DOE Bioenergy Technologies Office (BETO) 2019 Project Peer Review

Prevention of Low Productivity Periods in Large-Scale Microalgae Cultivation (PEAK)

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GLOBAL ALGAE

1 Project Goal

The goal is to reduce periods of unexplained low pond productivity by identification and control of microbiota cultivated with target algae

- Measure the microbiota (viral, bacterial, algae, protozoa, fungi)
- Develop a tool for low cost, rapid analysis of pond microbiota
- Utilize the tool and microbiota information to develop cultivation methods to achieve algal productivity of > 25 g/m^2d.

Relevance to bioenergy industry

- Crop protection and productivity is crucial to economic viability and sustainability of algal biofuel production
- Understanding and controlling microbiota are crucial to algae crop protection and productivity
- There is little publicly available information on microbiota control in algae cultivation
- A low cost, rapid analytical tool to measure microbiota would greatly accelerate development of cultivation advances and treatment protocols

Quad Chart Overview

Timeline

10/2017 – 9/2020
40% Complete

Barriers

Aft-B. Sustainable Algae Production

Aft-C. Biomass Genetics and Development

Aft-A. Biomass Availability and Cost

Budget

	2017	2018	2019 +
DOE	65	1193	1442
Cost Share	16	298	360
Partners • Scripps Institution of Oceanography, UCSD • J Craig Venter Institute • Sandia National Lab. • Scott Fulbright			32% 13% 11% 2%

Objective

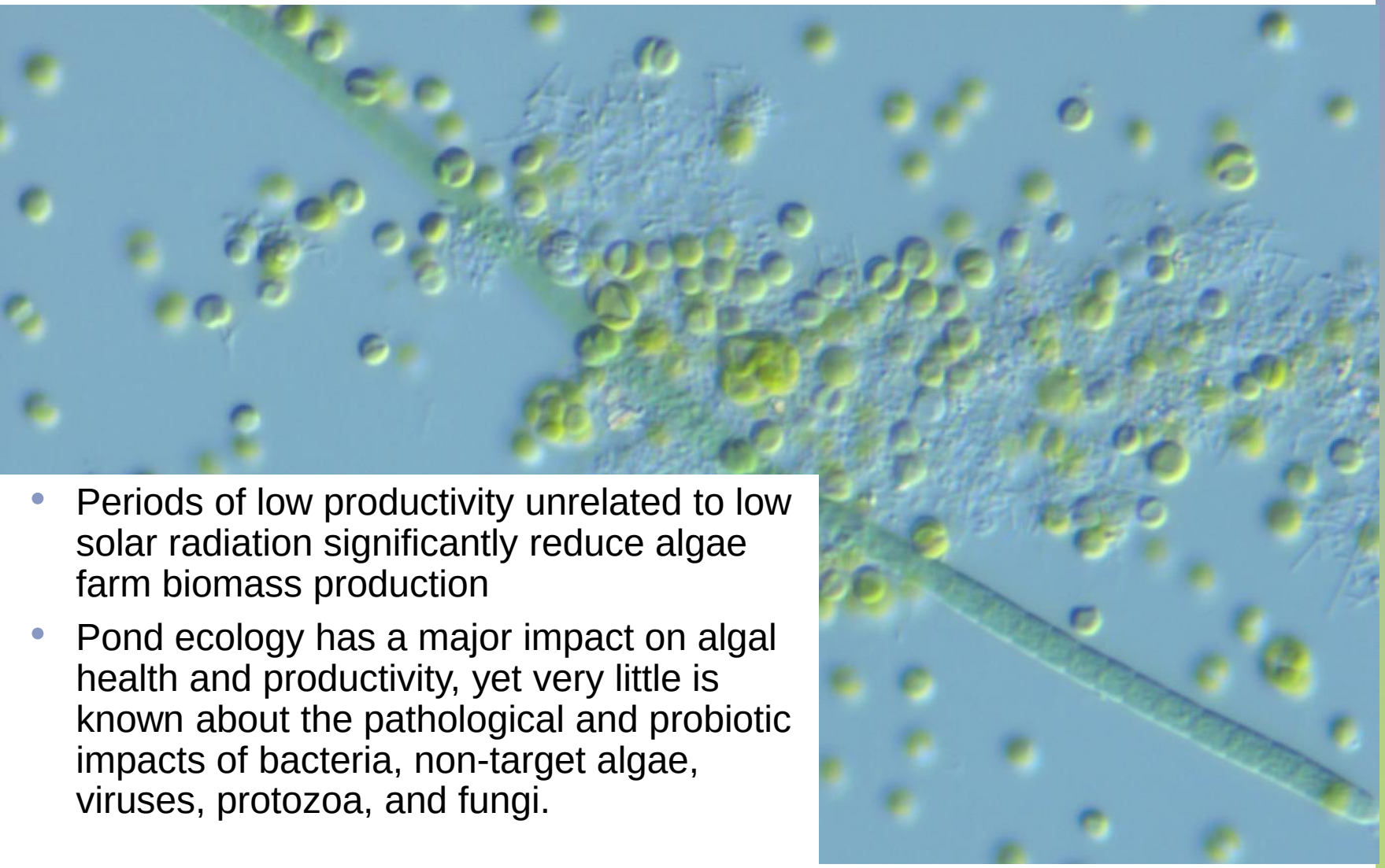
Achieve systematic high algal productivity through analysis and control of pond microbiota

End of Project Goals

- Data on pond microbiota and correlations with productivity
- Low cost, rapid analytical tool for measuring microbiota
- New cultivation methodology resulting in algal productivity of >25 g/m^2 day on an ash-free dry weight basis

GLOBAL ALGAE INNOVATIONS

General Project Overview



- Periods of low productivity unrelated to low solar radiation significantly reduce algae farm biomass production
- Pond ecology has a major impact on algal health and productivity, yet very little is known about the pathological and probiotic impacts of bacteria, non-target algae, viruses, protozoa, and fungi.

1 – Project Overview

Advanced, scalable outdoor cultivation system

- High productivity and high concentration
- Robust biofuel strains
- Relevant to future large-scale industry

Experts in genomic sequencing & analysis and isolation

- Viruses – JCVI, Dr. Zeigler
- Bacteria – USCD/SIO, Dr. Allen
- Eukaryotes – Sandia, Dr. Lane & Dr. Mahan

Tool development for fast/low cost microbiota analysis

- SpinDX

Develop and apply method to reduce dissolved organic material

- Proof-of-principle test shows reduction increased productivity in laboratory

2 - Approach (Management)

Team communication

- Well defined roles and milestones
- Bi-weekly conference call
- Data exchange through box.com
- Bi-monthly review: budget, milestones, issues, opportunities, risks

Milestones

- Initial sampling and analyses
- Isolations
- Challenge testing
- Control methods
- SpinDX successfully deployed and application for control
- Dissolved organic material control laboratory & outdoors

Project team responsibilities

GAI (cultivation, sampling, analyses, data integration & testing)

- Outdoor cultivation, sampling, non-genomic analysis, Spin DX testing
- Data integration, isolation of eukaryotes, testing of microbiota control

JCVI - Dr. Lisa Zeigler (sequencing, algal viruses)

- Genomic sample preparation, sequencing, and data processing
- Viral data analysis, isolation, challenge testing and control methods

SIO - Dr. Eric Allen (bacteria, DOM control, baseline sequencing)

- Genome sequence of two cutivated strains (green GAI-247 and diatom GAI-229)
- Bacterial data analysis, isolation, challenge testing and control methods
- Reduction of dissolved organic material (DOM) during lab-scale cultivation

Sandia - Dr. Todd Lane and Dr. Krissy Mahan (eukaryotes, SpinDX)

- Eukaryotes data analysis, isolation, challenge testing and control methods
  Development of **SpinDX**

Dr. Scott Fulbright (data integration & analysis)

- Consultation on genomic data integration and data analysis

Project Risk Management

Risk	Pr - Sv	Mitigation	Pr - Sv
Sampling problems & coordination issues	5 - 5	All subs assist with first sampling for real-time solutions and coordination	1 - 5
Can't identify cause and effect for microbiota	3 - 4	Forcing off-normal operation, multiple seasons, many targets (bacteria, fungi, viruses, protozoa)	1 - 2
Can't complete project within budget	4 - 4	Combine genomic analyses; division of data analysis to leading experts; bimonthly reviews – status, synergy opportunities, & adjustments	1 - 2
Can't make SpinDX work	3 - 5	Early test; accelerate feedback loop: two prototypes for outdoor testing in parallel with Sandia modifications	1 - 5

2 – Approach (Technical)

Microbiota information and treatments

- Build broad genomic database
- Identify potential key organisms
- Isolate organisms of interest and test for cause/effect on productivity
- Develop treatment methods

Spin DX instrument to measure microbiota

- Develop instrument and protocol for rapid, simple measurements
- Utilize genomic information to develop target sequences to identify organisms in microbiota
- Demonstrate use of the instrument for identification of culture microbiota

Broad-spectrum treatment

- Develop method to reduce dissolved organic material in the algal cultures
- Test for impact on algal growth in laboratory
- Deploy and test in outdoor cultivation for impact on productivity

Build Genomic Data Set

Broad data set

- Daily genomic and analytical samples following culture from laboratory inoculum preparation through large-scale raceways
- Large-scale grow-outs
- Four seasons
- Two biofuel strains, a green and a diatom
- Stress and different nitrogen sources
- A high bacteria – lower growth rate condition
- High and low productivity

Broad microbiota data

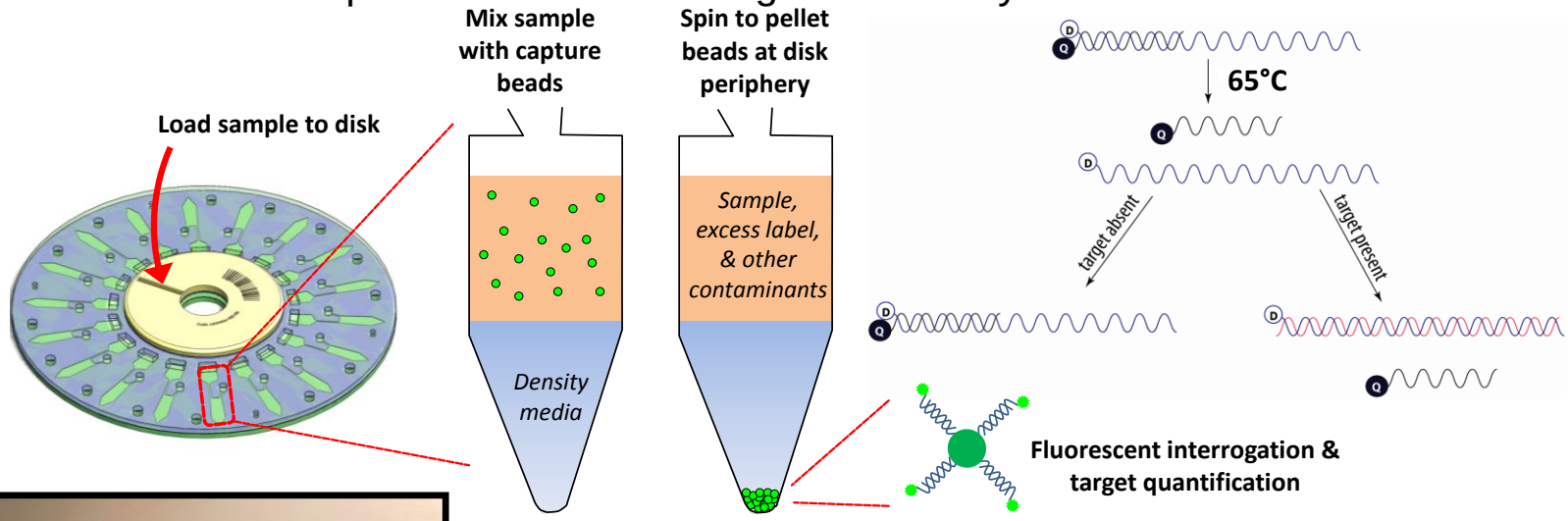
- Full genome sequences of the two biofuel strains of to separate microbiota sequences from algae sequences
- Sampling to collect viral DNA and RNA, bacterial DNA, and eukaryote DNA – fungi and protozoa

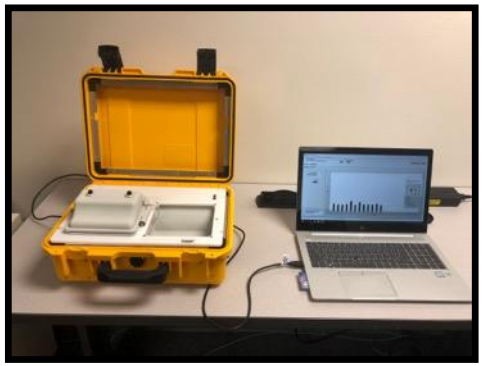
Microbiota treatments

- Correlate pond productivity with genomic organism database to identify potential target organisms affecting algal growth
- Isolate and test organisms to identify potential cause and effect interactions with algae (positive or negative)
- Learn to control key organisms affecting algal productivity through new cultivation strategies or treatments
- Test control strategies in the laboratory
- Deploy and test control strategies outdoors

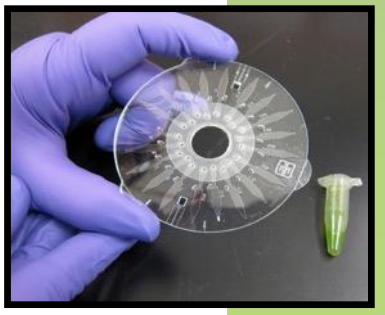
SpinDX

- Fluorescence Resonance Energy Transfer (FRET) -based bead hybridization assay enabling capture and quantification of pathogen-specific RNA/DNA signatures
- Goal is to use SpinDx to provide early and rapid detection of positive and negative members of the pond microbiome using FRET assay.





- **Assay time: approximately 30 min**
- **20 channels per disc**
- **Potential for multiplexed assays in each channel**
- **Low reagent costs**
- **Low material costs**
- **Low instrument cost**
- **Fieldable**



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Challenges

Pinpointing and isolation of key organisms

- Isolation is necessary to conduct cause and effect testing and to develop treatment methods.
- Correlation does not necessary mean causation
- Many of the contaminates are a challenge to isolate

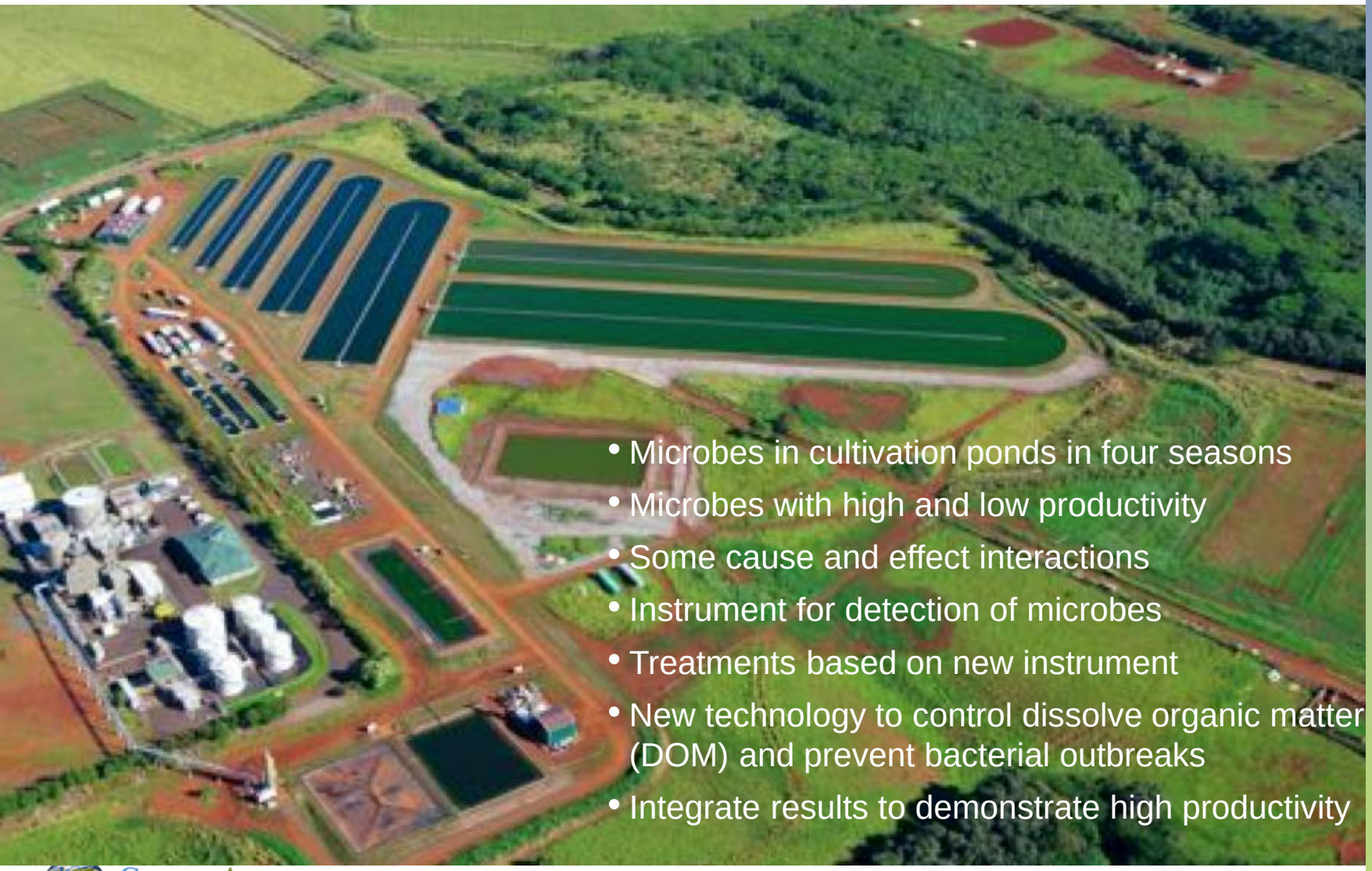
Large data sets

- Overwhelming amount of data from sequencing and limited time to analyze it
- Need to prioritize which data sets should be worked on and which will be left if future funding is available

Translating lab results to large-scale outdoor cultivation

- Application of dissolved organic material control in an advanced cultivation system
- Application of probiotics or treatment protocols
- Application of SpinDX on outdoor raceways

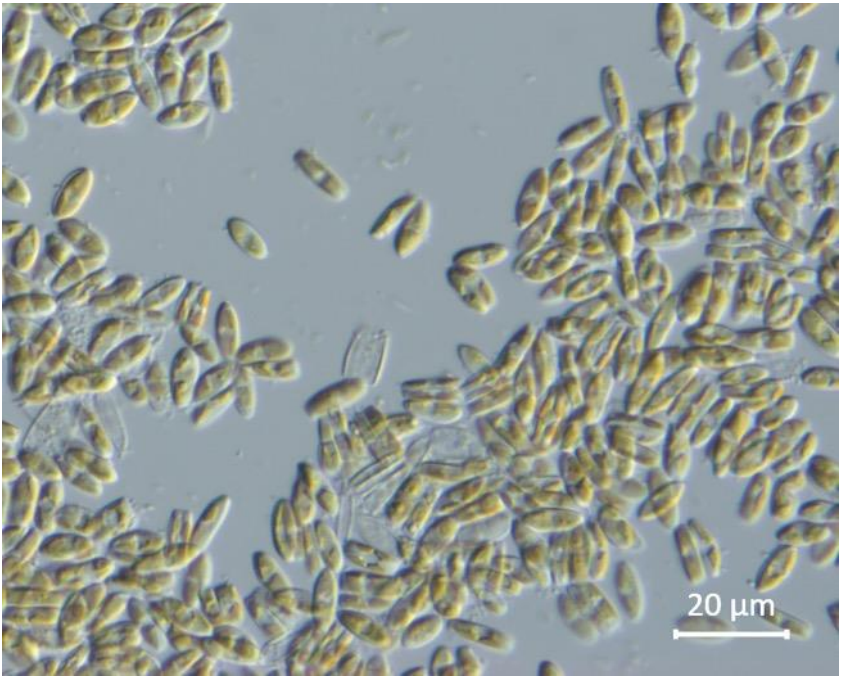
Utilize advanced outdoor cultivation system to generate relevant data and results



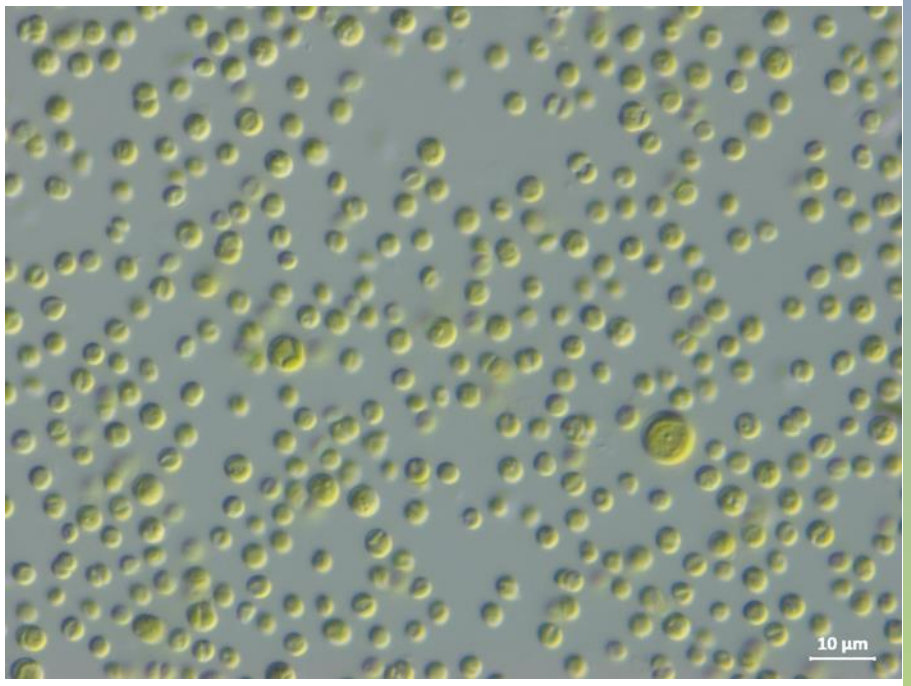
- Microbes in cultivation ponds in four seasons
- Microbes with high and low productivity
- Some cause and effect interactions
- Instrument for detection of microbes
- Treatments based on new instrument
- New technology to control dissolve organic matter (DOM) and prevent bacterial outbreaks
- Integrate results to demonstrate high productivity

3 – Technical Accomplishments

GAI-229



GAI-247



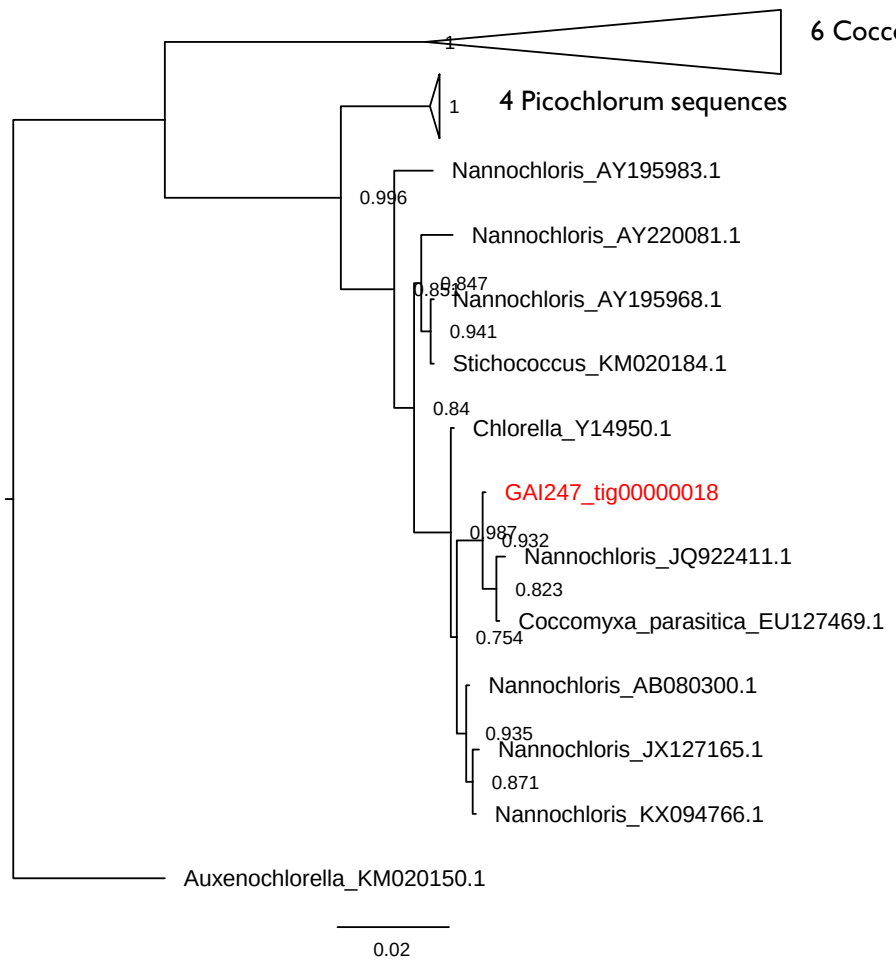
GAI-247 and GAI-229 genome sequence

Draft genome assemblies were constructed.
GAI-229 is diploid and GAI-247 is also most likely diploid

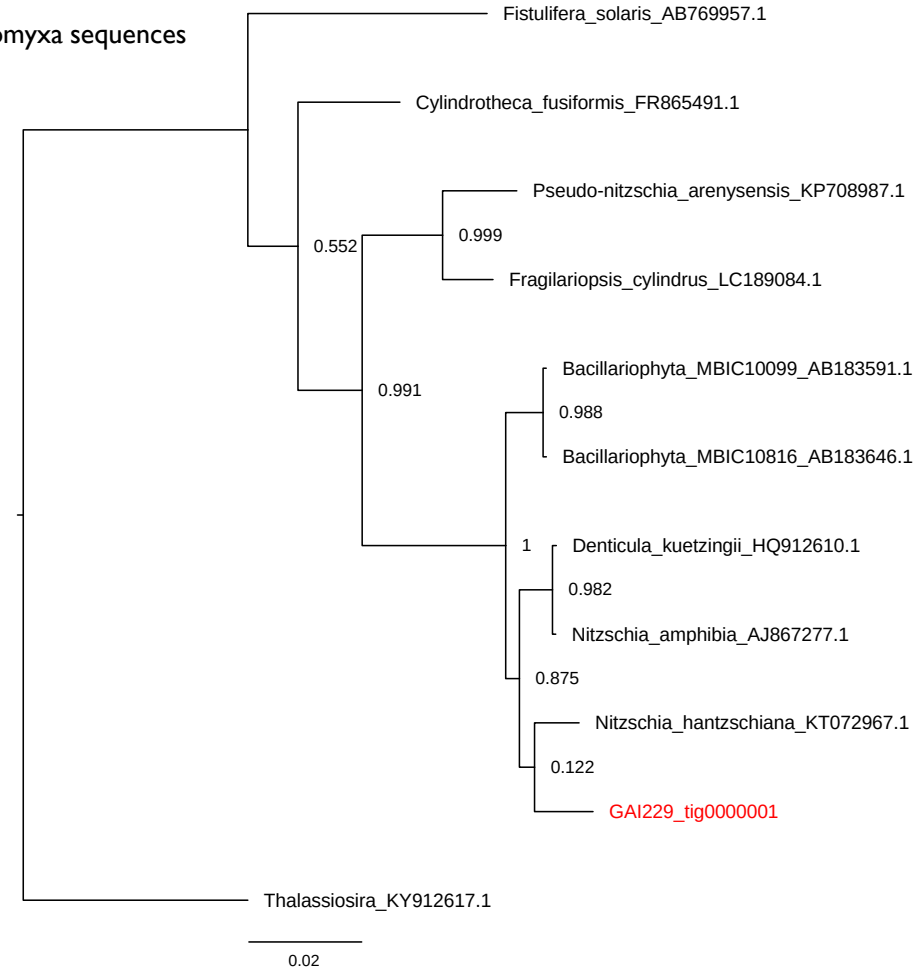
	GAI-247	GAI-229
Nuclear genome size	**35,759,286**	**99,706,970**
Num nuclear scf	164	123
Max scf length	2,850,407	6,574,884
N50	346,905	3,618,388
Nuclear GC content	44.9	45.4
Nuclear coverage (fold)	33.9	59.2
Plastid genome size	165,171	264,596
Mitochondrial genome size	85,836	97,106

PacBio single-molecule DNA sequencing was applied to generate high quality draft genomes for GAI strains 247 and 229

GAI-247 is Coccomyxa or Nannochloris 18S rRNA gene tree



GAI-229 is Nitzschia 18S rRNA gene tree

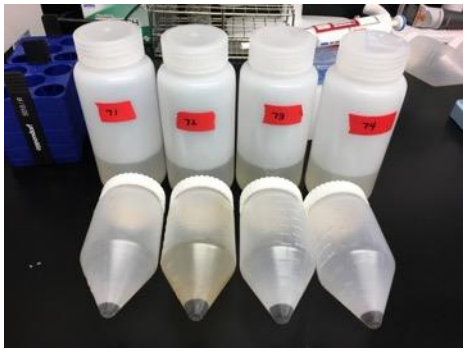


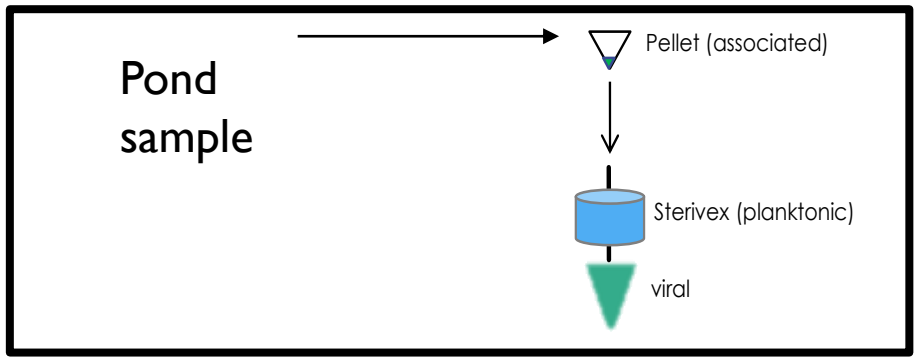
Both are new species.

Microbiota associated with algae ponds

We developed a sampling protocol for collecting genomic sample from algae associated microbiota (pellet), planktonic fraction (Sterivex 0.22µm filter) and viruses. Samples are flash frozen in LN2 and shipped on dry ice

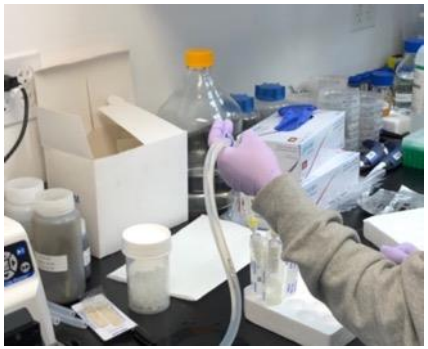
Pond Sample and pellet





Final Pellet

Collect <0.22 µm filtrate (Virus)



Filter through 0.22 µm Sterivex filter

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Grow-outs and sampling

March: Both species, nitrogen sources, induced stress
June/July: Green, high productivity
October: Green, crash
Jan/Feb: Diatom, high bacteria-low growth
May: Diatom, TBD, probably high productivity

Grow out	Days of genomic sampling	Max scale	Species	Samples	Sequencing schedule
March 2018	5	1,214L	229, 247	84	Mostly completed
June/July 2018	32	101,705L	247	67	March 2019
October 2018	19	2,500L	247	34	March 2019
January/February 2019	In progress		229		August 2019
April/May 2019	tentative		229		August 2019

Initial Analyses Completed

Genetic sequencing (selected samples)

- 16S/18S – ribosomal sequences of Eukaryotes and Prokaryotes
- Viruses
- metaG – DNA metagenomics

  Identify RNA viruses and active viruses; active is an indication of actual infection at the time of sampling rather than standing stock or ambient numbers. Provides whole community and data on the physiology of cellular organisms
- metaT – RNA metatranscriptomics

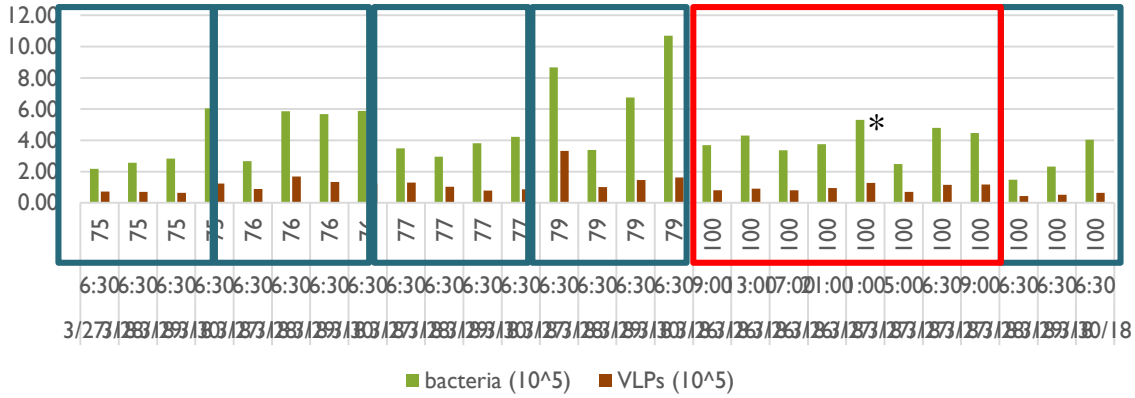
  Assess community and get genomic context of organisms/viruses of interest

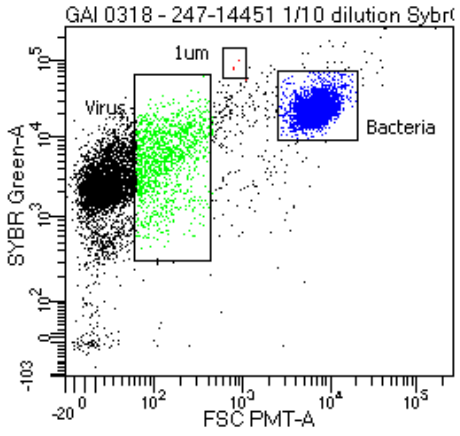
Non-genomic analysis

- Microscopy
- Ash-free dry weight, optical density, Nile red, algae CHN, nutrient concentrations
- Lipid content via gas chromatography for lipid phase samples
- Virus, bacteria and eukaryote contaminant counts (selected samples)

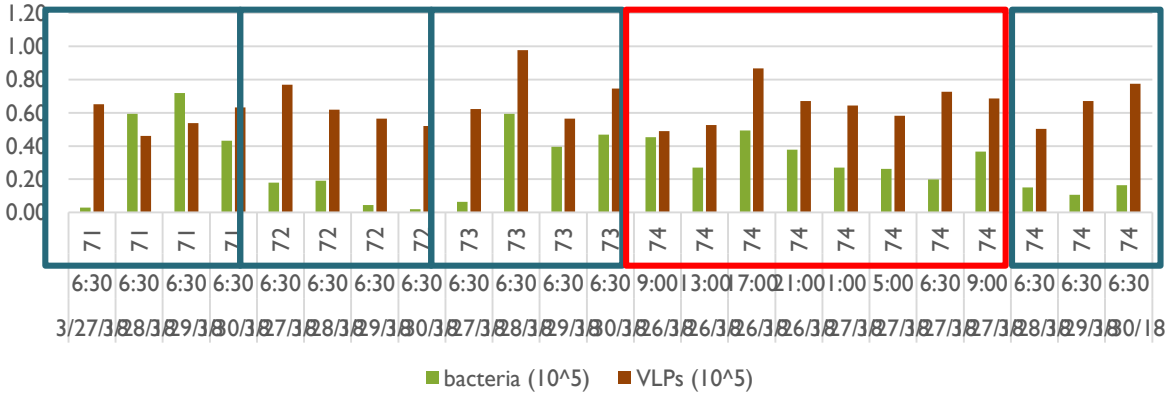
Viral and bacterial count protocol

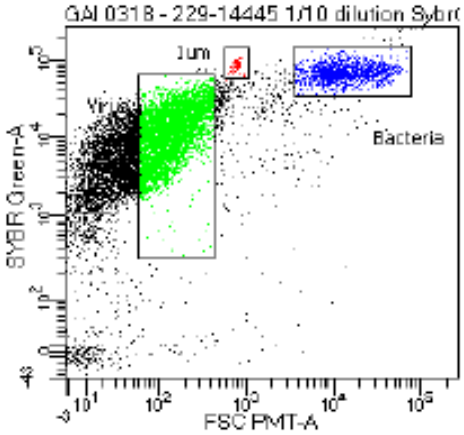
GAI247





GAI229





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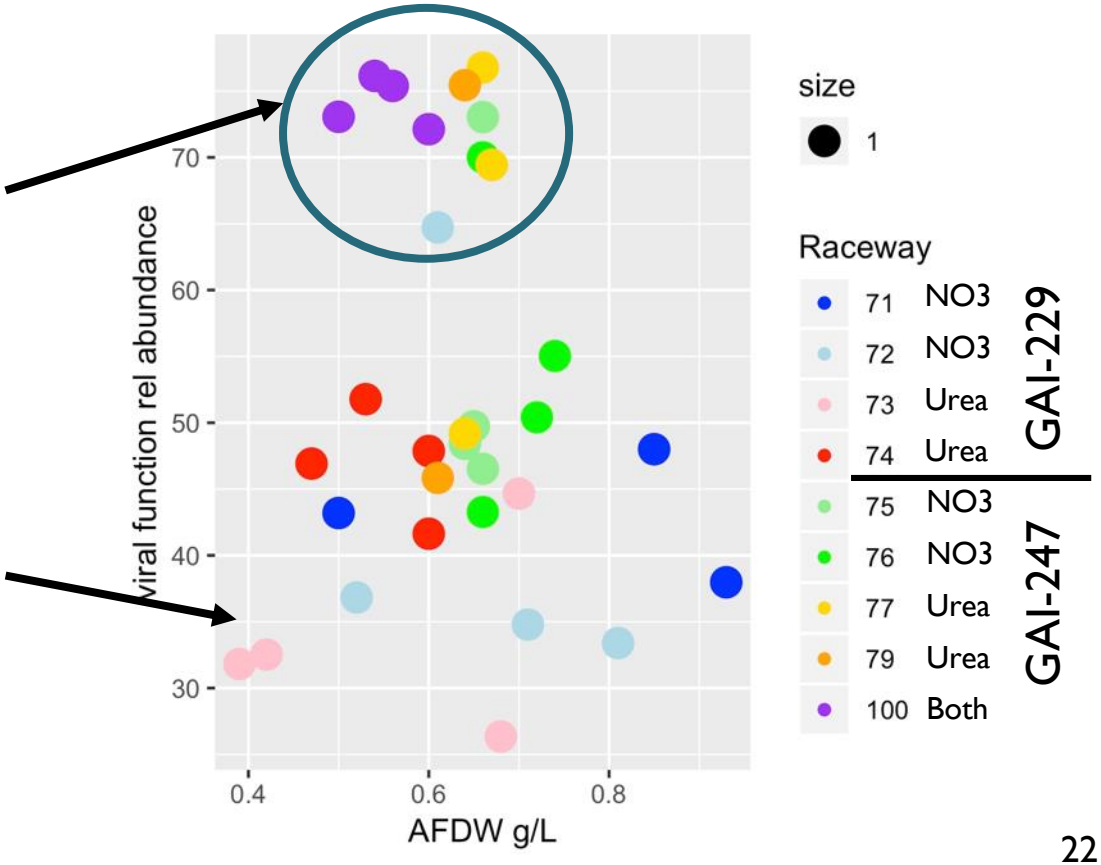
Preliminary Viral Count Results

Important Caveat

- RNA sequence libraries are underway
- RNA viruses are major components of diatom (GAI229) infectious particles and well as other unicellular eukaryotes.

Highest viral abundances primarily for GAI-247 (RNA results not added yet)

GAI-229 had higher levels of biomass (AFDW g/L); however viral sequences were lower relative to GAI247



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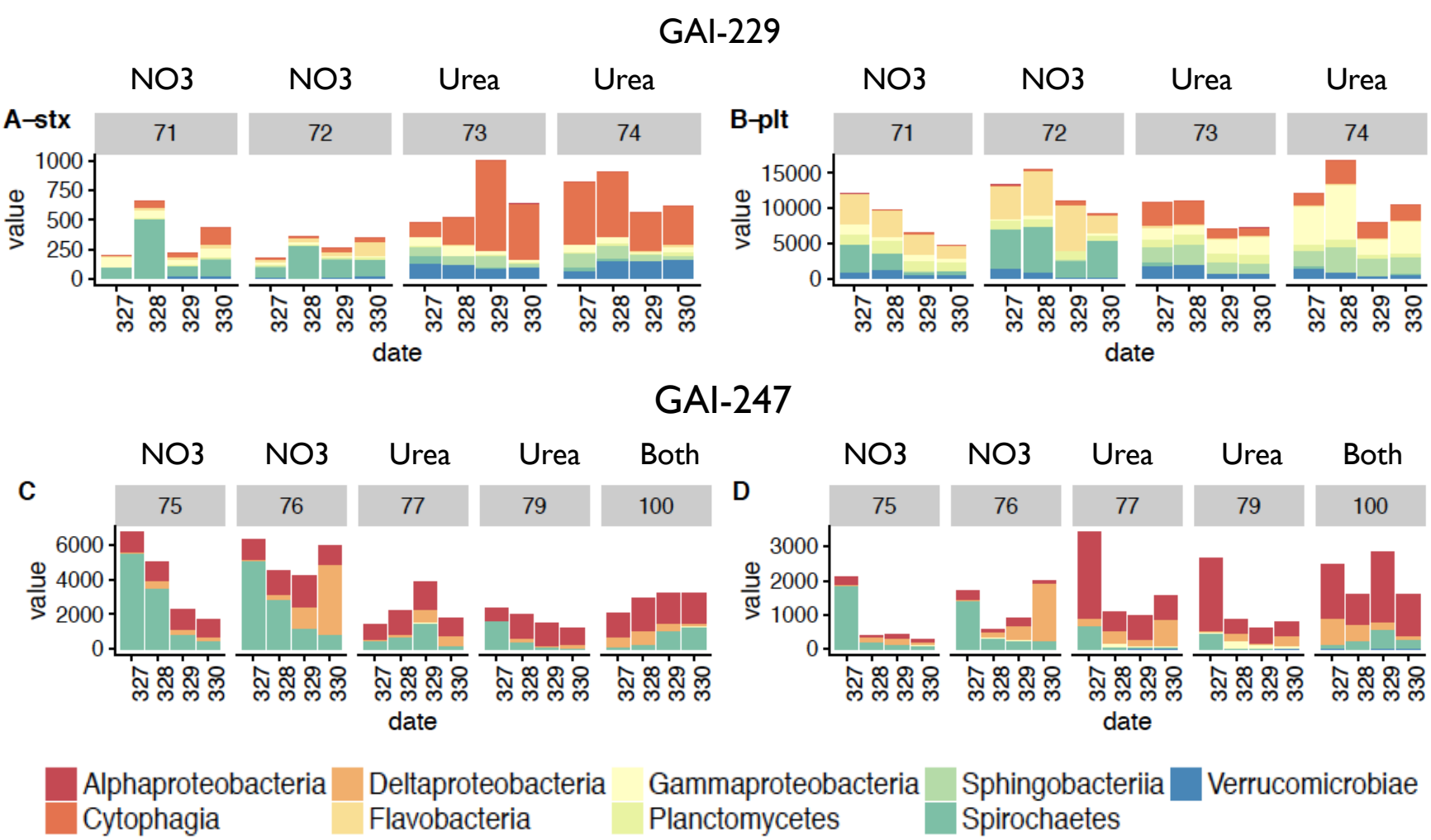
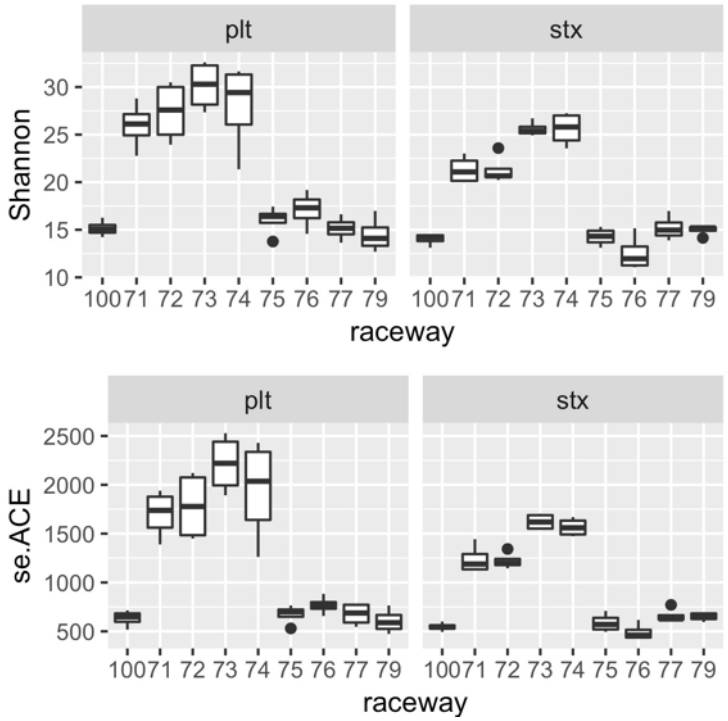
Bacteria and Eukaryotes



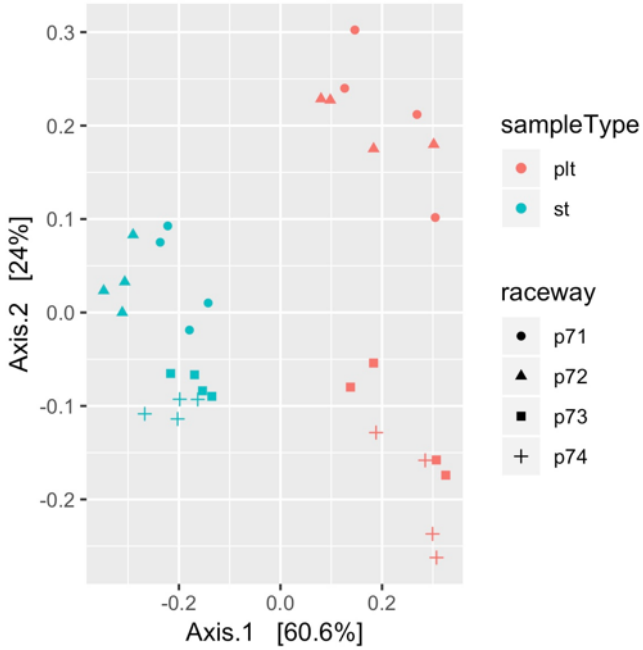
Bacteria and Eukaryotes

Ordination (NMDS) again indicate that the bacteria and eukaryotes segregated based on sampling scheme or culture conditions, urea vs $NaNO_3$.

Diversity measures

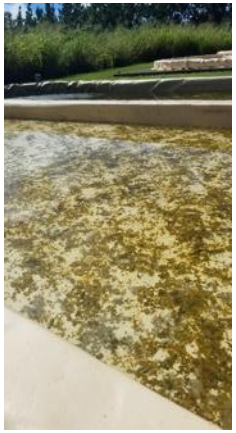


GAI 229 only

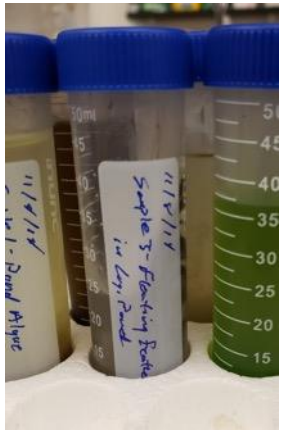


Diversity higher within GAI229 ponds vs GAI247

Dissolved Organic Material


Farm samples with high DOM



Inoculation samples

Baited enrichments

- Samples for isolation were collected in March 2018, November 2018, and February 2019 to test for dissolved organic material (DOM) control potential
- Samples are being processed, but significant challenges have been encountered
- Two key issues identified, so trying new approaches
- Preliminary evidence supports likely success for the new protocol

SpinDX

- Two SpinDx devices were designed and fabricated
- Software was designed and implemented
- Initial testing at the algae farm identified several issues with the protocol and device
- Device/protocol modifications and testing are in-progress
- Probes for viral, bacterial, and eukaryotic targets have been designed from DNA sequences obtained in the genomic screening

4 - Relevance

- Sustainable high algal productivity without pond crashes and periods of low productivity is crucial for algae cultivation economics and viability of algae based biofuels
- Productivity in algal systems is the most important factor in achieving economical, sustainable algal biofuels and products
- Uncertainty around microbiota during raceway algae cultivation is the key unknown to to attaining higher and more reliable algal productivity
- This project will improve the understanding of the microbiota by collecting extensive data during variable cultivation conditions on all key components of the microbiota – viruses, bacteria, algae, fungi, and protozoa
- This data will also be used to develop a specific cultivation method or treatment protocol to increase the productivity to greater than 25 g/m^2d

4 - Relevance continued

- The data will also be used in the development of an effective, affordable and rapid detection instrument and protocol for algal pond microbiota
- Global Algae Innovations is planning to commercialize the instrument and probe development so a farmer friendly tool is available to the entire algae community
- This capability to rapidly and inexpensively identify microbiota is the first step in establishing pathogen and probiotic controls for algae farming
- Large scale agriculture and algae cultivation facilities in longer operation demonstrate that new contamination problems will arise, so having an instrument to quickly identify and address them will be important

5 - Future Work

1. Fully assembled genomes of GAI-247 and GAI-229

- Transcriptomes of both strains under nutrient replete and starvation conditions
- *Ab initio* gene prediction and functional annotation of both genomes

2. Building of genomic database of pond microbes

- High productivity grow out with diatoms
- Finish sequencing of samples from grow outs

3. Use sequencing data to identify potential key organisms for isolation

4. Isolate pond organisms of interests

- Eukaryotes, bacteria, viruses
- Test for cause/effect on algal productivity in lab

5. Develop treatment methods

- Use list of potential treatments previously tested and new ones on the new isolates
- If probiotics identified, culture and test for improved productivity in laboratory
- Utilize robotic system and microplates for rapid testing of treatments
- Deploy treatment or cultivation technique in an outdoor raceway

5 - Future Work

6. Adapt DOM control to our cultivation system

- Apply new approaches to adapt DOM control to our cultivation system
- Validate productivity improvement in the lab
- Deploy in an outdoor raceway

7. Spin DX

- Complete modifications to the instrument
- Complete development of the protocol for sample lysis and nucleic acid extraction
- Optimize and validate the assays against key species
- Test the device and assays with pond cultures at the farm

6 - Summary

1. **Overview:** Addresses key BETO targets in productivity, robustness and reliability
2. **Approach:** Use advanced cultivation system and robust biofuel strains
   Generate broad genomic database on all pond microbiota
   Isolate key organisms, measure cause/effect, and develop treatments
   Develop SpinDX for rapid, cost effective measurement of microbiota
   Develop method for controlling dissolved organic material level
3. **Technical Accomplishments/Progress/Results:**
   - Developed sampling and sequencing protocols
   - Completed four of five grow-outs and sampling for genomic database
   - Identified microbe populations associated with species of algae, nitrogen source, productivity and growth phase
   - Designed, built and completed initial testing of SpinDX instrument
   - Experiments on DOM control adaptation identified path forwrard
4. **Relevance:** Address microbiota using outdoor cultivation and robust strains
   Target is commercially-available, farmer-friendly tool
5. **Future Work:** Isolate key organisms and develop control methods
   Complete SpinDX development and validation