SAND2011-4212C

Pond Crash Forensics

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4th Congress of the International Society for Applied Phycology

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Sandia National Laboratories

Sandia National Laboratories is a multi-program laboratory operated by Sandia Corporation, a wholly owned subsidiary of Lockheed Martin Corporation, for the U.S. Department of Energy's National Nuclear Security Administration under contract DE-AC04-94AL85000.

Project goals

Rapidly Identify biological agents that play a role in pond collapses by next gen DNA sequencing

- **Compare healthy ponds to crashed**
- **Compare time series in ponds leading to crashes**

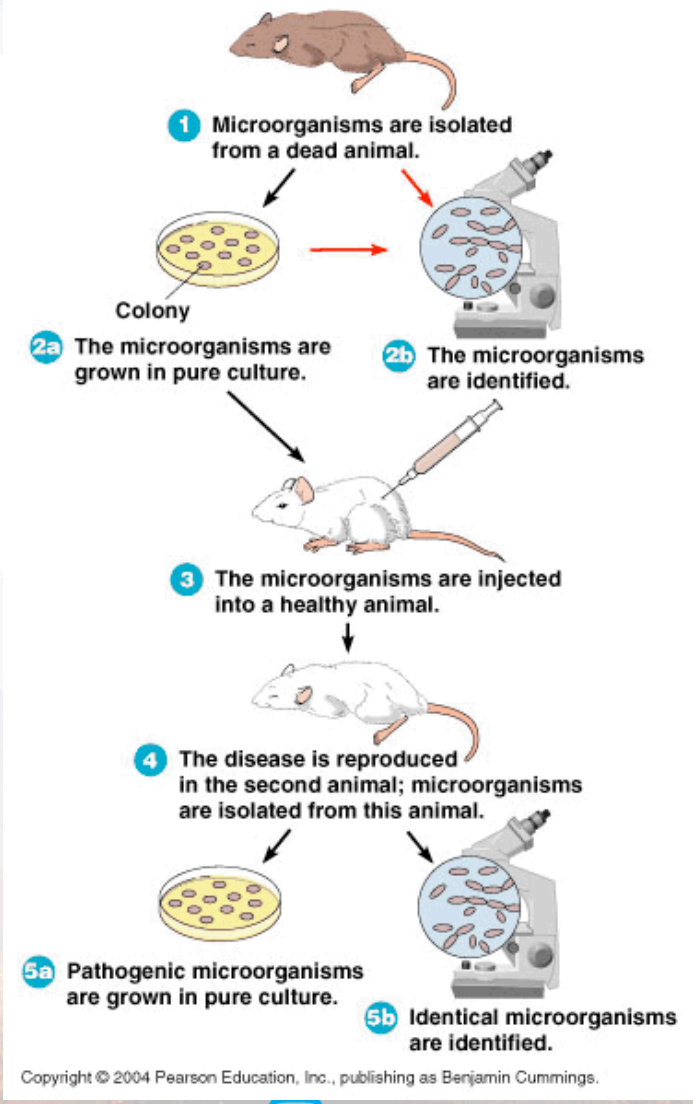
Goal is to complete this analysis in <24 hrs

Drive down costs

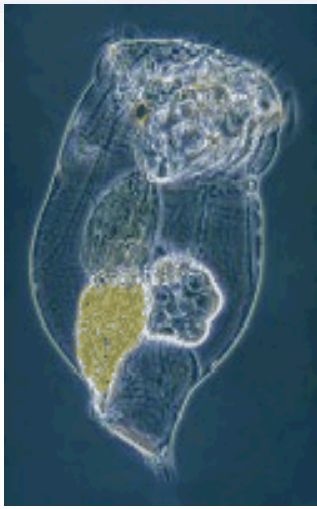
Create molecular assays against these agents.

When possible isolate agents and reconstitute crashes

- **Confirm the role of the suspected agent (Koch's postulates)**
- **Determine the role of abiotic factors in modulating the crash**



A rogue's gallery of threats confront mass algal culture



Predators: Several literature reports can require expertise and be difficult ID at low density **Lincoln *et al* *1983,***

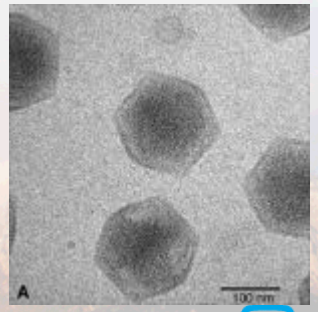
Fungi: Reported in literature, confidential reports. **Jia *et al* *2010***

Bacteria: Reported in literature, Confidential reports
Difficult to identify. **Mayali and Azam 2004**

Viruses: Species specific, Reported agent of bloom collapse, Control biomass **Lange *et al* *2009***

Weed species:

Abiotic threats: pH, temperature, nutrient depletion



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

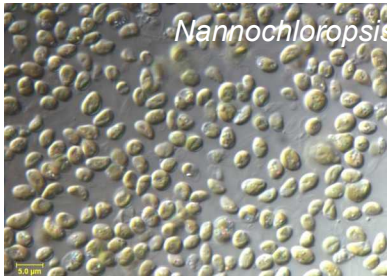
Agent *Algae* *Environment* *Collapse*

Virus

Bacteria

Predators

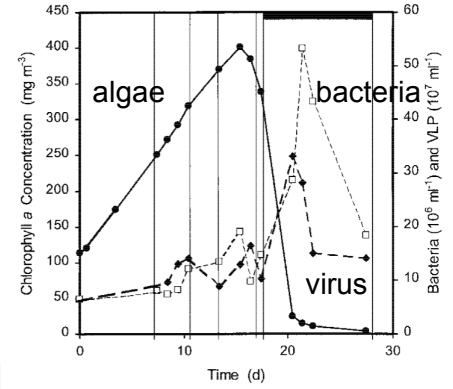
+



+

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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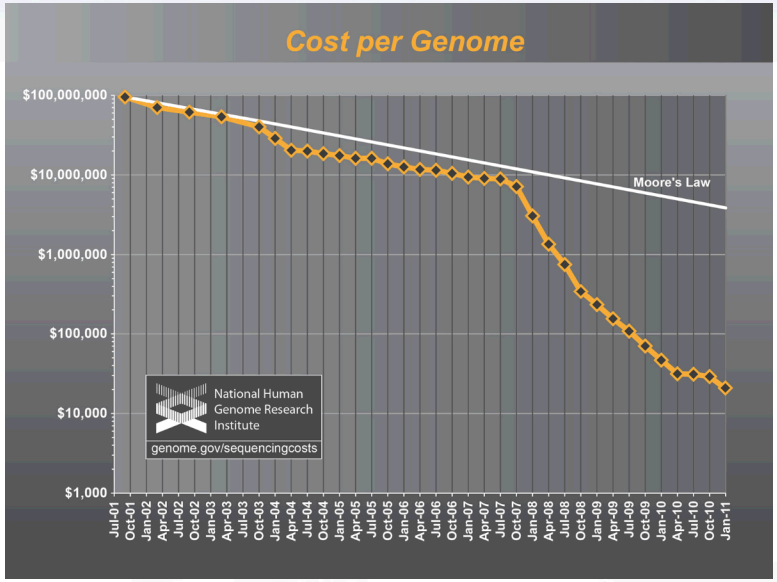
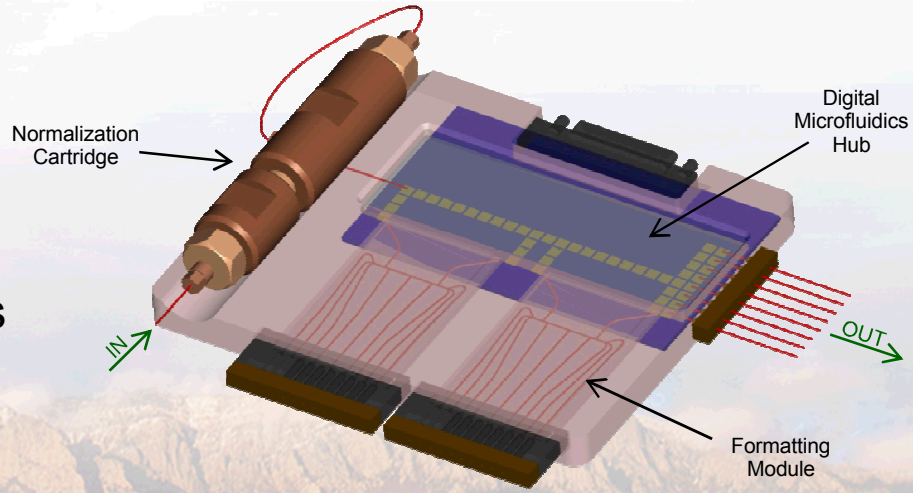
Next Generation Sequencing is becoming a cost effective assay

- 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



- Sample prep has been time consuming and technically challenging
- Sandia has devised automated methods to simplify and accelerate sample prep



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Identifying biological agents is complicated by the metagenomic background

Metagenome

Host background
- Can overwhelm signal from pathogen (~100% of signal)
- Individual variation
- Disease state variation

Normal flora
- Could be mistaken for pathogen
- Could be as large a contributor as human
- High diversity
- Individual variation
- Disease state variation

Pathogen
- Can be very minor contributo
- Hard to detect or characterize

Two problems:
Signal to noise (Molecular biology)
Signal interpretation (Informatics)

Pathogen characterization is possible after background suppression

Host background can overwhelm pathogen signal

We plan to discard or ignore host nucleic acids with varying levels of stringency according to the needs of the system

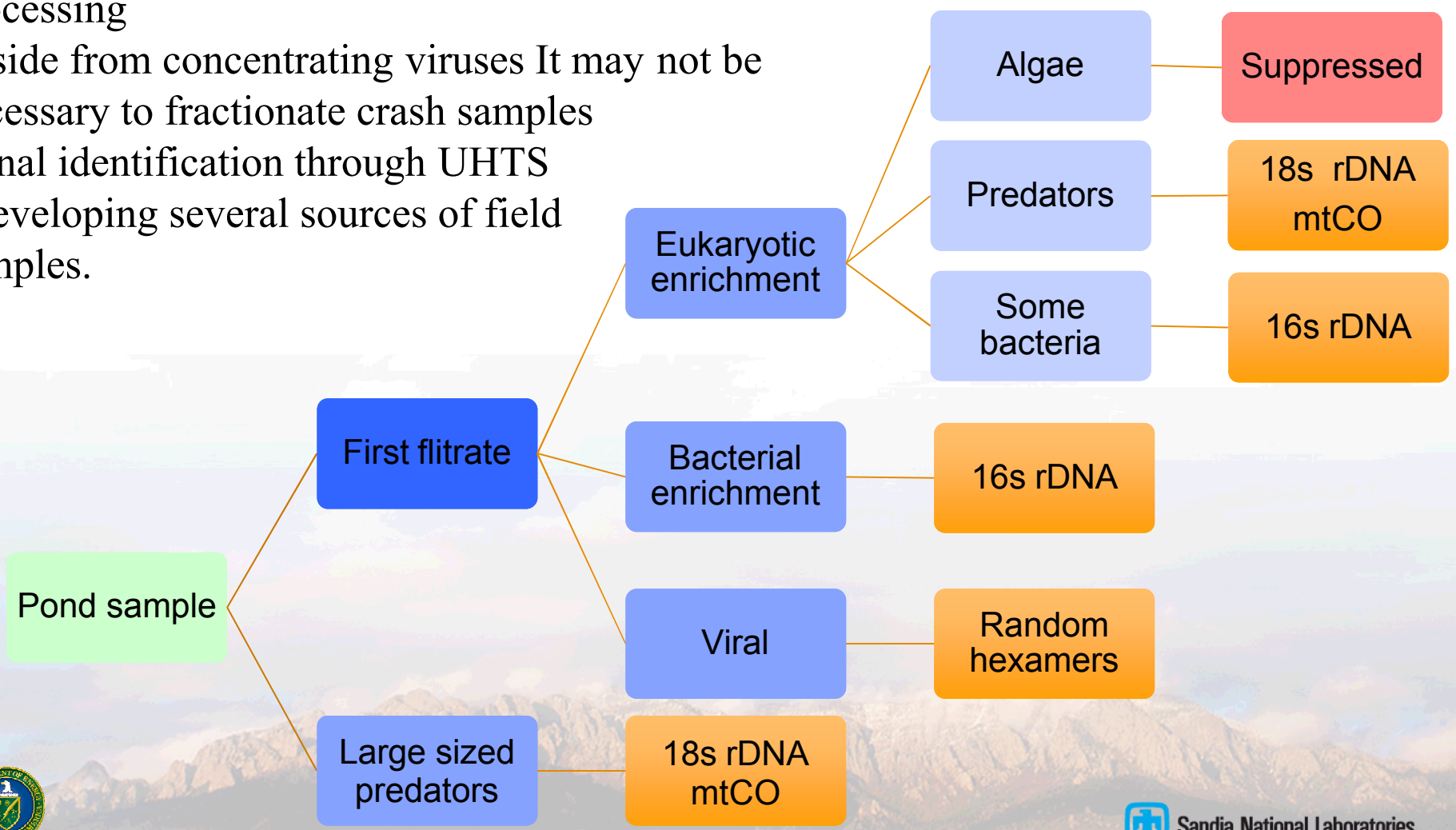
Suppression can reduce the background derived from the host and microbiome leaving a more complex and more informative derivative

Metagenome

Processed metagenome

Physical separations are 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.



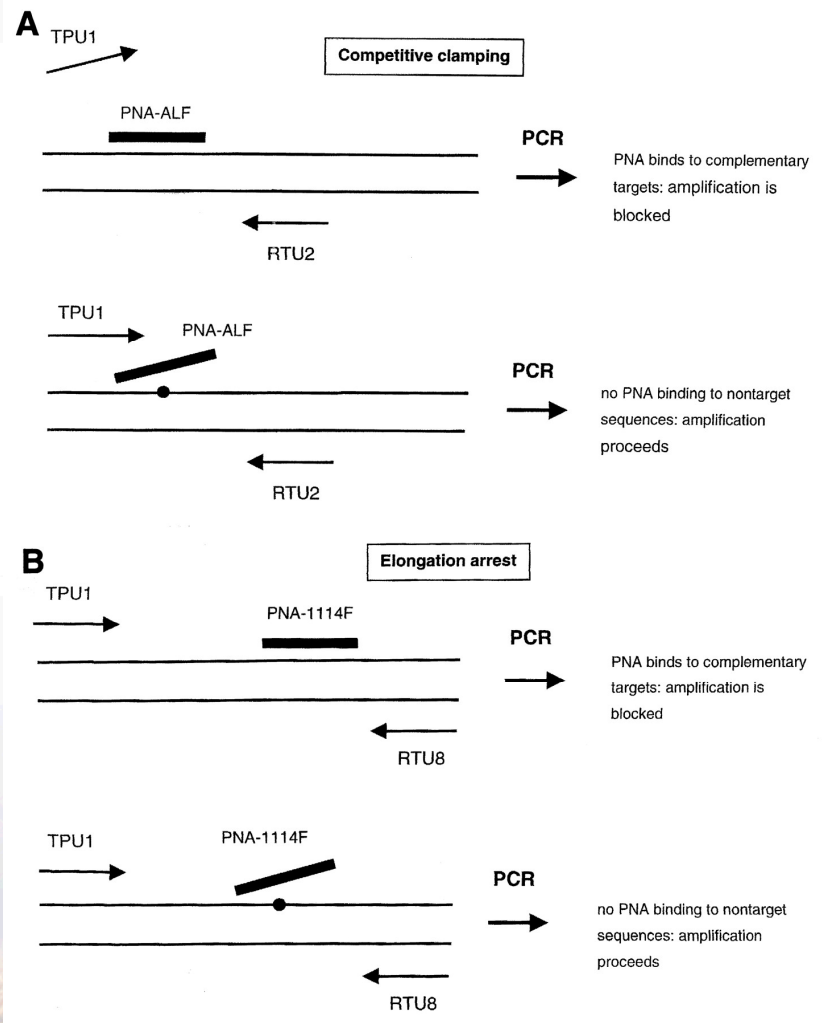
We have devised a suite of “suppression” strategies

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

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

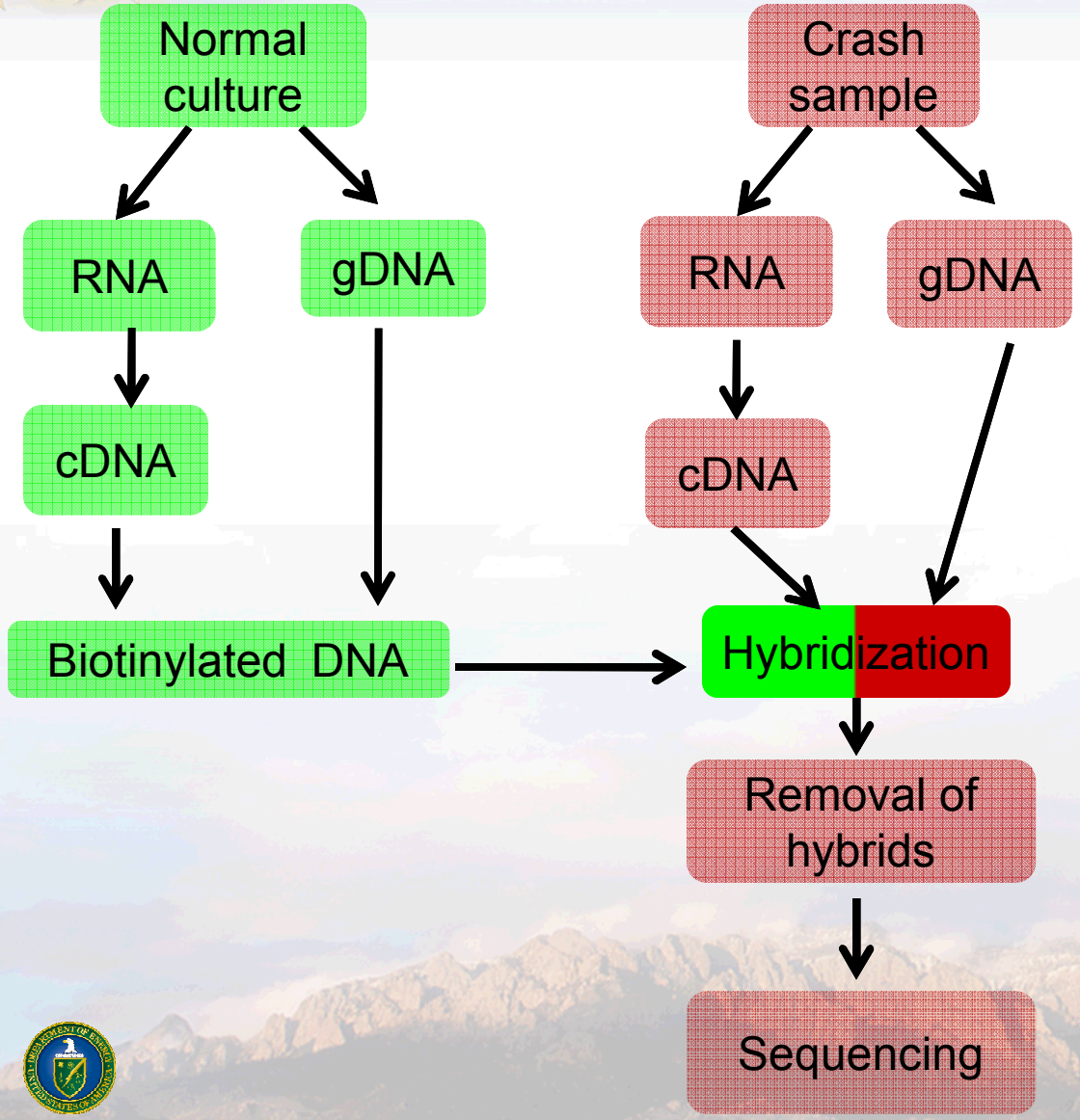
Subtraction:
rDNA analysis or metagenomic analysis
Physically removes unwanted sequenes

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



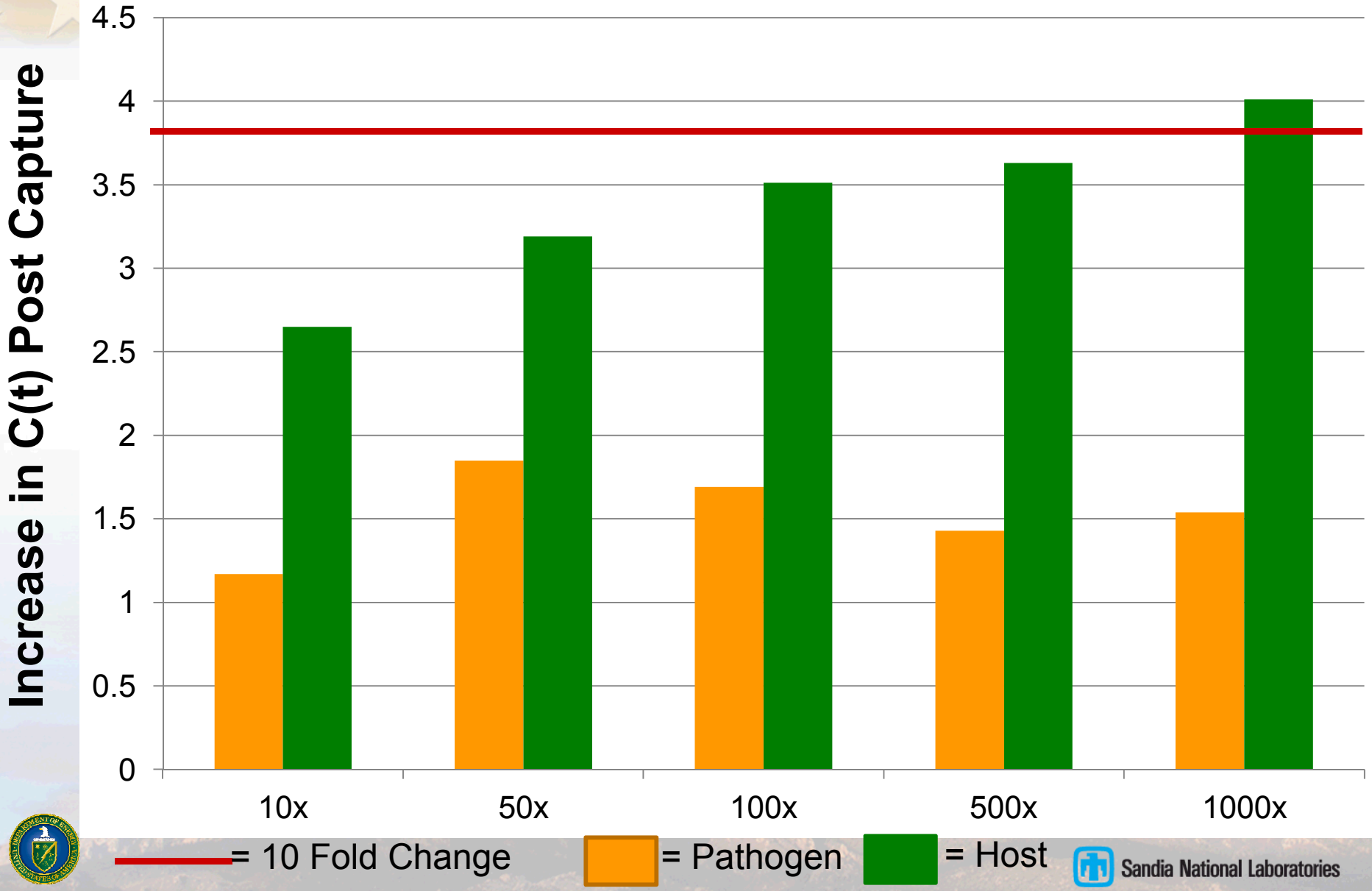
von Wintzingerode, F. et al. 2000. Appl. Environ. Microbiol. 66(2):549-557

Development of subtraction reagents

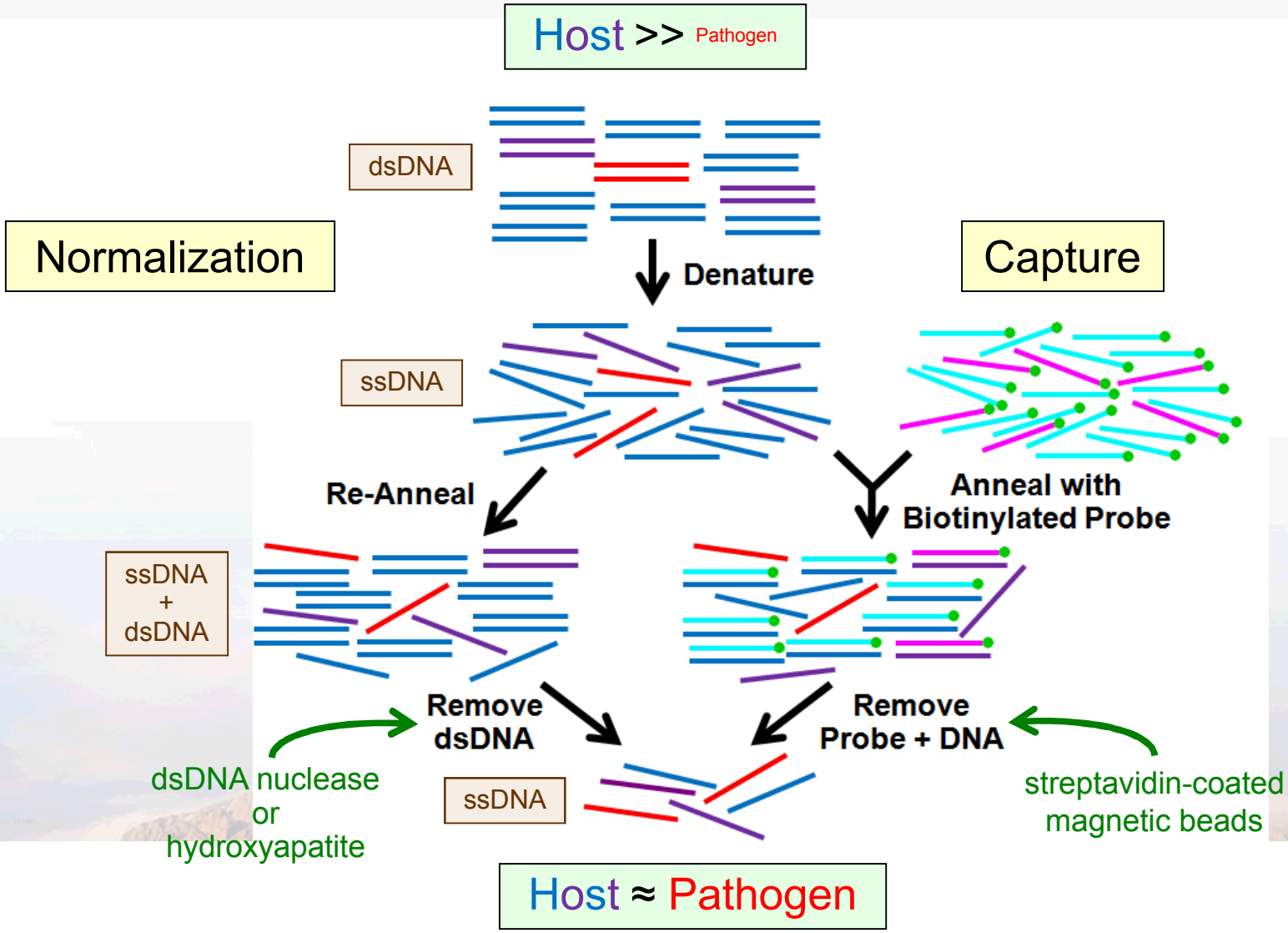


- Anything that is “normal” is depleted from the crash sample
- Enriches for sequences that are likely to be associated with the crash agent
- In the final manifestation these manipulations will be carried out in an automated fashion by RapTOR.

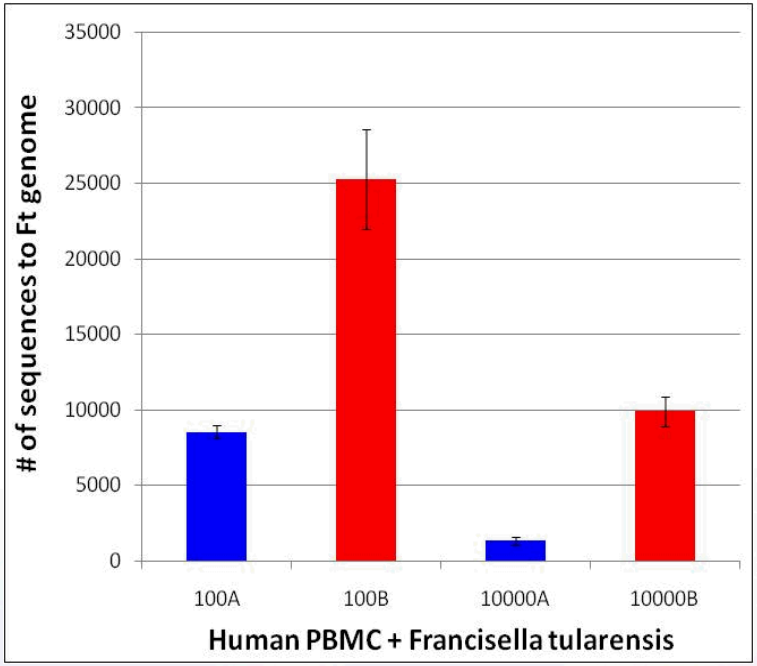
Capture probes effectively remove host sequences

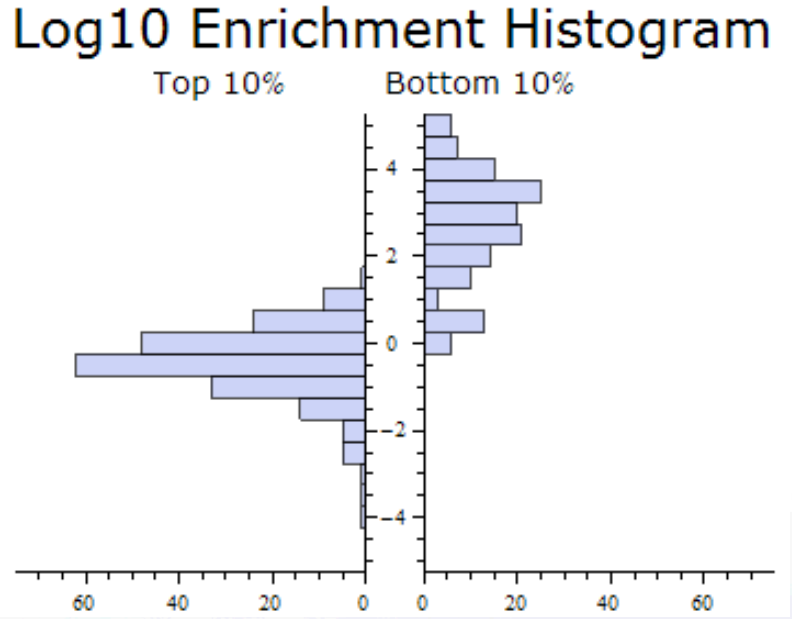


We are focusing on two complementary suppression methods for depleting algal NA



Normalization Yields more hits on rare targets





The bottom decade of least abundant initial FPKM values is enriched 2-3 log orders

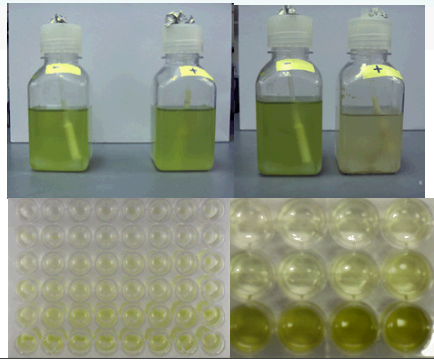
The top decade is depleted or suppressed

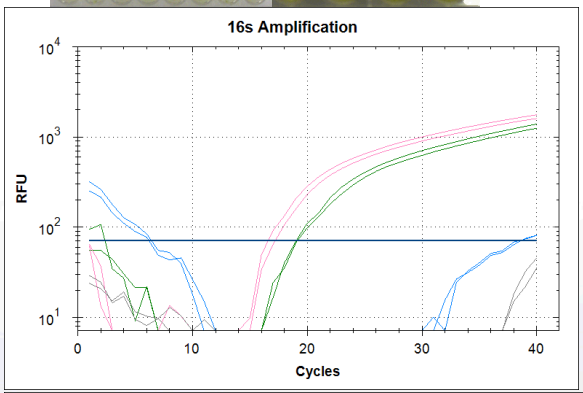
Typical sequencing dateset Identification of eukaryotes in a “Infected” sample

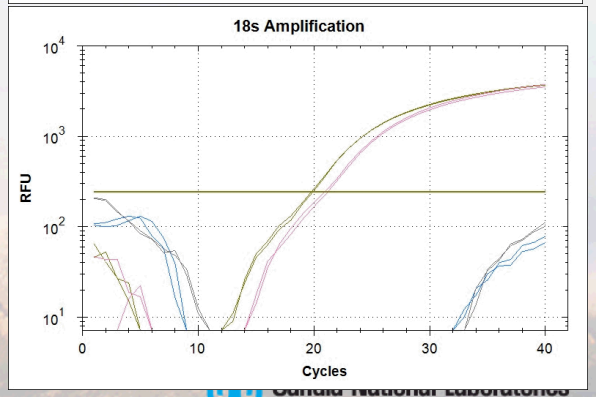
Sample	% hits	Best hit	type
Pond 10	29	*Cercomonas plasmodialis;*	flagellate
	21	*Aplanochytrium stocchinoi*	marine fungus
	16	*Chaetonotus neptuni*	unsegmented worm-like
	4	*Labyrinthuloides minuta;*	*plant pathogen (seagrass wasting disease)*
	4	*Platyreta germanica*	parasite/predator
	4	*Amphora cf. capitellata*	competitor

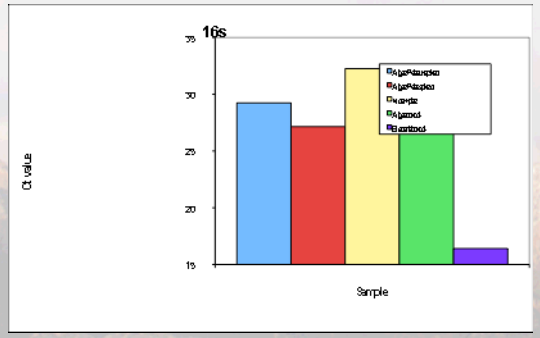
Isolation of putative biological agents

- **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





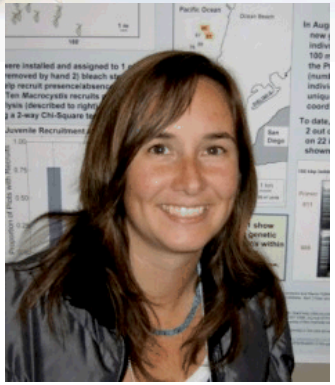




Conclusions

- **We have demonstrated the identification of grazers, fungi and weed algal species by DNA sequencing**
- **We have developed methods for the removal of background (algal) sequences**
  - Blocking primers
  - Subtraction
  - Normalization
- **We have developed methods for isolation and culture of agents.**

Acknowledgments



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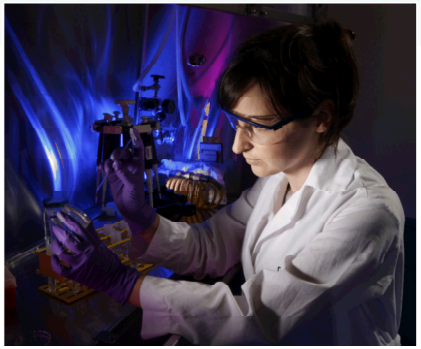

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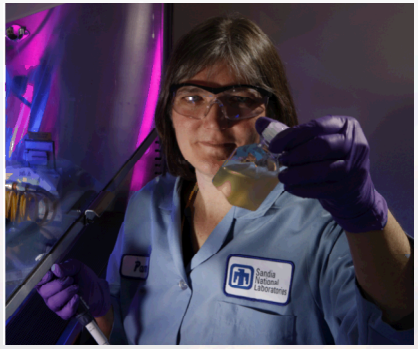

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RapTOR: A new approach to rapidly characterize unknown bioagents

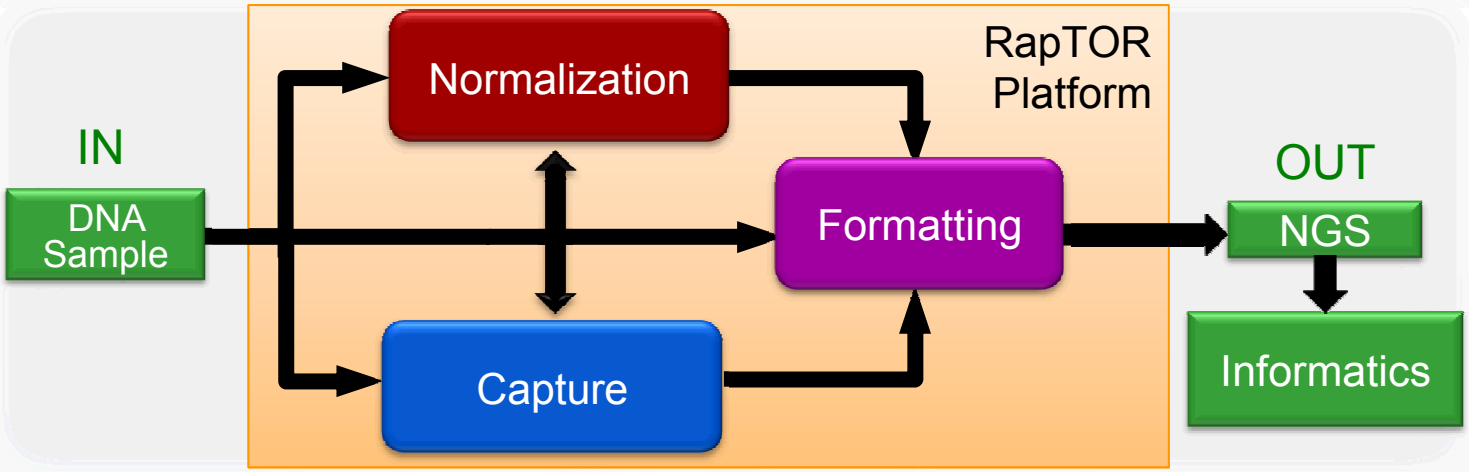
Rapid Threat Organism Recognition (RapTOR) system

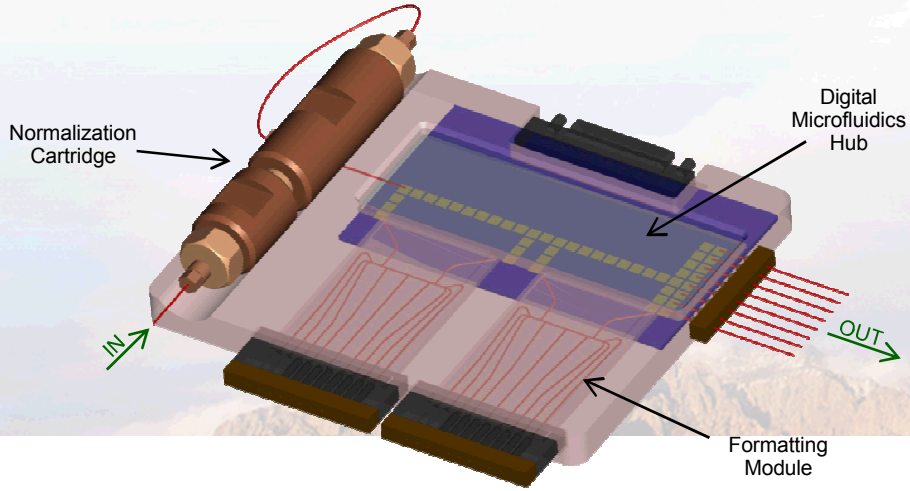
- **Goal:** Efficient analysis of pathogen nucleic acids (NA) in clinical samples *via* targeted Next Generation Sequencing (NGS)
  - **Key advance:** Automated microfluidic hardware to enable molecular suppression and preparation to improve signal-to-background (pathogen-to-host) NA ratio in samples

Drivers for our approach

- Identify and characterize unknown pathogens in a timeframe compatible with rapid disease outbreak detection and response
- No *a priori* knowledge of a pathogen or culturing of organism; (deep genomic sequencing)
- Automate the nucleic acid processing and integrate data analysis and knowledge discovery system inclusion at federal and state-wide laboratories

Microscale platform integrates suppression & formatting modules within a flexible architecture





Gen-1 design

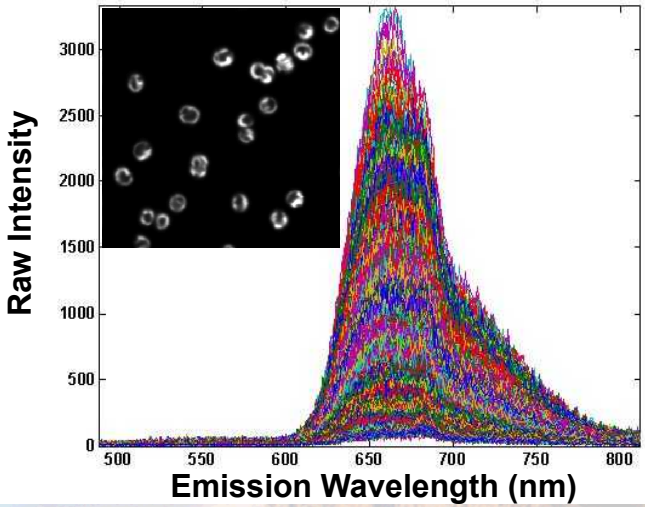
Microscale Platform Features

- 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

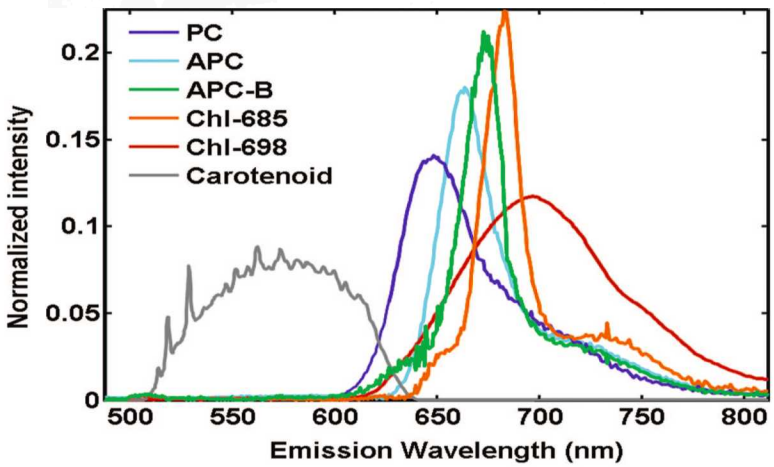
Inside an Algae Cell w/ Spectral Imaging & Multivariate Curve Resolution

- **Identify and quantify photosynthetic pigments and other fluorophores simultaneously in living cells**
  - Label-free
  - High spatial resolution in 3D (250 x 250 x 500 nm^3)
  - High spectral resolution and large spectral range

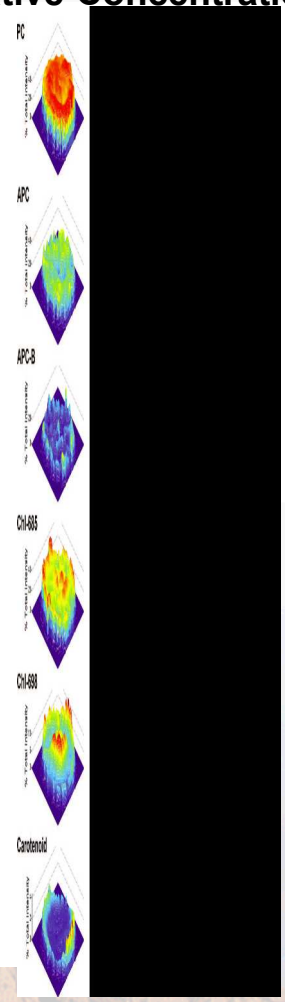
Raw Spectral Data



Identified Spectral Signatures



Relative Concentrations



WT Synechocystis

Vermaas, et. al. PNAS, 2008

Jones et al., J Chemom. 2008 and references therein

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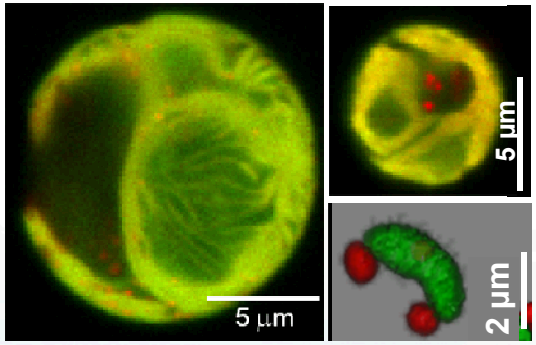
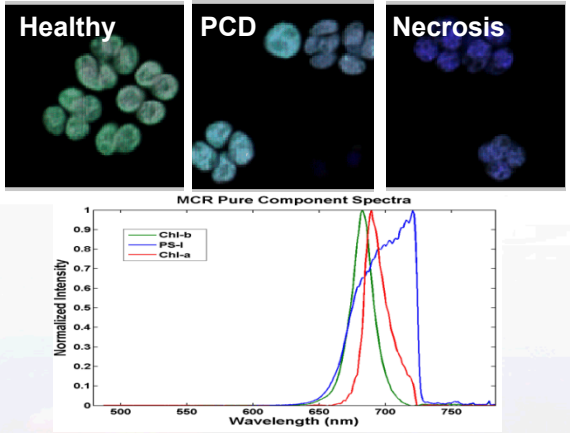
Advanced Imaging = Unprecedented View of Cell Processes

Hyperspectral Confocal Fluorescence Microscopy



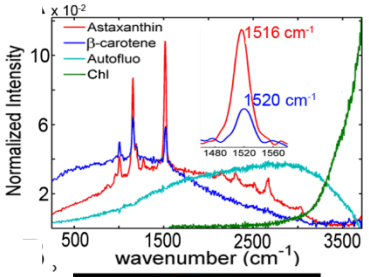
Subcellular localization, quantification of lipid and chlorophyll *w/o use of labels*!

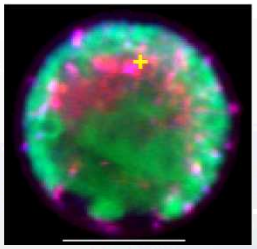
Two-Photon Hyperspectral Fluorescence Microscopy



Discrimination between cell death mechanisms *at early stage w/o use of labels*!

Hyperspectral Raman Microscopy





Subcell localization, discrimination of carotenoid, lipids, and precursors *w/o use of labels*!

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