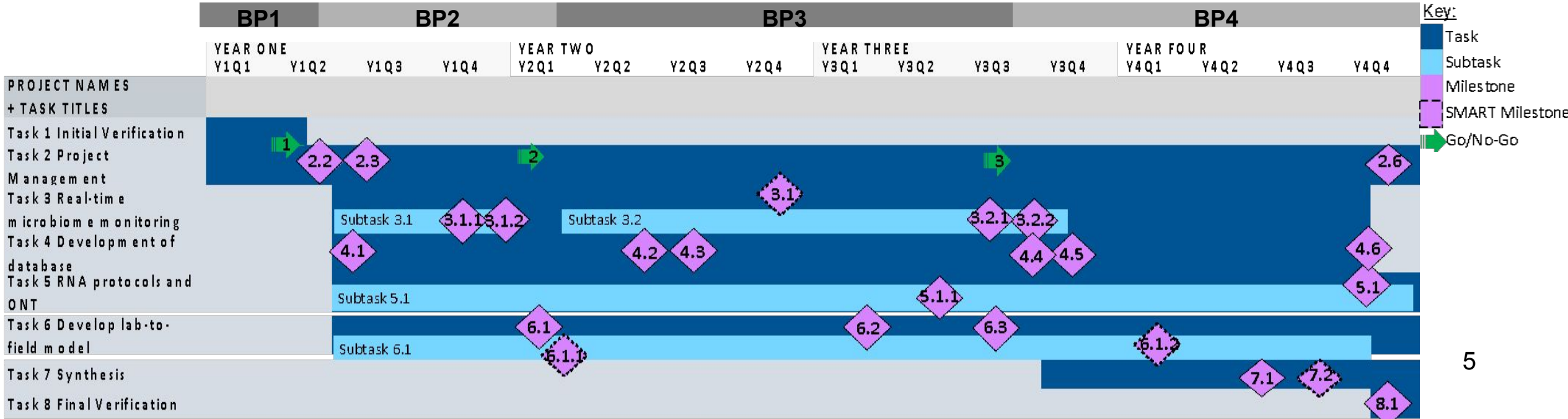
1 – Approach



- **Topic Area 2: Algae Productivity Exceeding Expectations (APEX)**
  - Enhance cultivation technologies and strategies to increase productivity of the industrial relevant diatom, *Nitzschia* capable of growth using a CO_2 supply derived from power plant flue gas and using recycled agriculture ditch water in large-scale outdoor facilities.
  - Integrate work across a ***lab-to-field model***. Controlled laboratory-scale experiments will simulate diurnal raceway conditions through computer controlled light level, heating, cooling, air addition, CO_2 addition, mixing level, media addition, and mimic current methods for pond transfer and scale up.
  - Our prior successes now make it possible to engineer polymicrobial systems (designed ecosystems) and test using our toolkit and shared learning deliverables.

1 – Approach

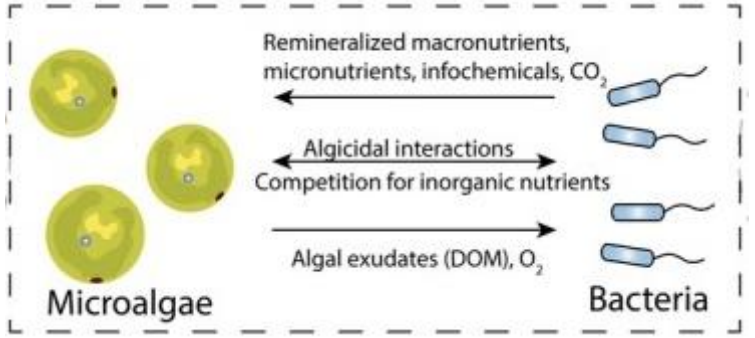
- ***Key decision points***
  - BP2: Develop and implement methods for real-time evaluation of GAI pond associated microbiome
    - G/NG#2: Successful implementation of ONT protocols at Global Algae Innovation (May 2023; *18 months (6-month extension))*.
  - BP3: Develop, curate, and contextualize database
    - *Achieving intermediate targets (18 months)*
  - BP4: Synthesize actionable information logistics framework
    - *Achieve end targets (15 months)*



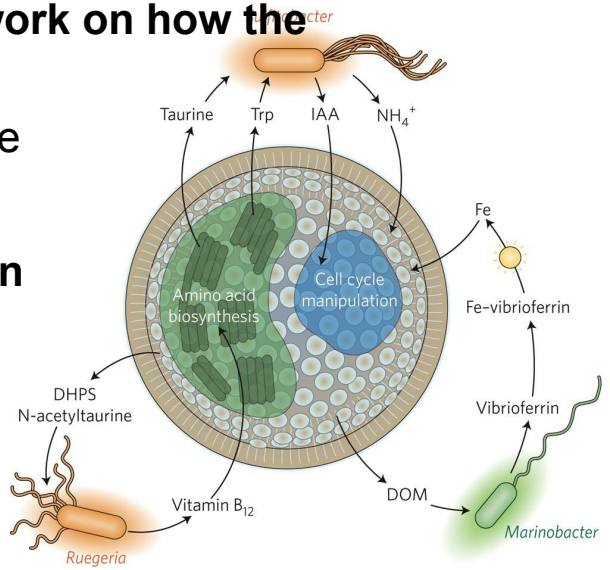
1 – Approach

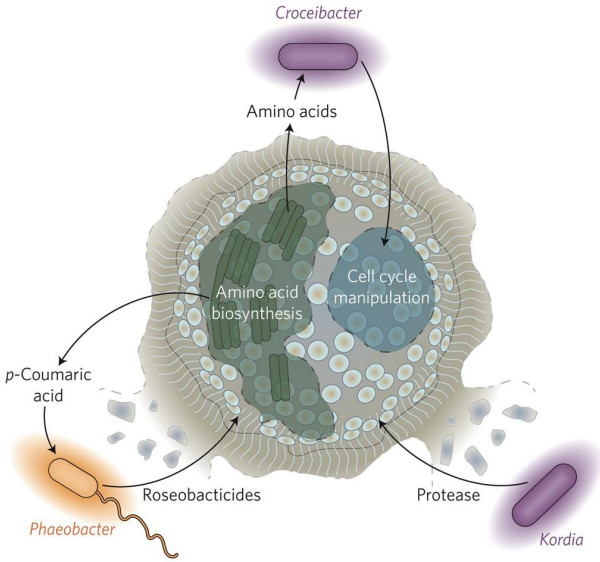
- ***What is the microvirome? Why is it important to pond productivity?***

- **Cultivation of algae in open ponds are at the mercy of the environment**
  - Impacted by adverse weather conditions and contamination
  - Understanding microbiome in algae cultivation is the new frontier that is going to have a major effect on improving algal productivity
- **Eukaryote, bacterial and viral co-inhabitants (microbiome) are a vital part of the mesocosms**
  - capable of impacting microalgae both positively and negatively leading to corresponding impacts on productivity
- **Identification and isolation of key organisms will facilitate work on how the algae pond ecology works.**
  - This is the first step to conduct any kind of pond microbiome manipulation to improve productivity.
- **Phycosphere related research is advancing its importance in**
- **varied environments.**
  - Therefore, data generated in this project may have a much broader application than originally considered



Lian, et al., Micro Biotech, 2018

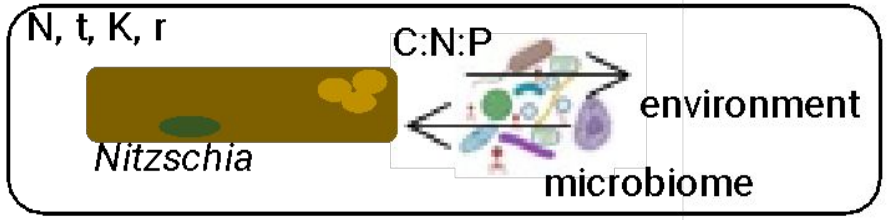


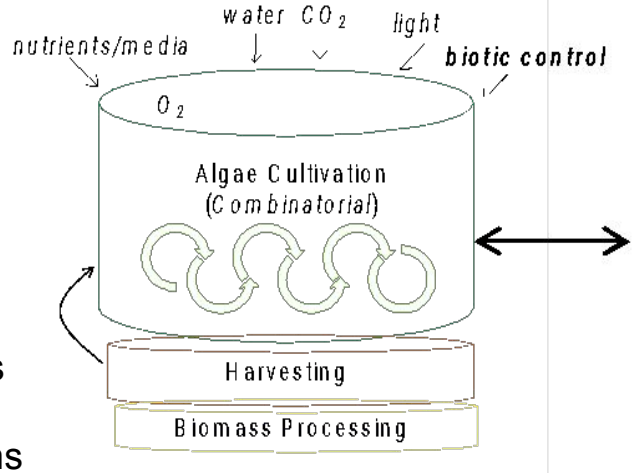


2017, Seymour et al., Nature Microbiology

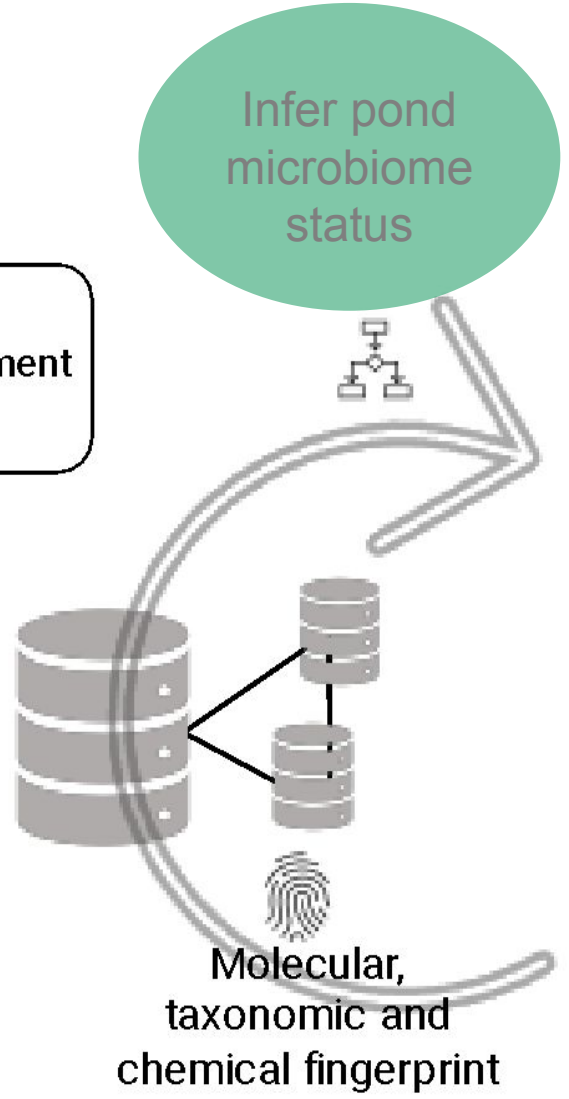
1 – Approach

Task 4. Development of relational database and analysis workflow (M4-M46).
Task 5. Molecular signatures of activity for toolkit development (M6-M46).
Task 6. Use lab-scale mesocosm and continuous culture experiments to develop *lab-to-field* model (M6-M46).
Task 7. Synthesis (M34-M46).

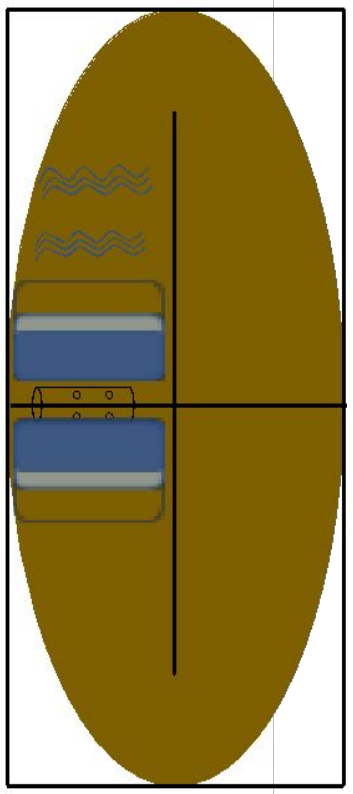




Empirical data:
Abundances
Transcript (activity)
Temporal dynamics
Diversity
Weighted interactions (spearman correlations)
Microbiome stability



- *How and when are taxa connected?*
- *What molecular fingerprints lead to greater algae productivity?*



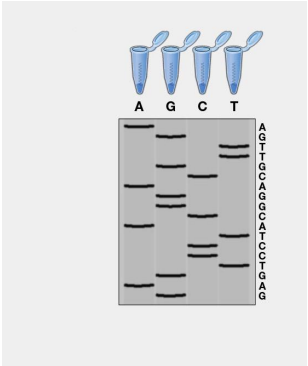
1 – Approach

- ***Task 3:*** **Real-time pond microbiome monitoring with Oxford Nanopore Technologies (ONT)**

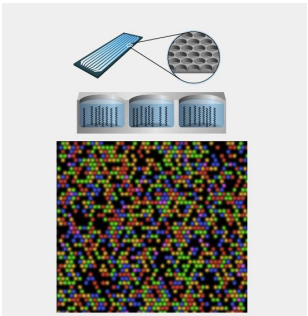
- The task will bring 3rd generation sequencing to the farm, linking state of the art technologies with agriculture practices.
- We will bypass classic approaches of detecting and tracking single organisms, e.g., quantitative PCR (qPCR) or loop mediated isothermal amplification (LAMP) assays.
- We will test and implement ONT to evaluate pond communities in real-time using both barcoded amplicon and full-length (or near) genome sequencing for ***unbiased monitoring of taxa***, not restricted to *a priori* knowledge.

DNA sequencing by synthesis

Sanger DNA sequencing

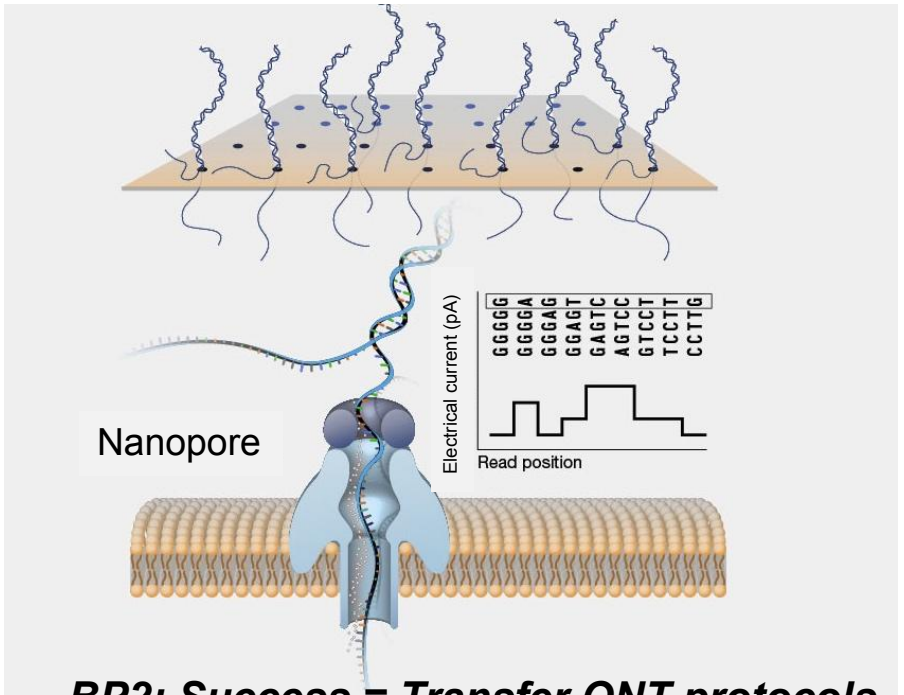


Massively parallel DNA sequencing (e.g., Illumina)



Single molecule DNA sequencing

Nanopore DNA sequencing



BP2: Success = Transfer ONT protocols to GAI; optimize time, cost and quality

1 – Approach

- ***Risk Mitigation Plan***

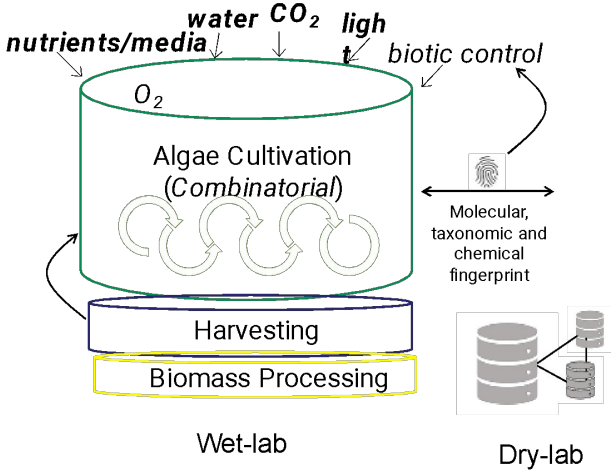
Project Risk	Planned Mitigation
Time – toolkit does not complete prior to having enough time for actionable information.	Reduce efforts on other tasks where enough data has been generated.
Acquisition of high quality and/or quantity of HMW gDNA from pond samples	Use established protocols to reduce contaminating chemicals or residuals from media.
Use of RNA on ONT as a proxy of algae stress	Use funds on Illumina based approaches to acquire RNA data as needed.
Cost – identify cost model that achieves desired outputs.	Plan and purchase flow cells in bulk, identify when a full flow cell is needed versus flongle. Weigh importance of time versus output. Identify computation protocols that reduce costs in the long-term.

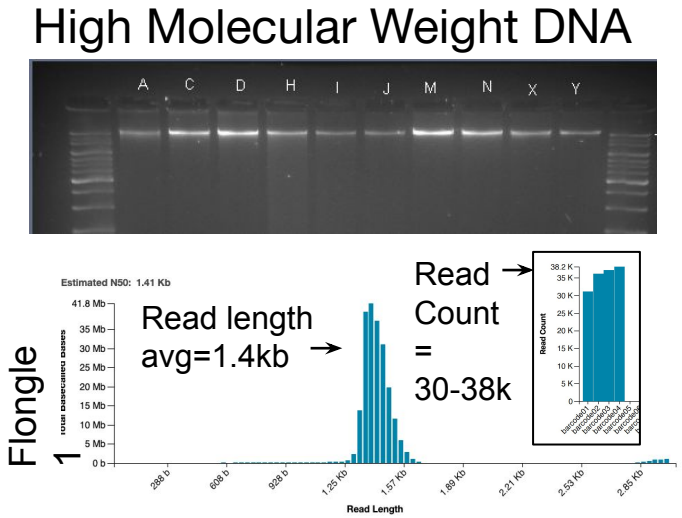
Project Communication Management

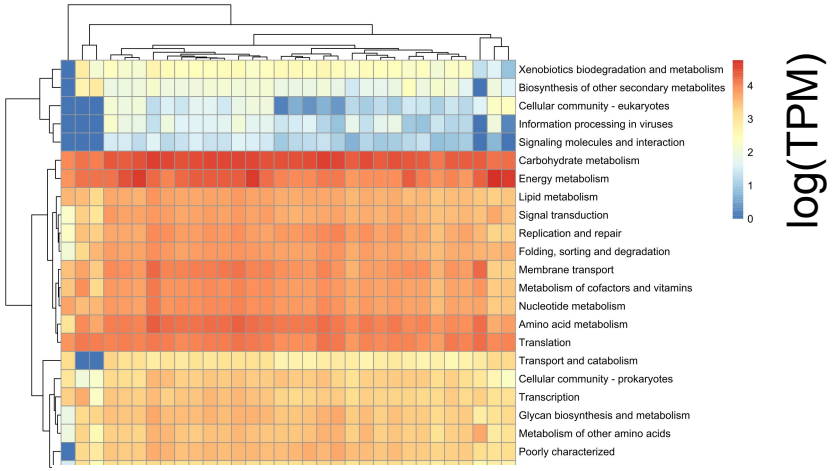
- Biweekly calls between SIO/UCSD and GAI (Allen, Pinowska, Traller, Zeigler and all personnel)
- File sharing of all information via email or google drive
- Monthly reporting and calls with Dan Fishman and Phil Lee (Zeigler and team when available)

1 – Approach

- ***Challenge/Risk***: Acquisition of high quality and/or quantity of HMW gDNA from pond samples
  - Planned mitigation: Use established protocols and reagents
- ***Challenge/Risk***: Identifying pond status and respond with combined mitigation strategies
  - Planned Mitigation: Use established protocols for assessing algal stress using standard sequencing approaches and/or combine current mitigation strategies and identify ways of mitigating influence upstream of ponds



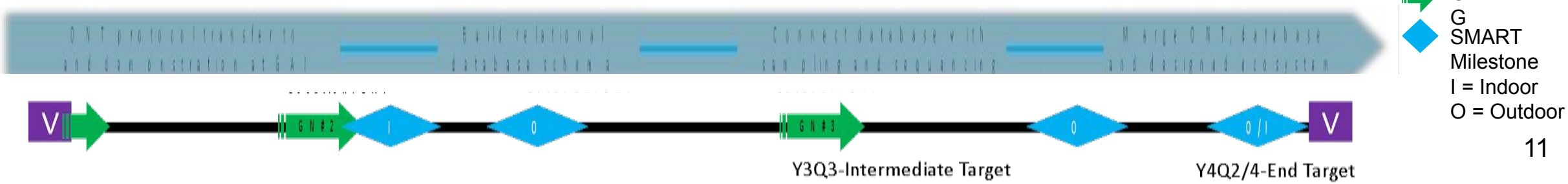




RNA sequencing = proxy for activity of cells

2 – Progress and Outcomes

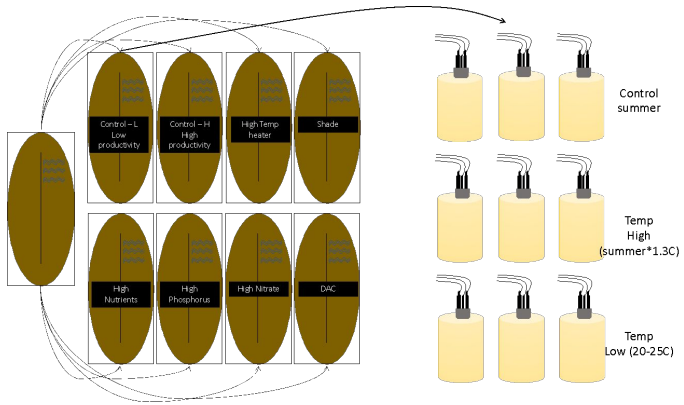
- ***Milestones***:
  - Develop and implement methods for real-time evaluation of GAI pond associated microbiome
    - ✔ Initial Verification (12/10/2021) (M2.1)
    - ✔ Kick-off meeting and sampling (June/July 2022) (M2.3)
    - ✔ Curate existing sequence data from previous project, both DNA and RNA (M4.1.1)
    - ✔ Identify appropriate field protocol from lab testing, including HMW DNA capture, library construction and sequencing. (M3.1.1)
    - ✔ Real-time pond microbiome monitoring with Oxford Nanopore Technologies (ONT) (SMART M3.1)
      - ***Go/No-Go 2 (05/08/2023)***

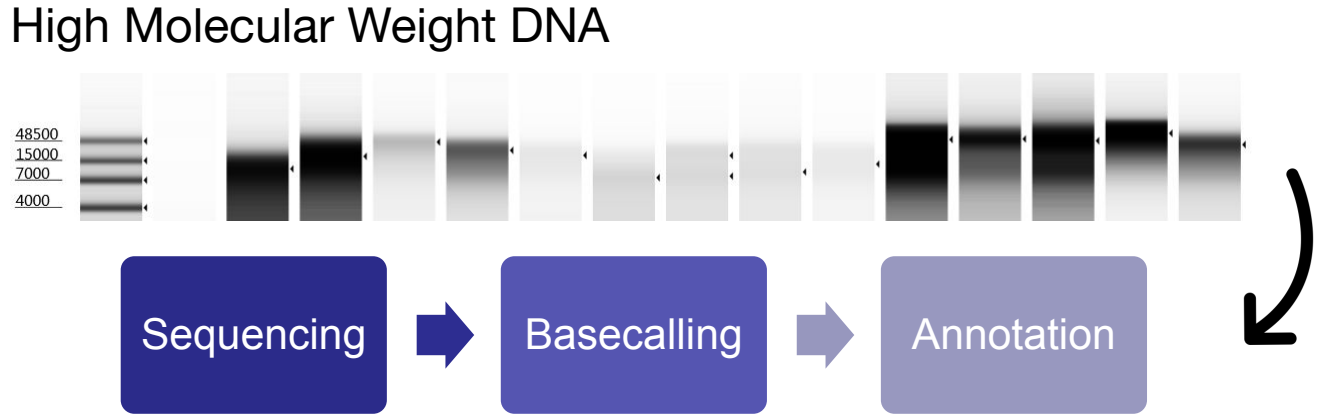


2 – Progress and Outcomes

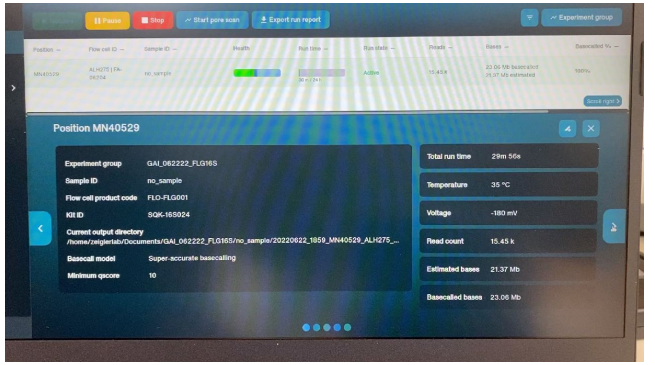
- Acquisition of high quality and/or quantity of HMW gDNA from ***lab-to-field*** samples while evaluating abiotic stressors
- Develop SOPs for time and cost efficiencies

June/July 2022 – Lab-to-Field Trial





✔ ***Reconfigured workflow basecall during sequencing in real-time***



2 – Progress and Outcomes

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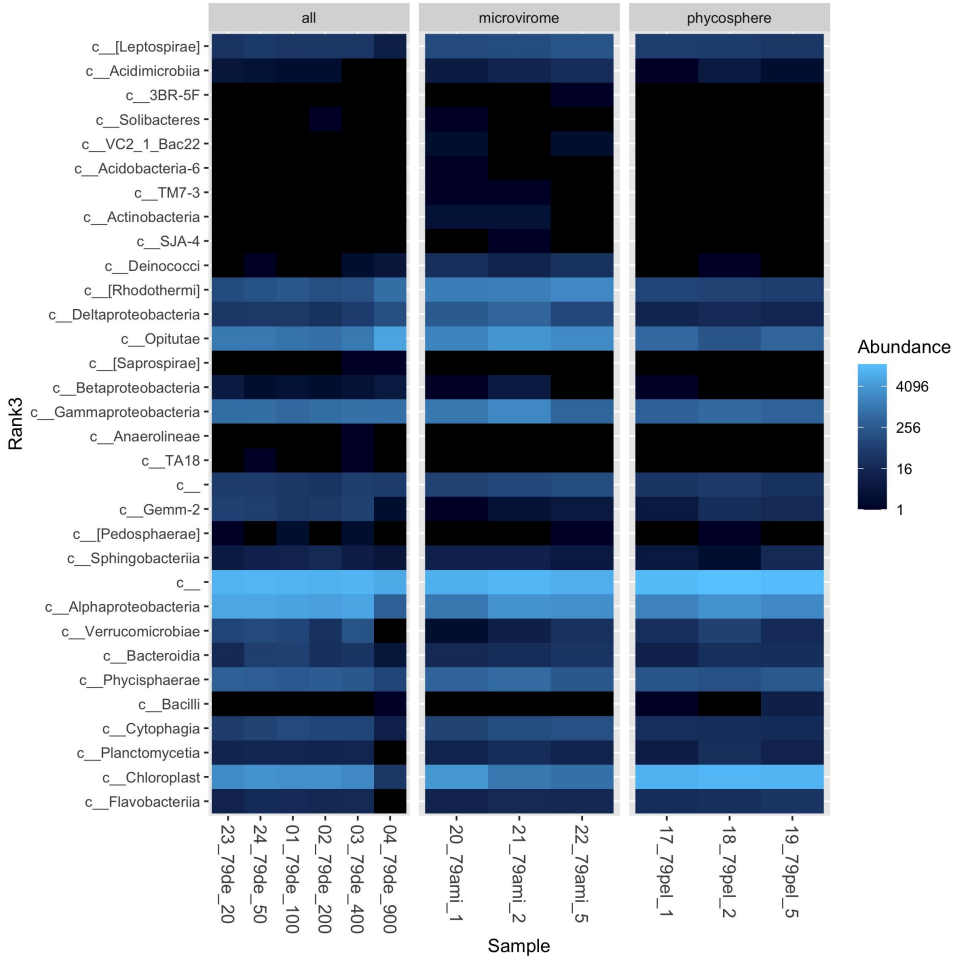
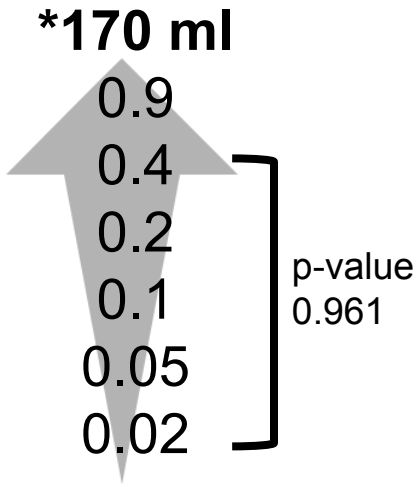
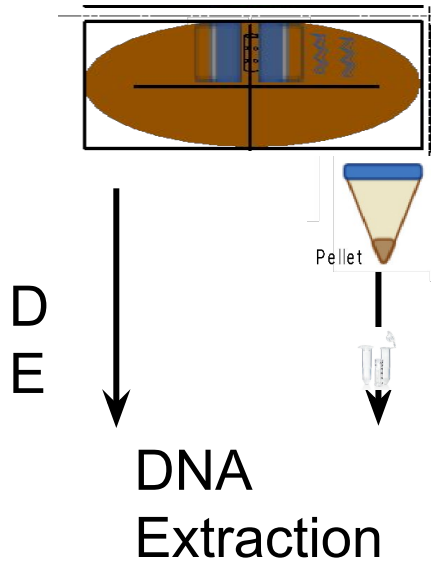




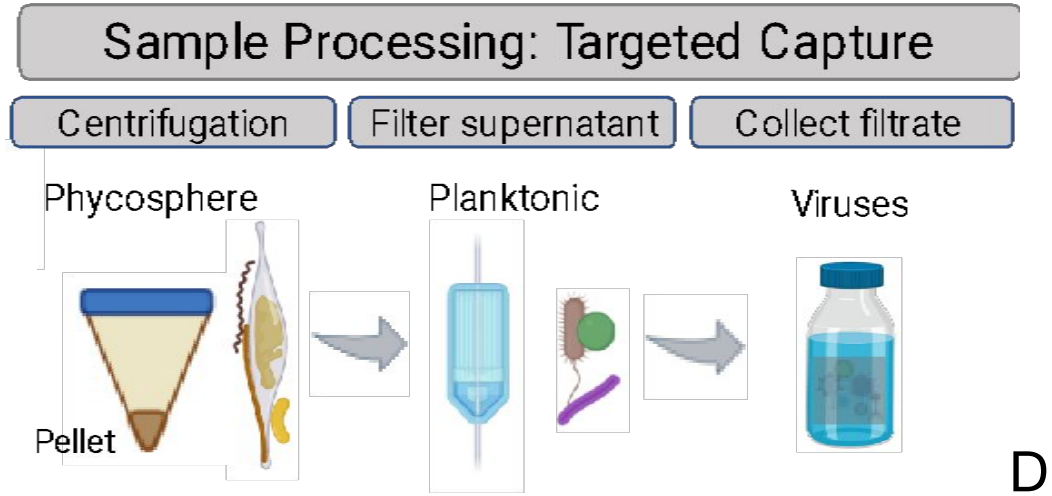


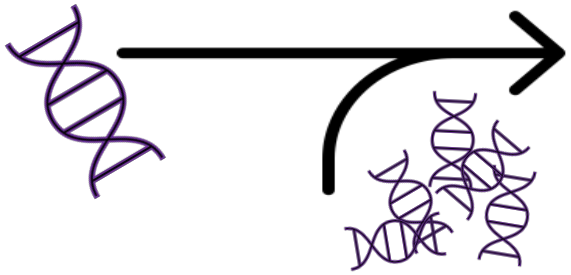
✔ ***Validated reduced biomass, sampling time does not affect DNA or microbiome analyses***

✔ ***Reduced processing time and cost***

2 – Progress and Outcomes

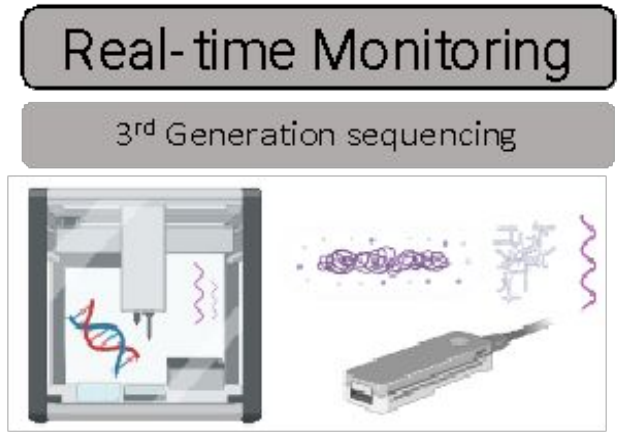
- Complete ***sample-to-sequence workflow*** on an actionable timeline.



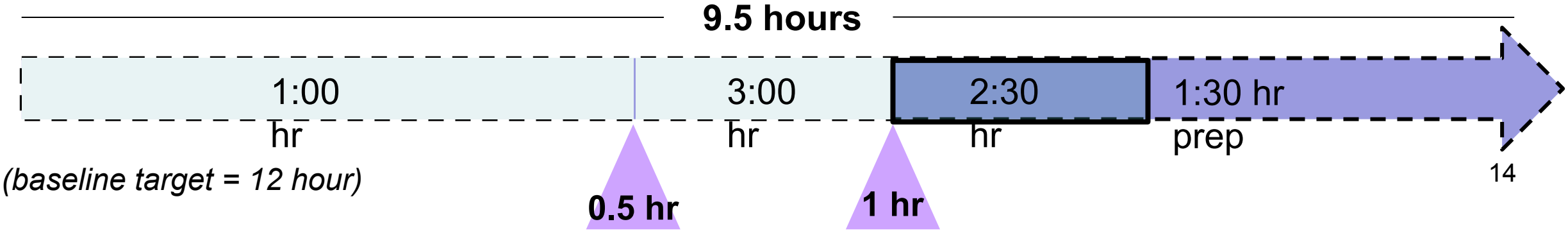


DNA extraction

PCR Amplification



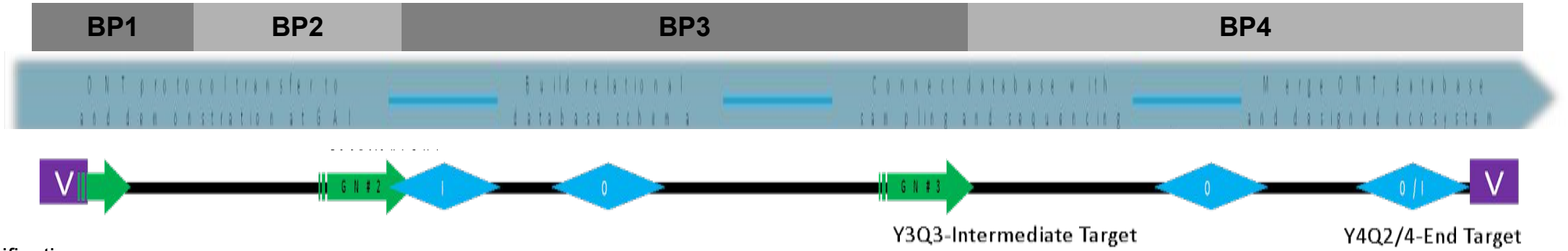
1. Direct sequencing HMW DNA
2. 16S rDNA amplicon sequencing



(baseline target = 12 hour)

2 – Progress and Outcomes

- ✔ Completed workflow for organizing larger datasets into a single analysis run
- ✔ Completed all taxonomic annotation of 16S and WGS
- ✔ Completed initial sequencing of 2022 RNA on Oxford Nanopore
- ✔ In progress – Identify molecular signatures of algae stress
- ✔ In progress – Continued analysis of microbiome data
- ✔ Finalize bioinformatic SOPs for transfer to GAI



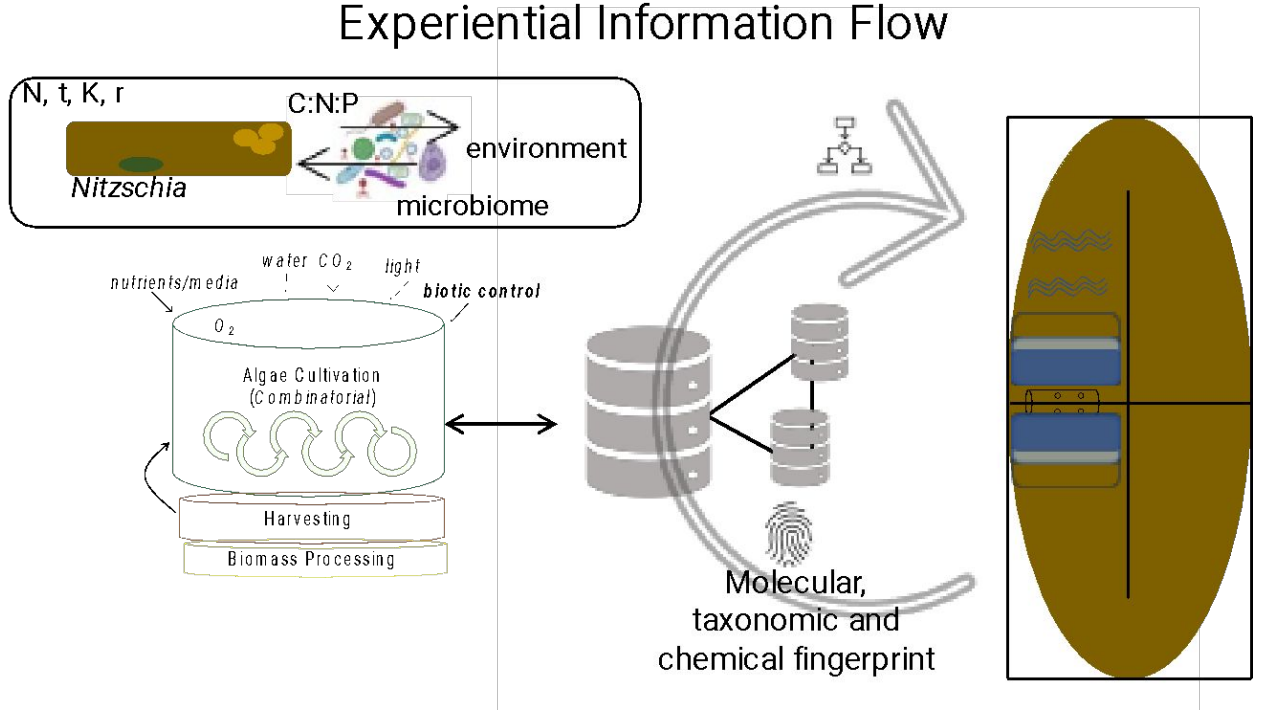
V Verification
G/N G
SMART Milestone
I = Indoor
O = Outdoor

3 – Impact

- ***Relevance to bioenergy industry***:
  - Elite algal strains have been targeted for the production of bioproducts, yet system-level ecological information is largely unknown.
  - Outdoor cultivation of algae necessitates ecological understanding.
    - The research and development of cultivation improvement strategies proposed here will use **real-world conditions, including using agriculture ditch water** needed for large-scale growth (rather than tap or other water sources) that directly link to economic viability and sustainability of algal biofuel production.
  - A low cost, rapid analytical tool to measure microbiota would greatly accelerate development of cultivation advances and treatment protocols.
- Our approach using combinatorial cultivation strategies concurrent with multi-omic detection methods enables evaluation of upstream stages that when balanced with current downstream harvesting technologies can reduce the total production cost.

Summary

- Toolkit and shared learning deliverables will include a combination of a curated algal microbiome database, analysis workflows, and a collection of mitigation strategies from an integrated systems-level approach
- This actionable information can alert farm personnel of ecological perturbations in real-time and be vital in mitigating non-directed algal stress leading to higher biomass quality and ultimately productivity.



Outdoor non-monoculture ponds are the most economically viable for industrial production of algal biofuels, therefore ecological monitoring is essential for achieving the full economic potential of outdoor-pond systems

Summary

Project Team

Scripps Institution of Oceanography, University of California San Diego

Lisa Zeigler
Eric Allen
Ariel Rabines
Laela Booshehri
Entesar Alrubaiaan
Aaron Oliver

Global Algae Innovations

Aga Pinowska
Jesse Traller
Dave Hazlebeck
Mark Hazlebeck
Paul Hazlebeck
Josh Brown Clay
Kailey Sager
Joel Burke
Shyla Villanuelva
Jeremy Frischknecht
William Demotta
Nick Vallatini
Jon Keating
Isaiah Dorsey

Quad Chart Overview

Timeline

- *10/1/2021*
- *09/30/2025*

	FY22 Costed	Total Award
DOE Funding	*(10/01/2021 – 9/30/2022)* *$271,450*	*(negotiated total federal share)* *$3,420,348*
Project Cost Share *		$690,328

Project Goal

Integrate and make actionable information about pond microbiota and genetic stress markers from real-time genomic-based monitoring to develop new cultivation strategies to achieve greater algal productivity (≥20%)

End of Project Milestone

Provide system-level functional relationships between elite algae and the microbiome. Toolkit and shared learning deliverables will include a combination of a curated algal microbiome database, analysis workflows, and a collection of mitigation strategies from an integrated systems-level approach that can be utilized in a decision tree model

Funding Mechanism

*FY21 BETO Feedstock Technologies and Algae FOA, T*opic Area 2: Algae Productivity Exceeding Expectations (APEX), *Subtopic 2a*

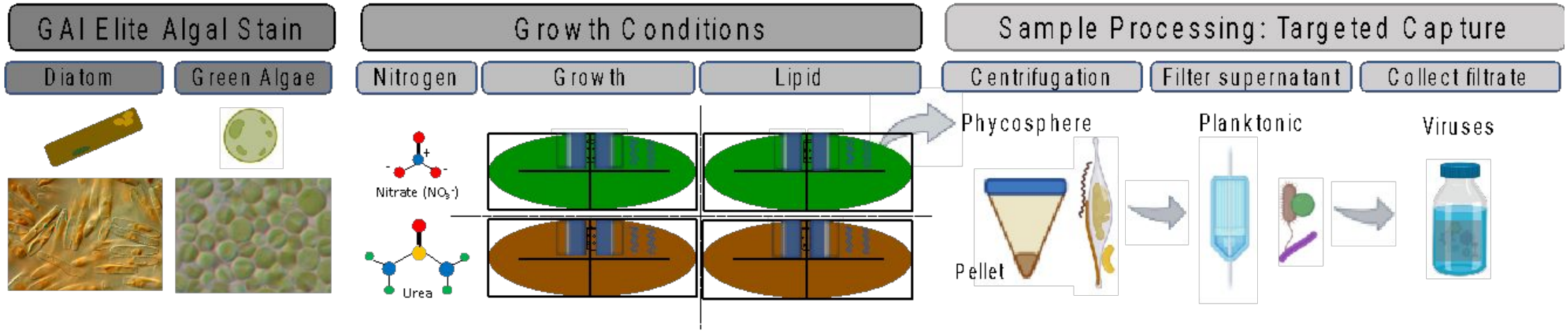
TRL at Project Start: 3
TRL at Project End: 4

Project Partners*

- Global Algae Innovations

*Only fill out if applicable.

Additional Slides



Sequencing

PacBio

Genomes:
Diatom – *Nitzschia**
Green - *Nannochloris*

*Oliver, Podell, et al., 2021

Oxford Nanopore

Real-time monitoring:
16S rDNA
WGS – bacteria and virus

Illumina

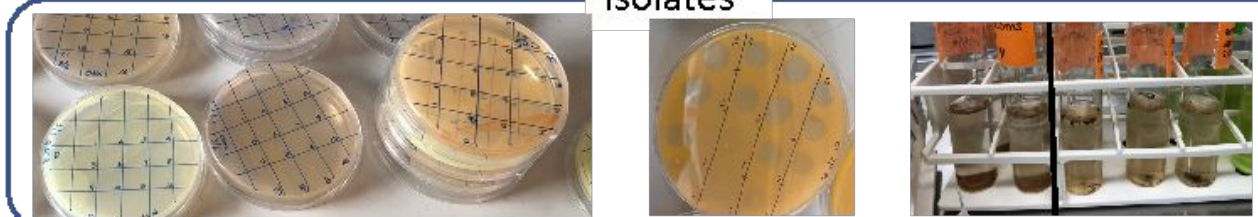
ASVs
Total RNAseq
*metaG

Viromics
Viruses binned from RNAseq

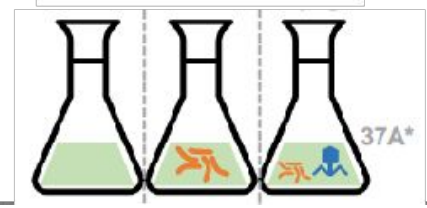
Rabines, et al., *in prep*

Cultivation

Isolates



Co-cultivation



Instrumentation acquisition

Quantification of MicroVirome

Attune Flow Cytometer
ThermoFisher

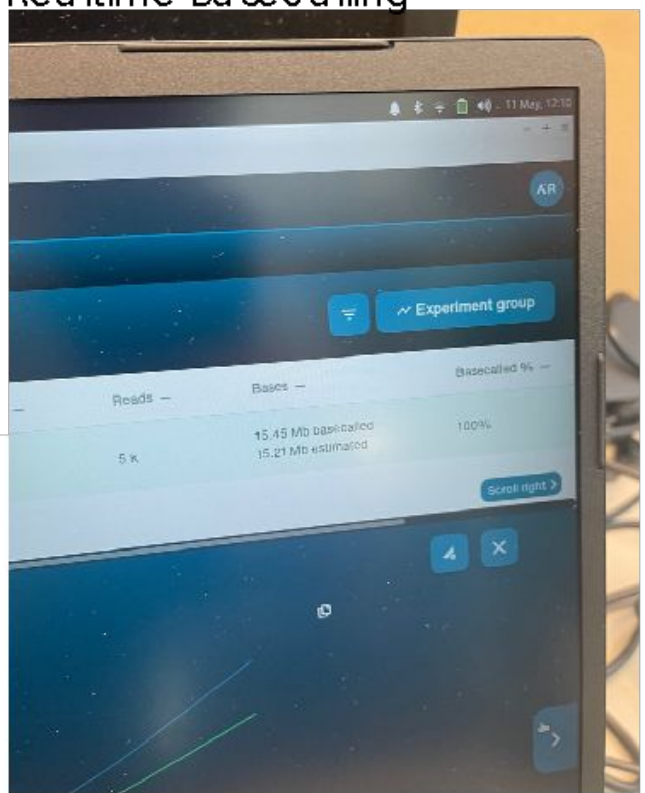
Graphics processor for increased sequencing/analysis throughput and accuracy

Juno Computers

Neptune 15" v3 × 1

Processor: Intel Eight-core i7-11800H (2.3GHz, 4.6GHz Turbo)
Display: 15.6" 240Hz Refresh Rate - Full HD 1080p Matte
Graphics Card: NVIDIA GeForce RTX 3080 Max-Q - 16GB GDDR6
RAM (DDR4): 32GB (2x16GB) 3200MHz SODIMM
1st SSD M.2 NVMe: 2TB (3480 MB/R, 3000 MB/W)
2nd SSD M.2 NVMe: 2TB (3480 MB/R, 3000 MB/W)
Keyboard: US English
Operating System: Ubuntu 20.04

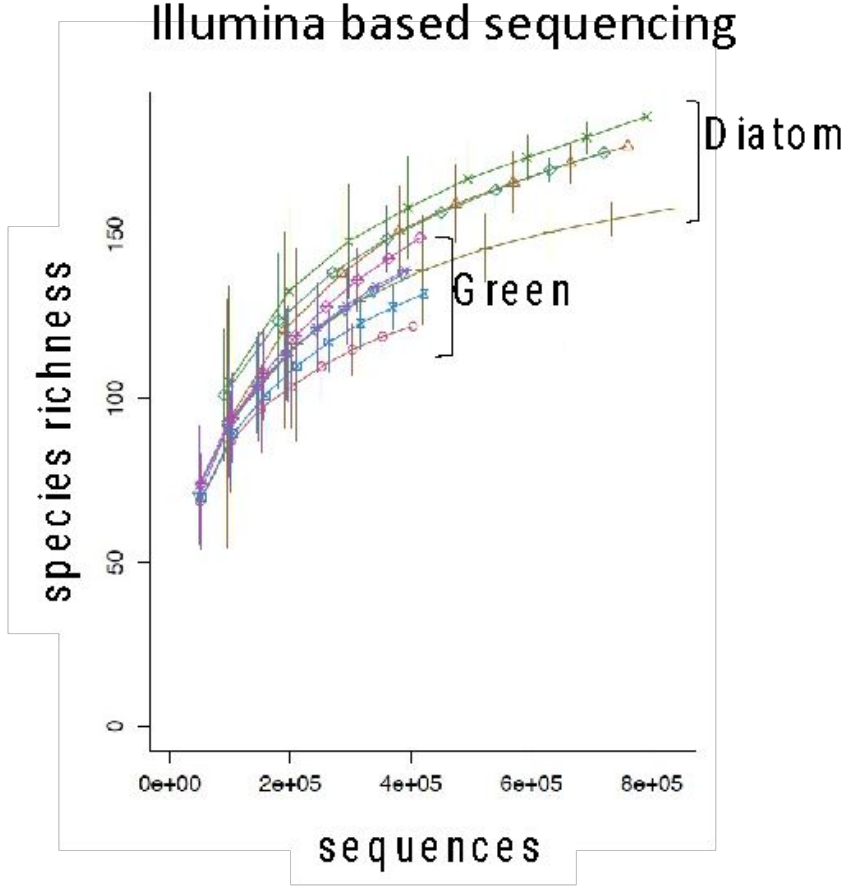
Realtime Basecalling



Amplicon sequencing coverage

What effects and risks are associated with reduced processing time when using ONT?

Illumina based sequencing

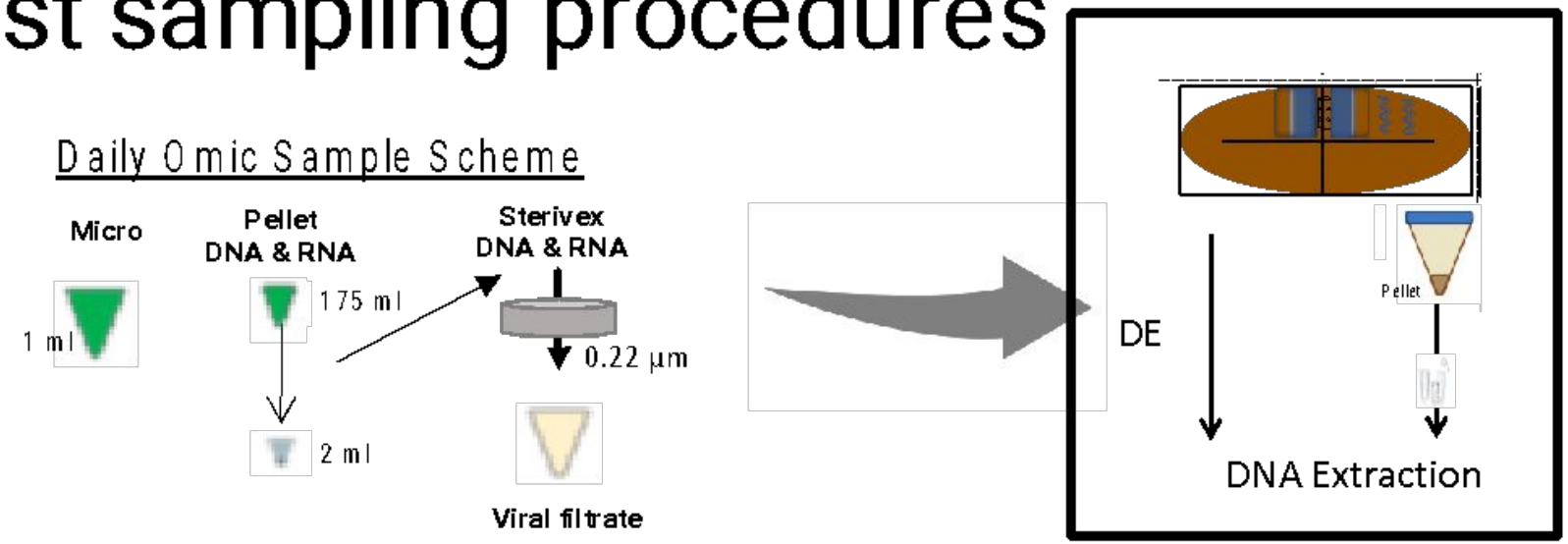


ONT based sequencing

Run		Flowcell	Run time	# reads	Avg/bar code (k)	N50 (kb)
Test 1	16S	full	1h 40m	250 k	110	1.53
Test 2	gDNA	Full	20h 50m	130 k	n/a	19.02
Test 3	16S	flongle	2d 18h	278 k	35	1.41
Test 4	16S	flongle	1d 22h	212 k	33	1.42
Test 5 (11/16)	16S	full	12 h	4.04 M	600-800	1.52
Test 6 (11/22)	gDNA	Full	12h	330 k	n/a	27.29

*testing indicates we are achieving saturation based on species richness. ONT provides full-length sequences giving better taxonomic resolution

Test sampling procedures



Pond 79 (6/24)

2. Sample Volume

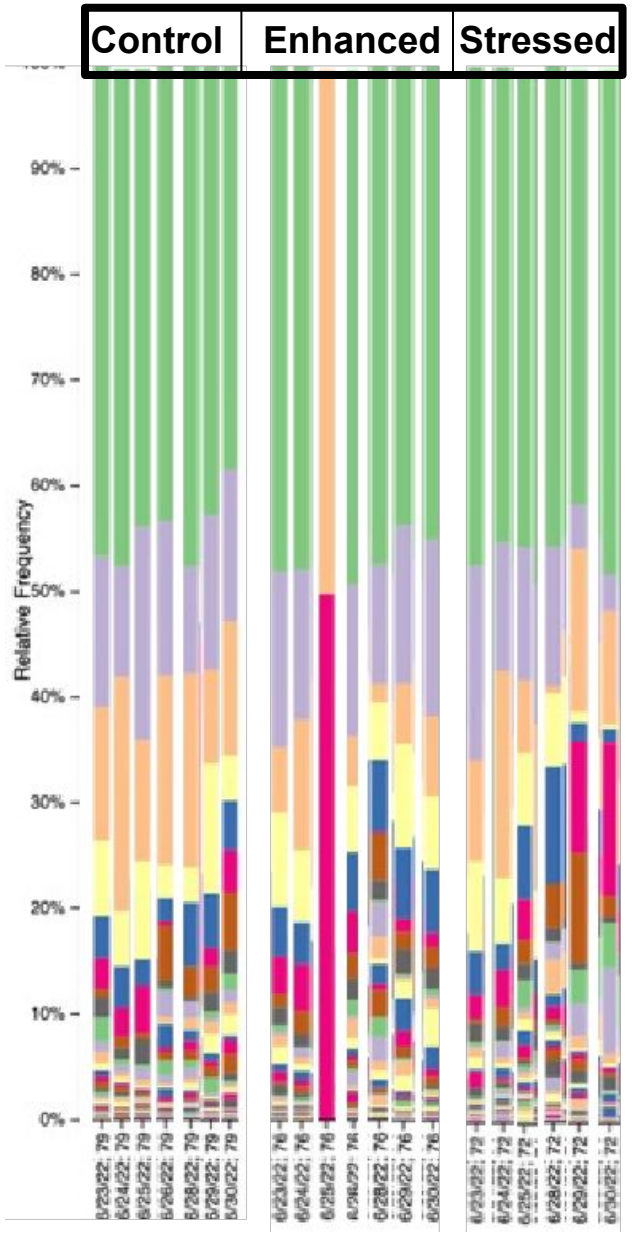
No centrifugation/filtration
Direct Extraction (DE)

Volume:	0.02 ml	0.05 ml	0.10 ml	0.20 ml	0.40 ml	0.90 ml
DNA yield	55 ng (31.8)	63.5 ng (34)	112 ng (74)	182 ng (130.4)	276 ng (204)	45.8 ng (21.4)

Extract the whole pellet

Concentrate Bacteria (planktonic) and viruses

Volume:	1 ml	2 ml	5 ml
DNA yield	1.1 ug pellet 47.5 ng ami	2.9 ug pellet 86 ng ami	4.9 ug pellet 274 ng ami

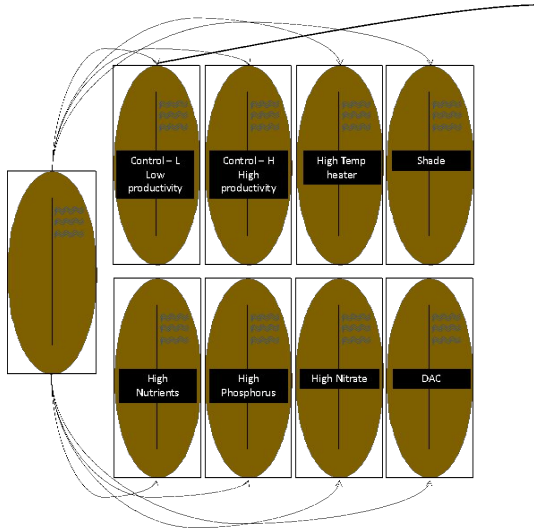


Unknown bacteria

Spirochetes

Pond	Condition
70	DAC
71	Control (L)
72	High Temp
74	Shade
76	High Nutrients
77	High Phosphorus
78	High Nitrate
79	Control (H)

Planktonic Microbial Fraction



- Completed workflow for organizing larger datasets into a single analysis run
- Completed all taxonomic annotation of 16S and WGS
- In progress – continued analysis of microbiome data
- Finalize analysis SOPs for transfer to GAI

Isolation of algae pond-associated bacteria

Isolation of Bacteria From Bulk Microbial Community

Xenic Diatom Farm Water

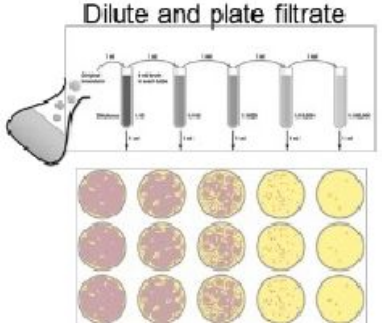
Transfer to sterile media

Batch farm water

Filter algae out and quick rinse

Bulk microbial community

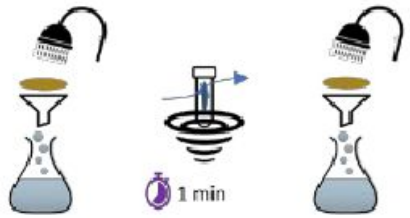
Dilute and plate filtrate



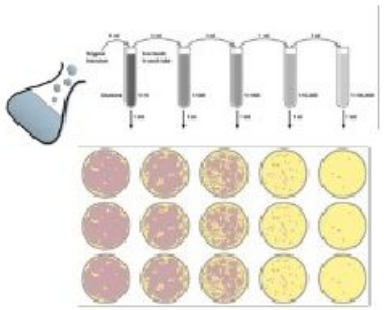
Isolation of Bacteria From Attached Microbial Community

Filtered Algae from Xenic Farm Water

Long rinse, vortex, rinse

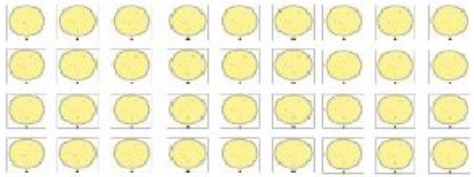


Dilute and plate filtrate

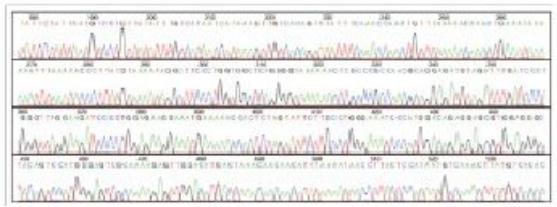


Purification and Identification of Bacterial Isolates

Purify colonies from bulk and attached mixed communities



Identify isolates using DNA extraction and 16s rRNA amplicon sequencing



Preserve isolates

Cultivation of relevant microbes and viruses

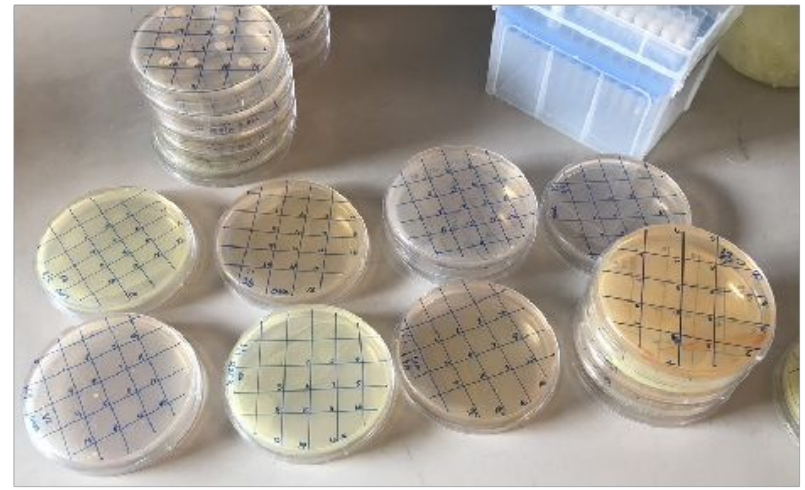
Diversity and characteristics of bacterial strains isolated from GAI ponds

	Isolate ID	Host	Phylum	Genus	Significance
	EA1	GAI-339	Actinobacteria	**Microbacterium**	Microbacterium can enhance growth of green algae; some strains competitive and inhibit growth
	EA6	GAI-339	Firmicutes	**Bacillus**	Related to *B. firmus* that can remove metal ions from water
*	**EA8**	GAI-339	Firmicutes	**Exiguobacterium**	Plant growth promoting bacteria.
	EA14	GAI-247	Gammaprot	**Halomonas**	Promotes growth of Nannochloropsis
	EA5	GAI-339	Bacteroidetes	**Arthrospiribacter**	Priority strain. Abundanat in farm water with diatom host. Can utilize diatom storage polysaccharides
	EA30	GAI-241	Alphaprot	**Rhodobaca**	Priority strain. Abundanat in farm water with diatom.
	EA13	GAI-247	Firmicutes	**Bacillus**	B. pumilus; inhibits growth inhibition of Nannochloropsis
	EA16	GAI-339	Gammaprot	**Halomonas**	Halomonas sp. HSB07; inhibits growth of the red-tide microalga Gymnodinium sp.
*	**EA32**	GAI-247	Alphaprot	**Paracoccus**	Does not promote algae growth in growth experiments at GAI farm
	EA2	GAI-247	Gammaprot	Luteimonas	
	EA3	GAI-339	Alphaprot	Alishewanella	
	EA7	GAI-247	Actinobacteria	Jonesia	
	EA9	GAI-247	Firmicutes	Planococcus	
	EA10	GAI-247	Actinobacteria	Dietzia	
	EA11	GAI-339	Alphaprot	Roseomonas	
	EA12	GAI-339	Gammaprot	Alkalimonas	
	EA17	GAI-247	Alphaprot	Arsenicitalea	
	EA18	GAI-229	Gammaprot	Pseudomonas	
	EA21	GAI-232	Actinobacteria	Microcella	
	EA24	GAI-235	Bacteroidetes	Belliella	
	EA28	GAI-239	Actinobacteria	Aeromicrobium	
	EA29	GAI-240	Alphaprot	Natronohydrobacter	

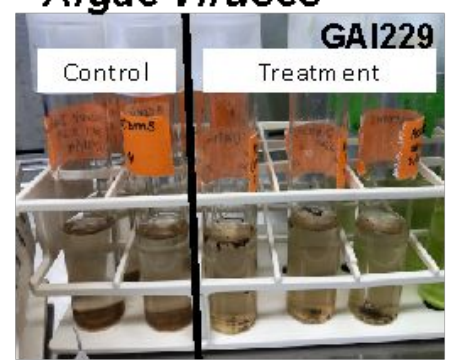
	Positive interaction
	Priority strain
	Negative interaction
*	Phenotype confirmed

- *Strains were isolated using multiple media formulations from GAI pond samples*
- *Hundreds of strains recovered representing >20 genera from diverse lineages*

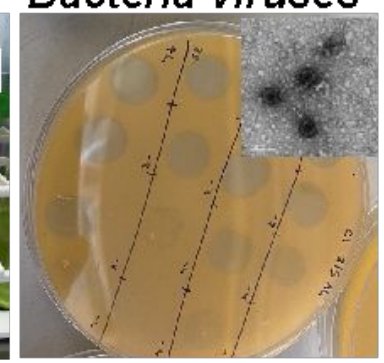
Bacteria



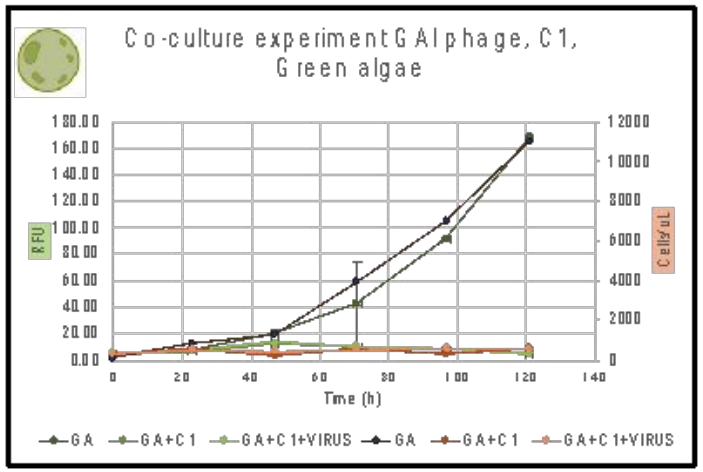
Algae viruses

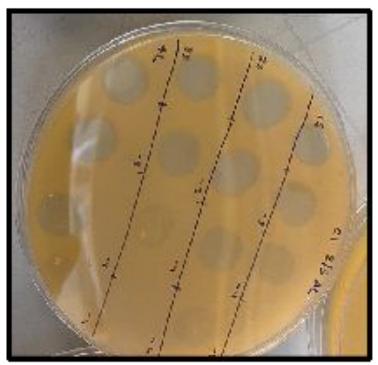


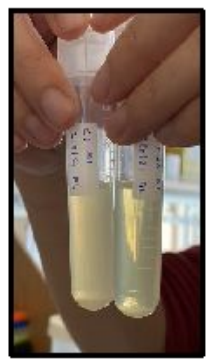
Bacteria viruses

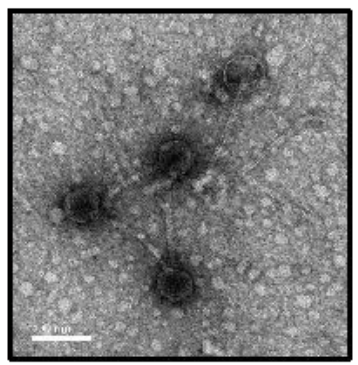


Laboratory-scale co-cultures





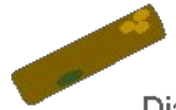


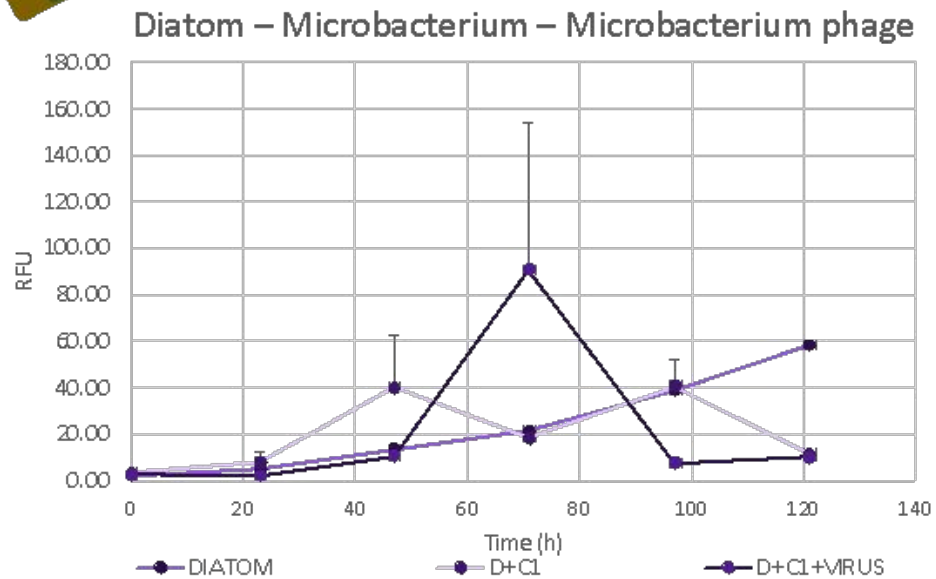


<u>Microbacterium phage characterized</u>

Complete genome, 53 kb

86 CDS; GC%: 67.78





<u>Diatom interactions</u>

- Diatom spike at 71 h (D+C1+V)
  - Indirect - re-colonization of DAB
  - Direct - removal of negative interactions
- Diatom spike at 42 and 99 h (D+C1)
  - C1 removing (–)DAB through antibiotic production*
  - Not as significant as removal of (–)C1:D interaction

**Free living Microbacterium inhibited the growth of known +diatom associated bacteria (Alteromonas, Pseudomonas species) but producing antibiotics*

Publications, Patents, Presentations, Awards, and Commercialization

- List any publications, patents, awards, and presentations that have resulted from work on this project
- Use at least 12 point font
- Describe the status of any technology transfer or commercialization efforts

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