SAND2011-7863C

Pond Crash Forensics

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Sandia National Laboratories

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Presence of the biological agent can be necessary but not sufficient to crash

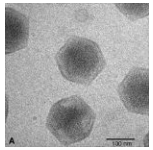
Agent + *Algae* + *Environment* = *Collapse*

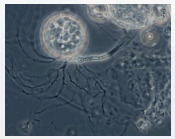
Viruses

Bacteria

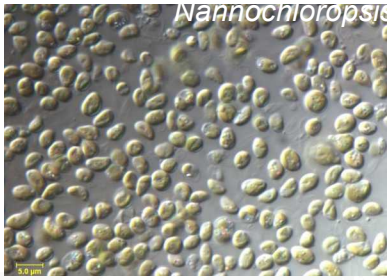
Predators

Fungi

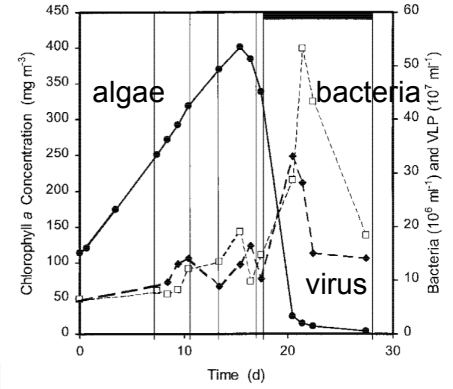




Patterson & Laderman, 2001.



Environment (Temp, salinity, pH, CO2, nutrients)



Herman Gons et al., Antonie van Leeuwenhoek, 81: 319-326, 2002.

"Perhaps the most worrisome component of the large-scale algal cultivation enterprise is the fact that algal predators and pathogens are both pervasive and little understood."

- DOE Draft Algal Biofuels Technology Roadmap (2009)

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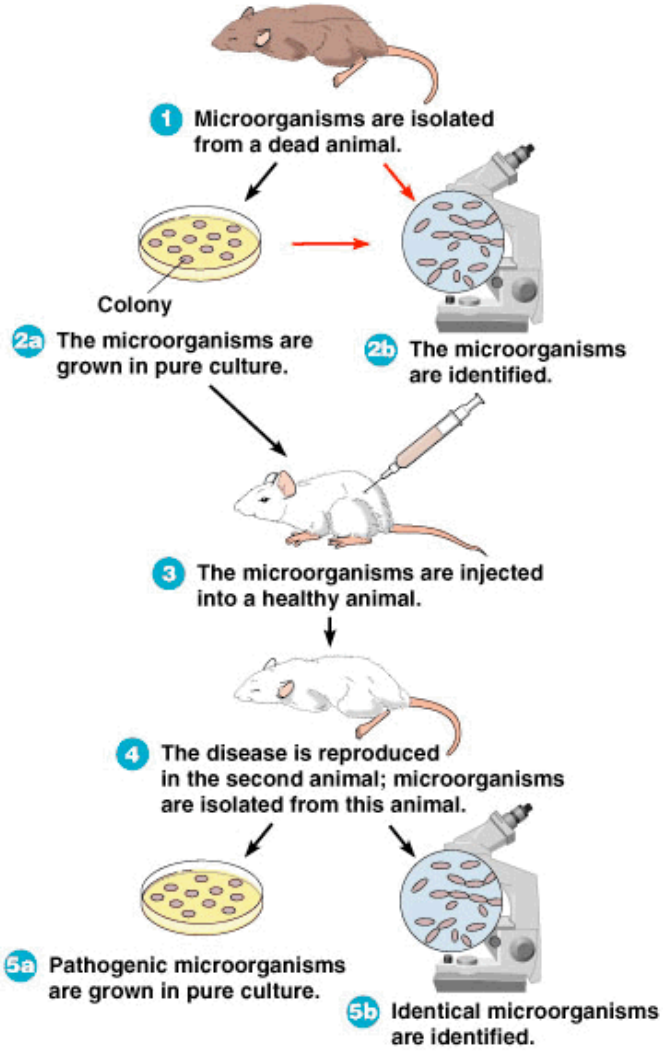
Project Goals

- **Rapidly identify biological agents that play a role in pond crashes**
  - Next gen DNA sequencing
  - Compare healthy ponds to crashed
  - Compare time series in ponds leading to crashes
- **Goal is to complete this analysis in <24 hrs**
- **Drive down costs**
  - Removal of non-informative nucleic acids
  - Multiplexing of samples
- **Create molecular assays against these agents**
- **Develop methods for routine isolation and culture of agents.**
- **When possible isolate agents and reconstitute crash**
  - Confirm the role of the suspected agent (Koch's postulates)
  - Determine the role of abiotic factors in modulating the crash

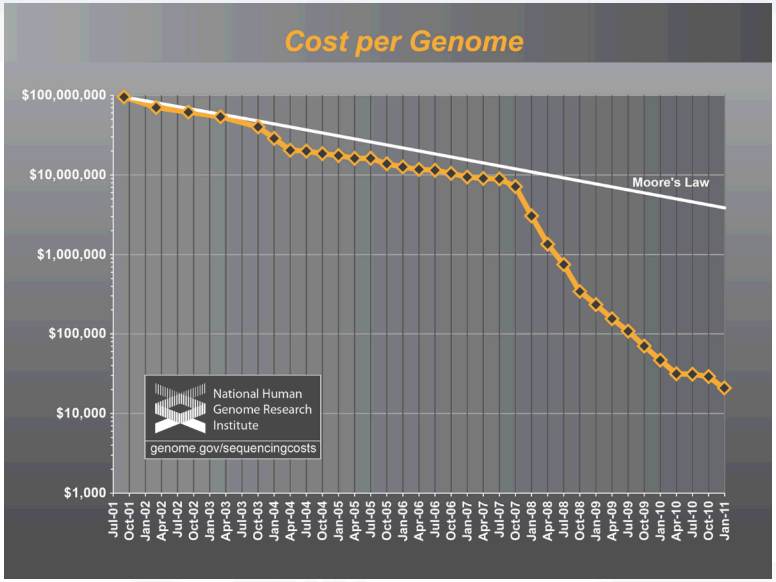
A staged approach to pond crash forensics

- **Presumptive Identification.**
  - Detect the presence of the agent crashed ponds.
  - Agent absent or in lower abundance in healthy ponds
  - Complicated by environmental parameters
- **Confirmatory Identification**
  - Isolate the agent(s) and recapitulate the crash
  - Complicated by environmental parameters
- **Development of Field Assay**
  - Quantitative yet rapid, simple and cheap (dipstick assay)
- **Early symptomatic or presymptomatic detection**



Sequencing can provide presumptive identification of pond crash agent.

- The cost of next gen sequencing is falling at a rate that outstrips Moores law
- Cost of human genome fallen by 1/2 – 2/3 since January
- The amount of data per run is increasing dramatically
- Bar-coding allows full advantage of this capacity
- Key is to get more sequencing hits on target reducing the cost of analysis to $10s



GS junior

1 X400nt 10 hrs
35 Mb

Ion Torrent

1 X100-200nt 2-4 hrs
10-100 Mb
Chip 314/316

MiSeq

1X 100nt: 12hrs
340 Mb
2X150nt: 27hrs
> 1Gb

HiSeq

2X150nt: 8days
540 -600Gb

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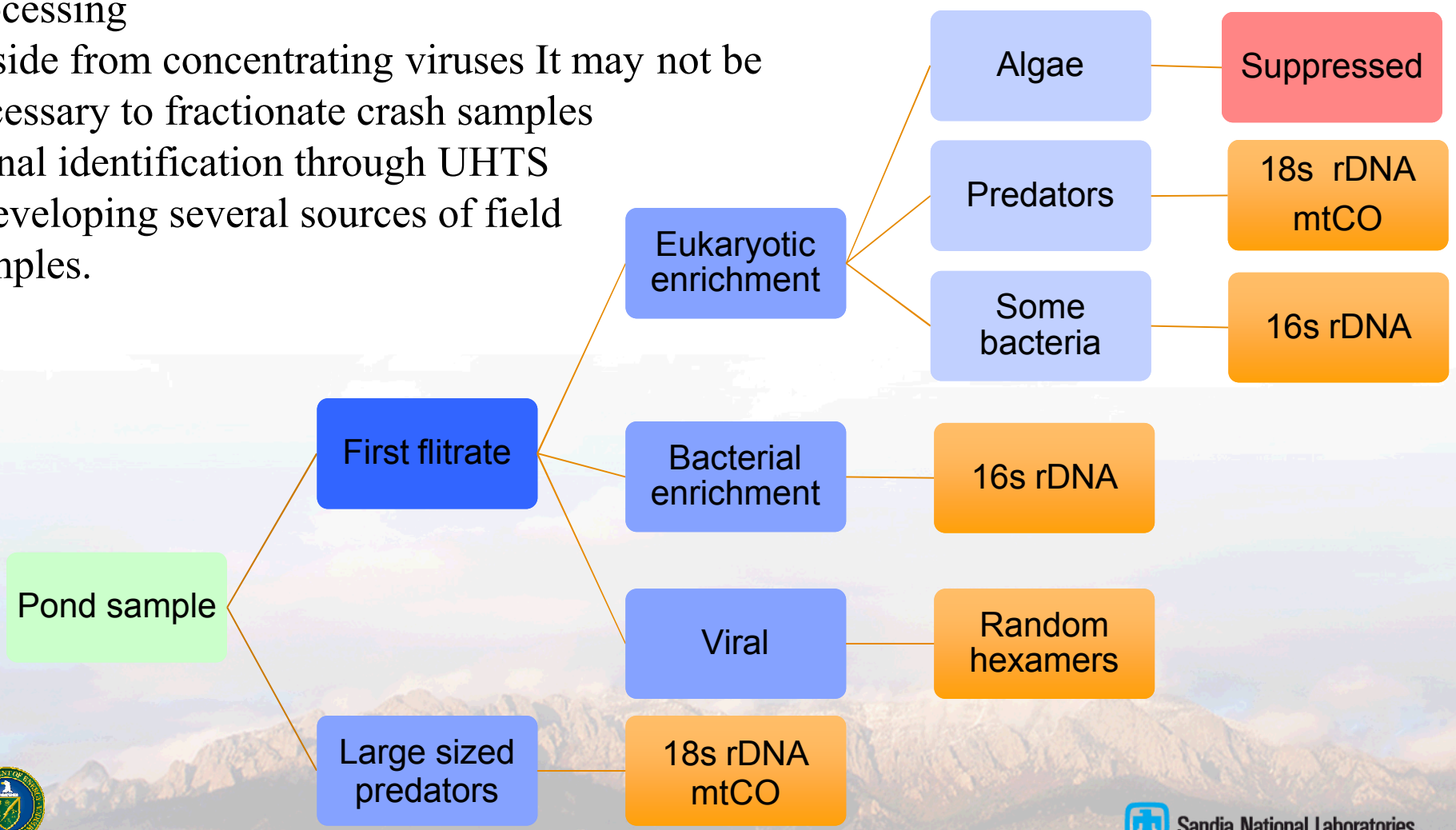
Physical separations can be time consuming and incomplete

- The final goal is to limit the extent of pre-processing
- Aside from concentrating viruses It may not be necessary to fractionate crash samples
- Final identification through UHTS
- Developing several sources of field samples.



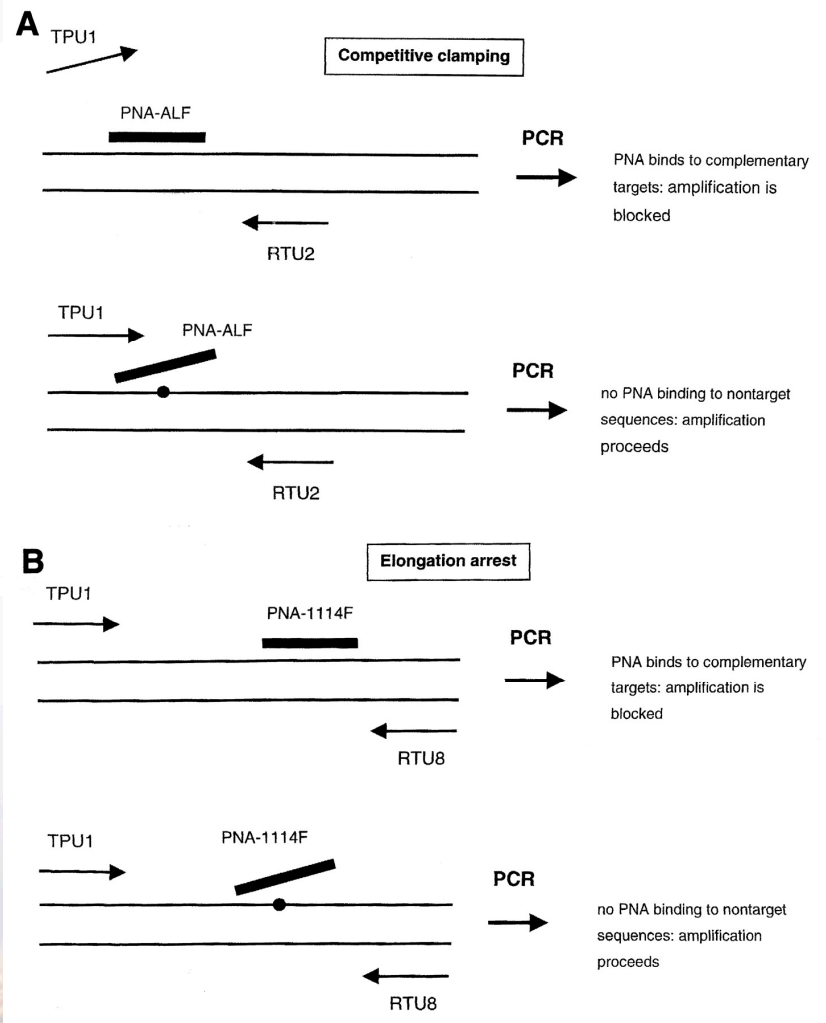
We have devised a suite of “suppression” strategies

Group specific primers:
rDNA analysis
Tested prokaryotic probe the exclude chloroplast.

Blocking primers:
rDNA analysis
3’ modified primers that prevent amplification from known targets

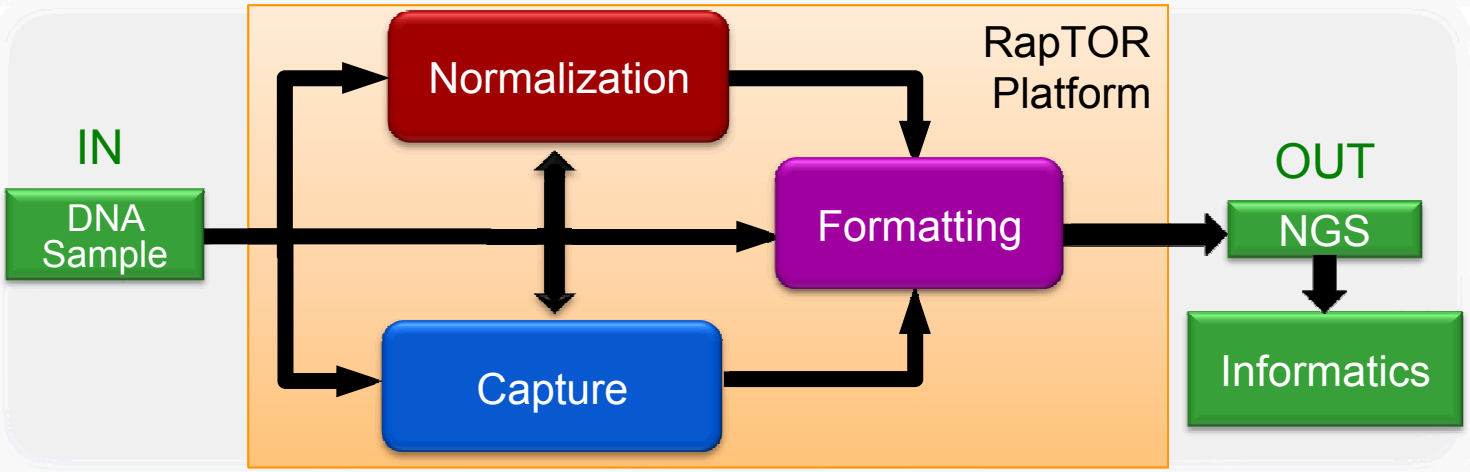
Subtraction:
rDNA analysis or metagenomic analysis
Physically removes unwanted sequenes

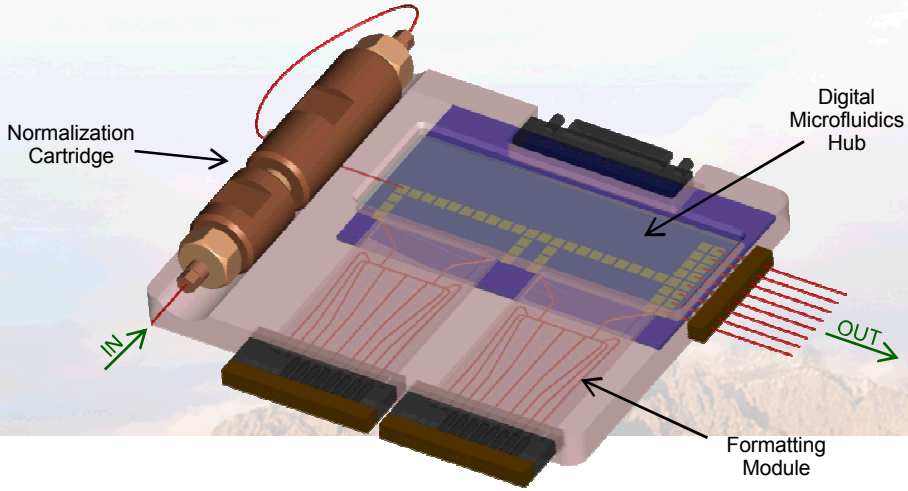
Normalization;
rDNA analysis or metagenomic analysis
Removes or destroys high abundance sequences



von Wintzingerode, F. et al. 2000. Appl. Environ. Microbiol. 66(2):549-557

Microscale platform integrates suppression & formatting modules within a flexible architecture





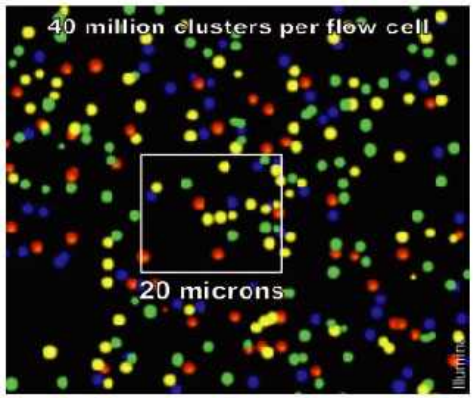
Gen-1 design

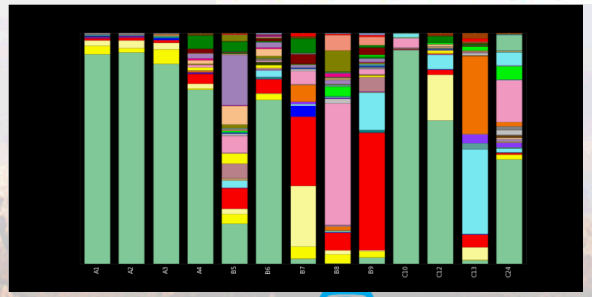
Microscale Platform Features

- Handles ng quantities of DNA
- Flexibility in processing path
  - Iterative cycles of suppression
    - Normalization +/- capture
- Rapid & reliable manipulations
  - Diffusion-limited reactions accelerated at microscale
  - Integration & automation
- Amenable to parallelization

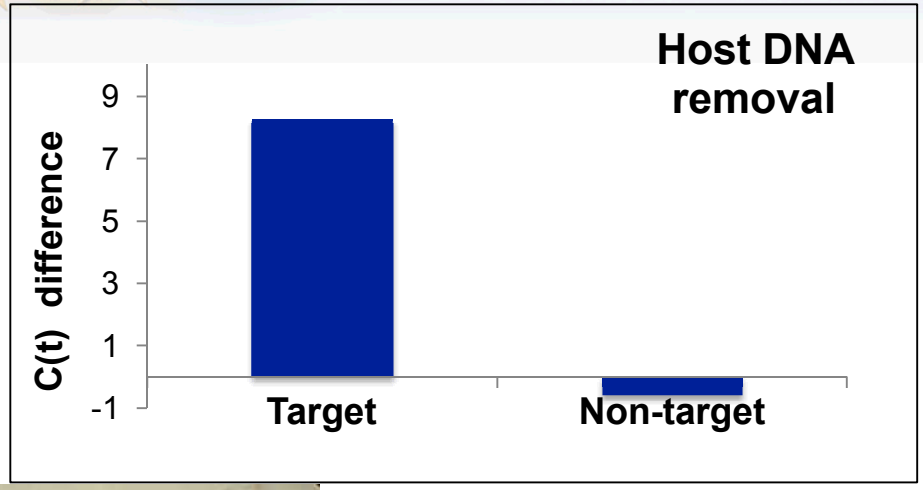
Sample processing/analysis pipeline

- **Pilot scale out door racetrack ponds**
  - Pond water shipped overnight on ice
    - Culturing of agents
  - Frozen biomass
  - Purified nucleic acids
- **Biomass harvested**
- **Viral fraction purified and concentrated**
- **Nucleic acids extracted**
  - Prokaryotic analysis
  - Eukaryotic analysis
  - Viral analysis –Random hexamer amplification
- **Library preparation and barcoding**
- **High throughput sequencing Illumina**
- **Bioinformatic analysis**





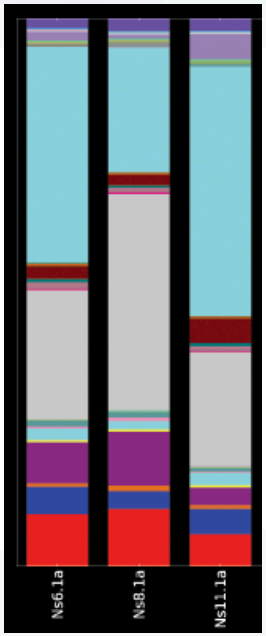
Presumptive Identification through sequencing

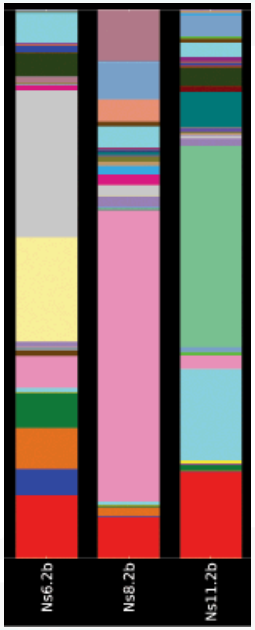


- ✓Probe independent: detection of unknowns
- ✓Physical removal of host DNA prior to sequencing *up to 250 fold*
- ✓No dependence on microscopic analysis/expertise
- ✓Effective for a wide variety of algal pathogens/predators

% hits	Best hit	type
29	*Cercomonas plasmodialis;*	flagellate
21	*Aplanochytrium stocchinoi*	marine fungus
16	*Chaetonotus neptuni*	unsegmented worm-like
4	*Labyrinthuloides minuta;*	plant pathogen
4	*Platyreta germanica*	parasite/predator
4	*Amphora cf. capitellata*	competitor

Profiling of Microbiome and Virome





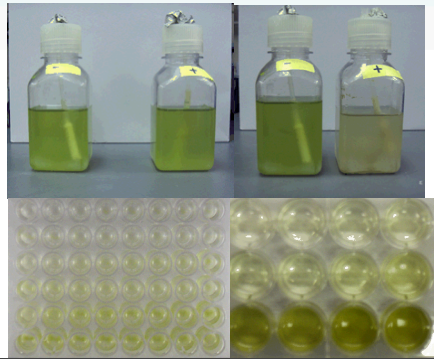
?

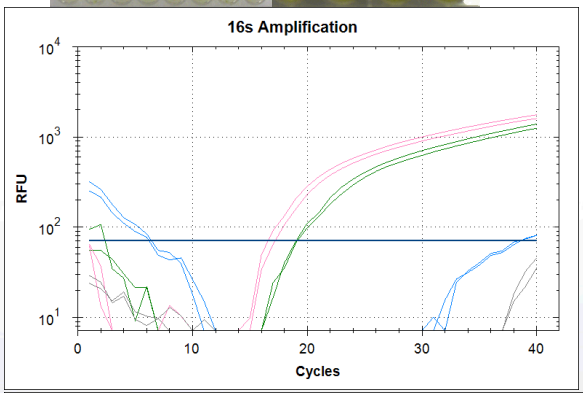
- Short term batch method pilot ponds
- Some variation in community structure at early timepoints.
- Significant variation at later timepoints.
- Significant variation post crash

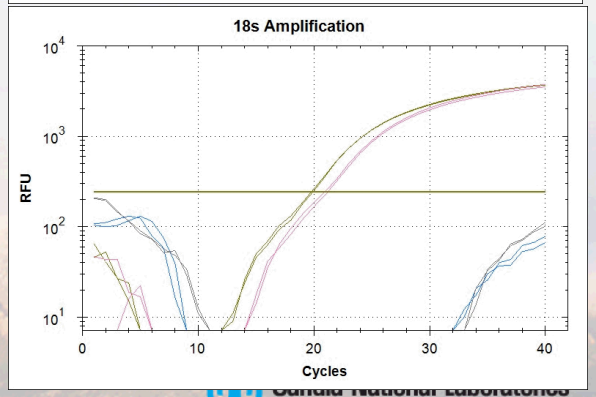
- Concentration by ultrafiltration
- Amplification by random hexamers
- Sequencing
- Assembly?

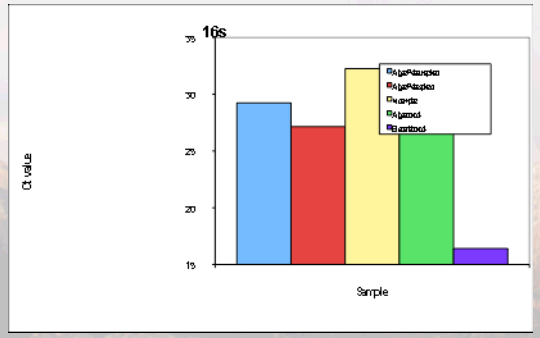
Confirmatory isolation of biological agents

- **Agent isolation**
  - Developed methods of isolation by terminal dilution.
  - Developing novel alternative isolation methods
- **PCR assays for model agents**
  - Developed assays to quanititate and amplify nucleic acids from various classes of agents
  - Developed species specific assays
- **Development and validation of fractionation and nucleic acid extraction methods**
  - Utilized species specific and general assays to track how agent "behave" during physical fractionation
  - Allow us to measure extraction and recovery of nucleic acids



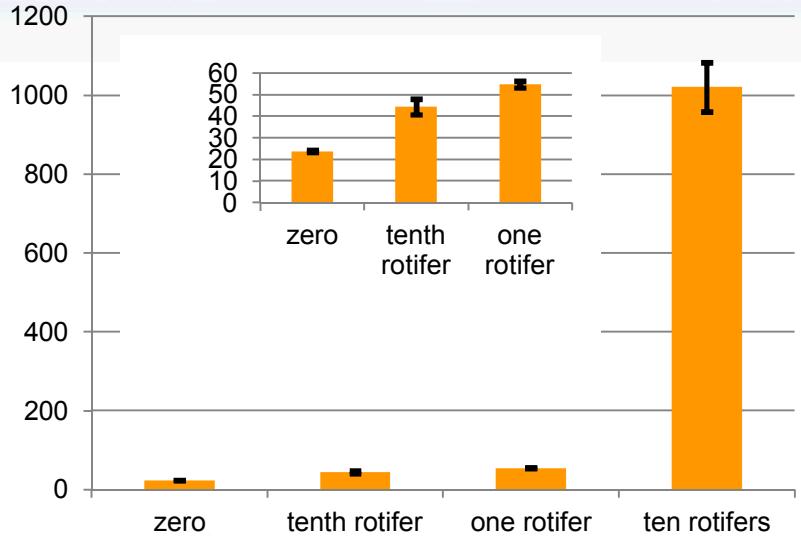


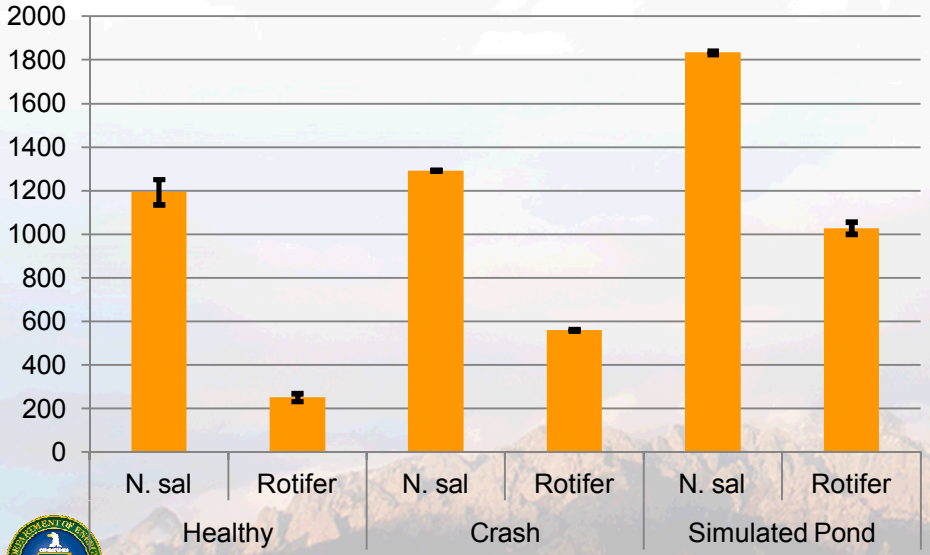


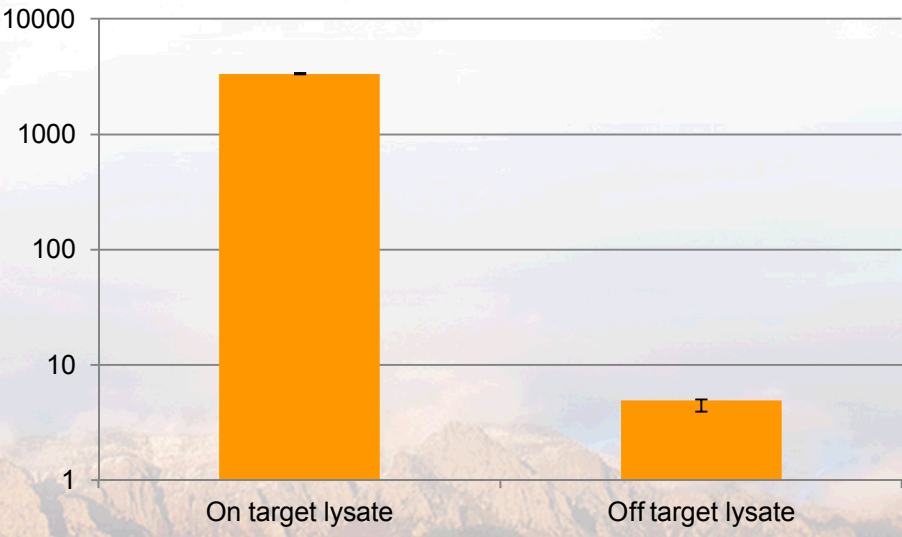


Development of a low cost fieldable assay

- ✓Time required: 30 min
- ✓Capable of multiplexing
- ✓Assays against new agents can be created in days
- ✓Low cost
- ✓Single cell sensitivity
- ✓High signal to noise







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Multivariate Curve Resolution

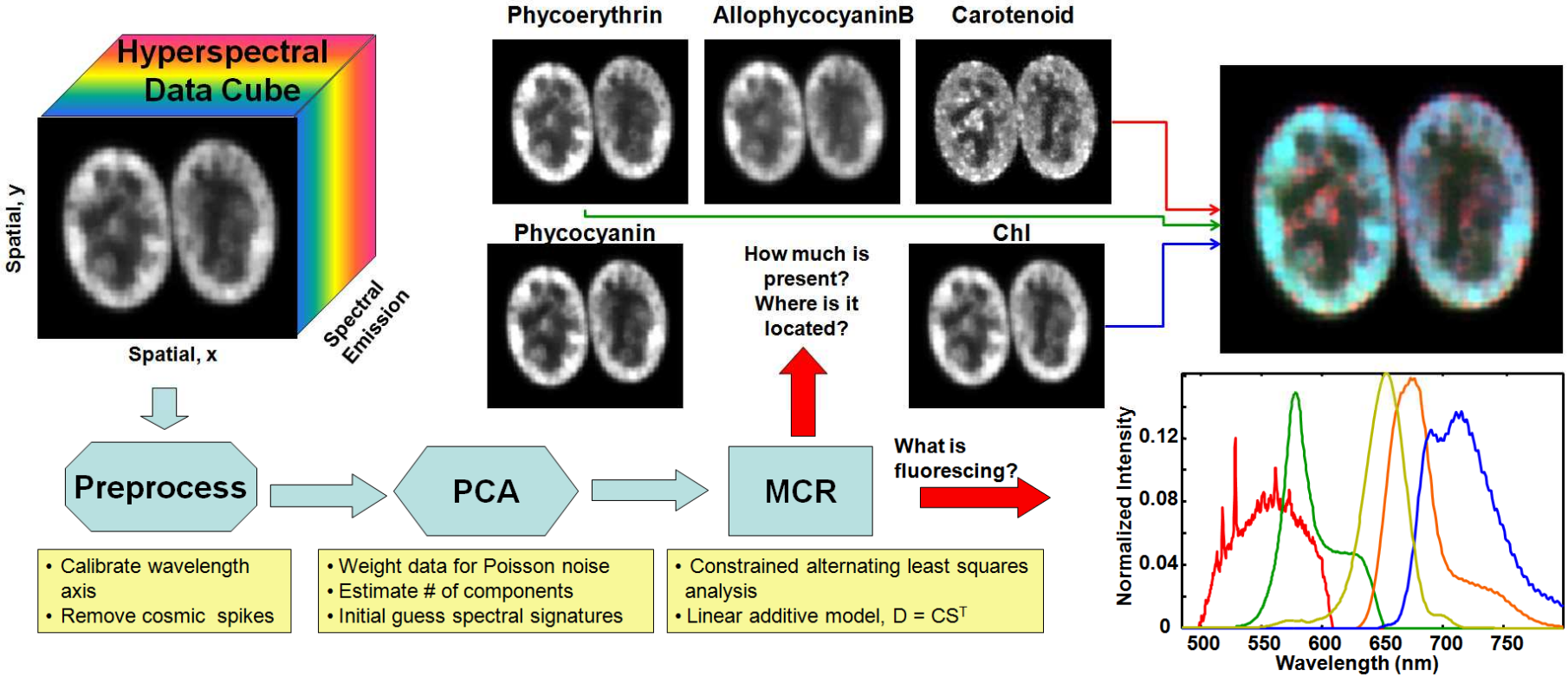


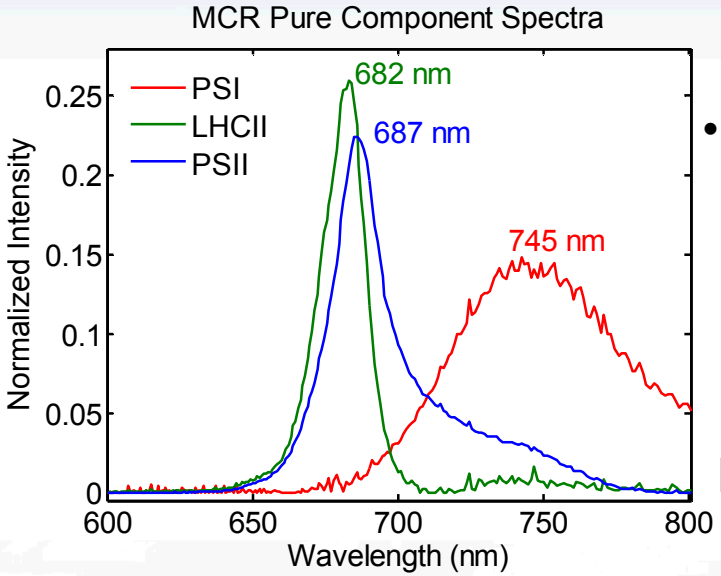
Figure 2. Mathematical isolation of independently varying chemical species is accomplished using a fast multivariate curve resolution algorithm with robust constraints. Example shown: hyperspectral imaging of endogenous pigments in the cyanobacterium *Cyanothece* sp. PCC 7822.

Hyperspectral fluorescence images and MCR of viral infection

Can we develop spectral signatures for the early detection of viral infections?

Cells of *Chlorella variabilis* NC64A infected with PBCV-1

Multivariate analysis yields pure spectral components for LHCII, PSII, and PSI



Viral infection leads to:

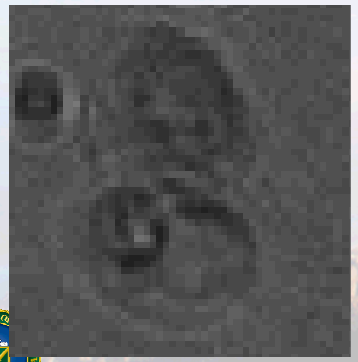
- altered chloroplast morphology
- decrease in PSII and LHCII

Possibility to monitor these pigment changes remotely

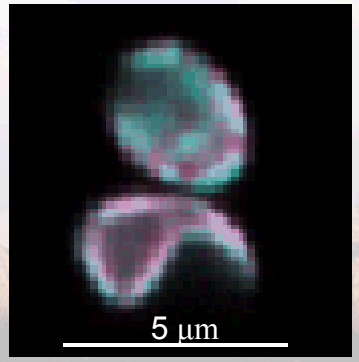
Next steps: time course of viral progression

Control

widefield

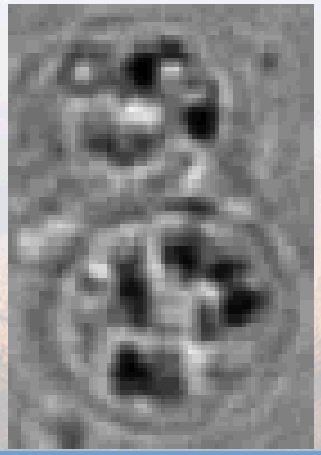


RGB composite



16 hours post infection MOI = 0.1

widefield



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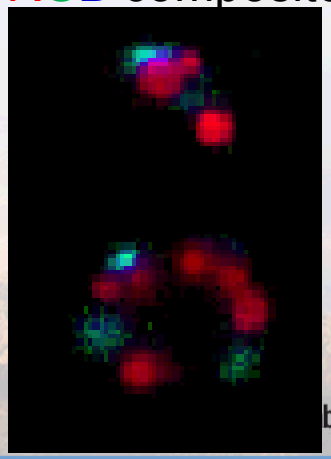

Algae and virus courtesy of the Van Etten laboratory

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Conclusions

- We have demonstrated the identification of grazers, fungi, bacterial and weed algal species by next gen DNA sequencing.
- We have demonstrated methods for the removal of background (algal) sequences.
- We have developed methods for isolation and culture of agents.
- We have developed a fieldable, quantitative assay for predator/pathogen detection.
- We are developing optical signatures of pond health

Acknowledgments

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