Single cell analysis of bacteria: activity and genomics

- Single cell activity - CTC
- Lysotracker for heterotrophic protists
- Matching phylogeny and metabolism - one cell at a time

Conceptualization

- Most marine bacteria are inactive at any given time and place (refuge: "hiding out in the open")
- These bacteria form an "information database" of phylogenetic and metabolic diversity.
- Substrate sources are patchy in space and intermittent in time.

Conceptualization (2)

- Patches of active bacteria form in microzones of high substrate (DOC) supply.
- These activity patches resupply the background of dormant bacteria.
- State-of-the-art techniques now allow us to test hypotheses associated with these concepts.
  - Single cell activity measures
  - Molecular genetic techniques

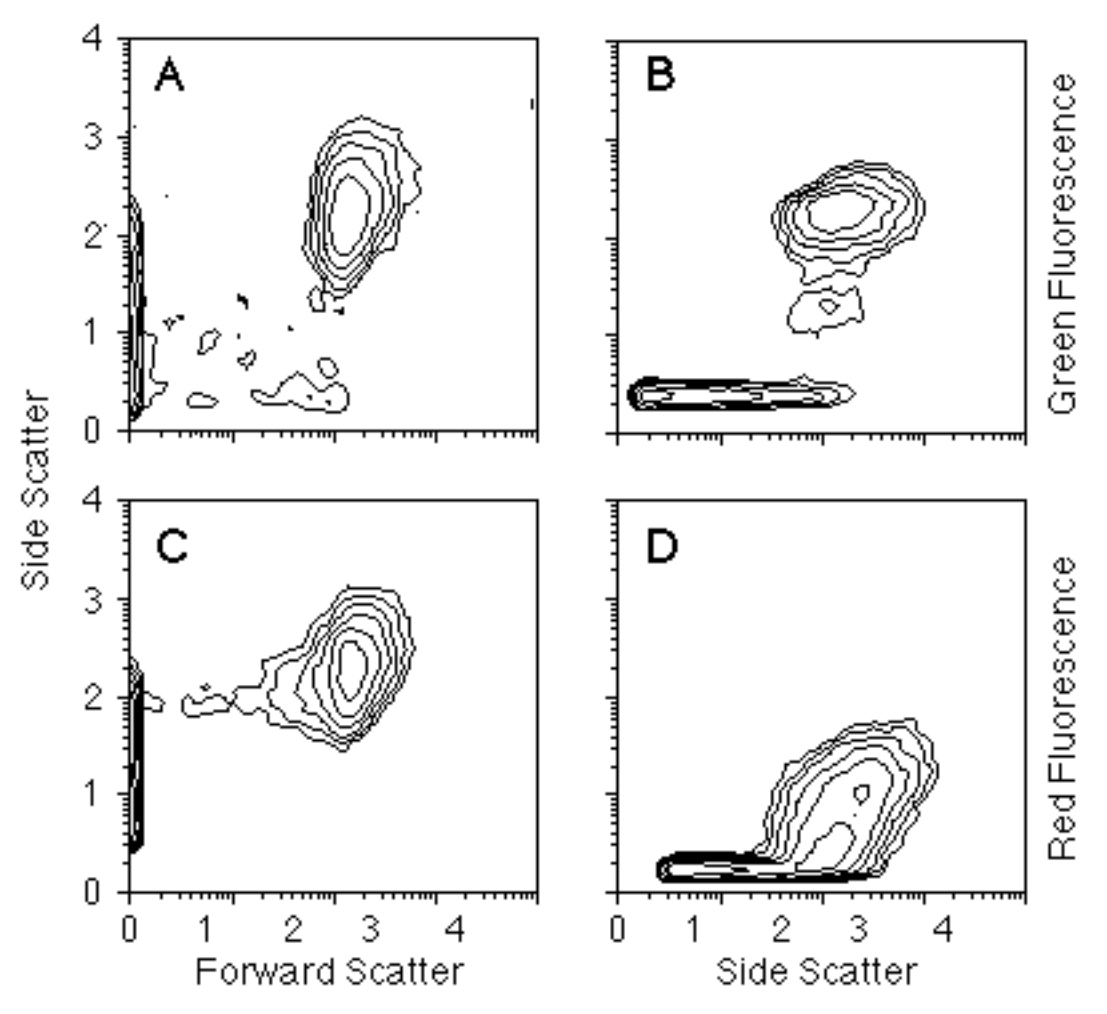
The CTC Method

- 5-Cyano-2,3-ditoyl Tetrazolium Chloride
- CTC is reduced intracellularly in respiring cells to form an insoluble, fluorescent precipitate (formazan, CTF)
- Individual active cells can be identified by microscopy or flow cytometry
- Counting CTC+ cells and total cells yields "% actively respiring cells"

CTC Controversy

- CTC active cells are a small proportion of the total (mostly less than 10%, rarely over 30%)
- CTC is toxic to cells (Ullrich et al.)
- Microautoradiography shows >90% of cells take up labeled "goodies" (sugars, amino & nucleic acids)
- CTC indicates respiration - not cell growth or productivity - what is "active"?
- All marine bacteria tested reduce CTC in culture (Sherrs)
- Detection limit for weakly active cells

CTC Detection by Flow Cytometry

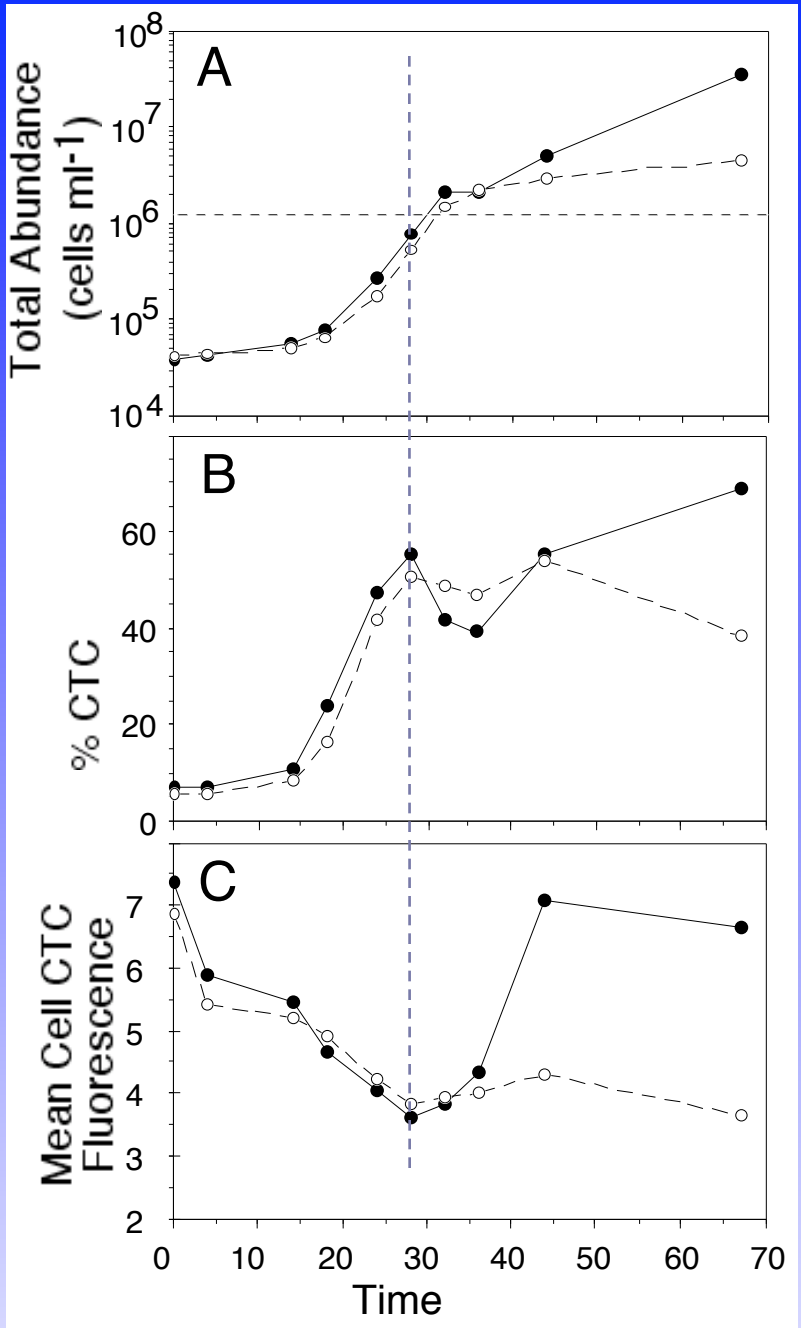


Total bacteria (PicoGreen)

CTC+ bacteria

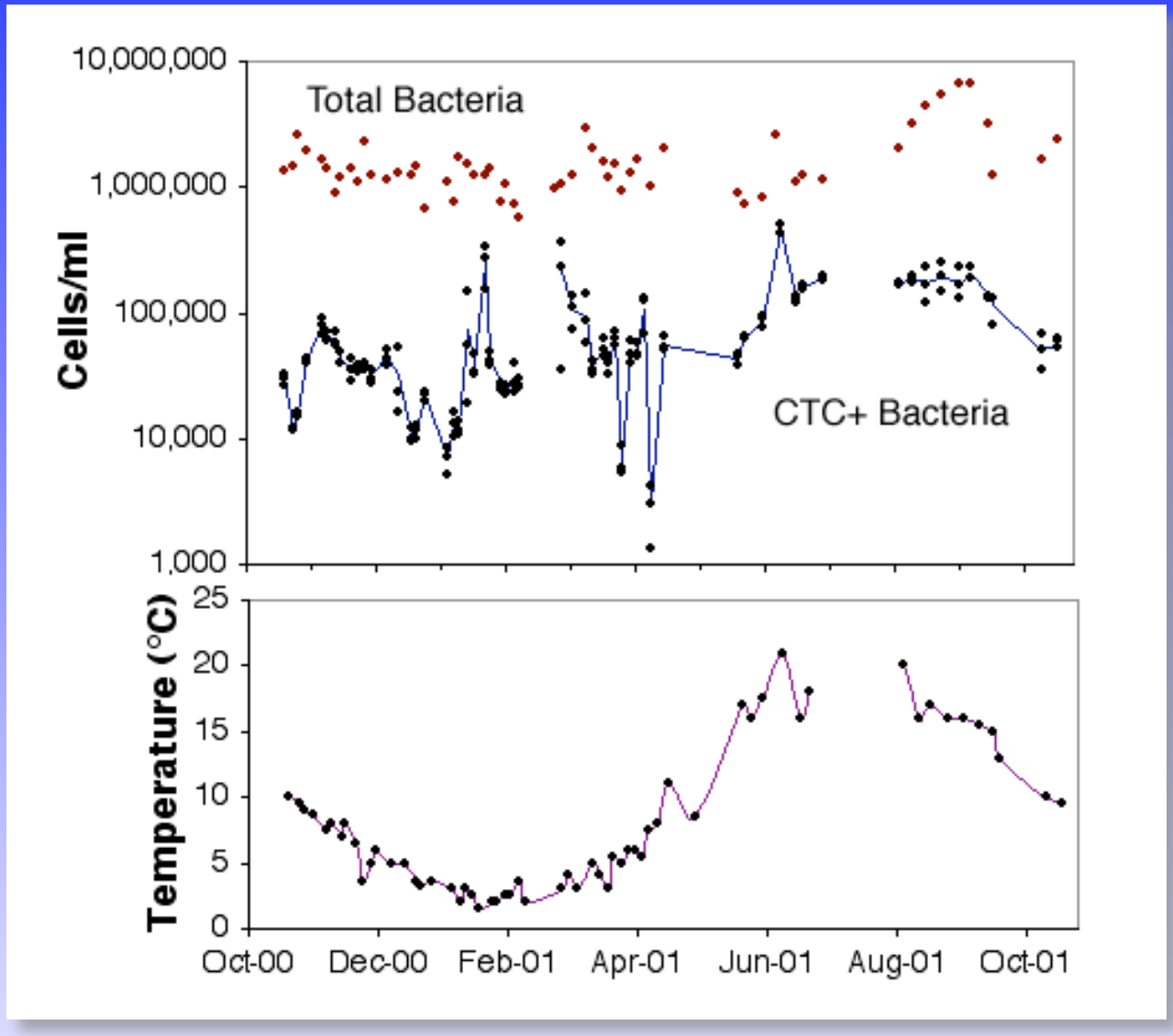
Cell Activity CTC

- Dilution/growth experiment
- % active peak at max growth rate
- Mean CTF fluorescence lowest at max growth rate



Sieracki et al. 1999. AEM 65:2409-2417

Seasonal Cycle
Boothbay Harbor

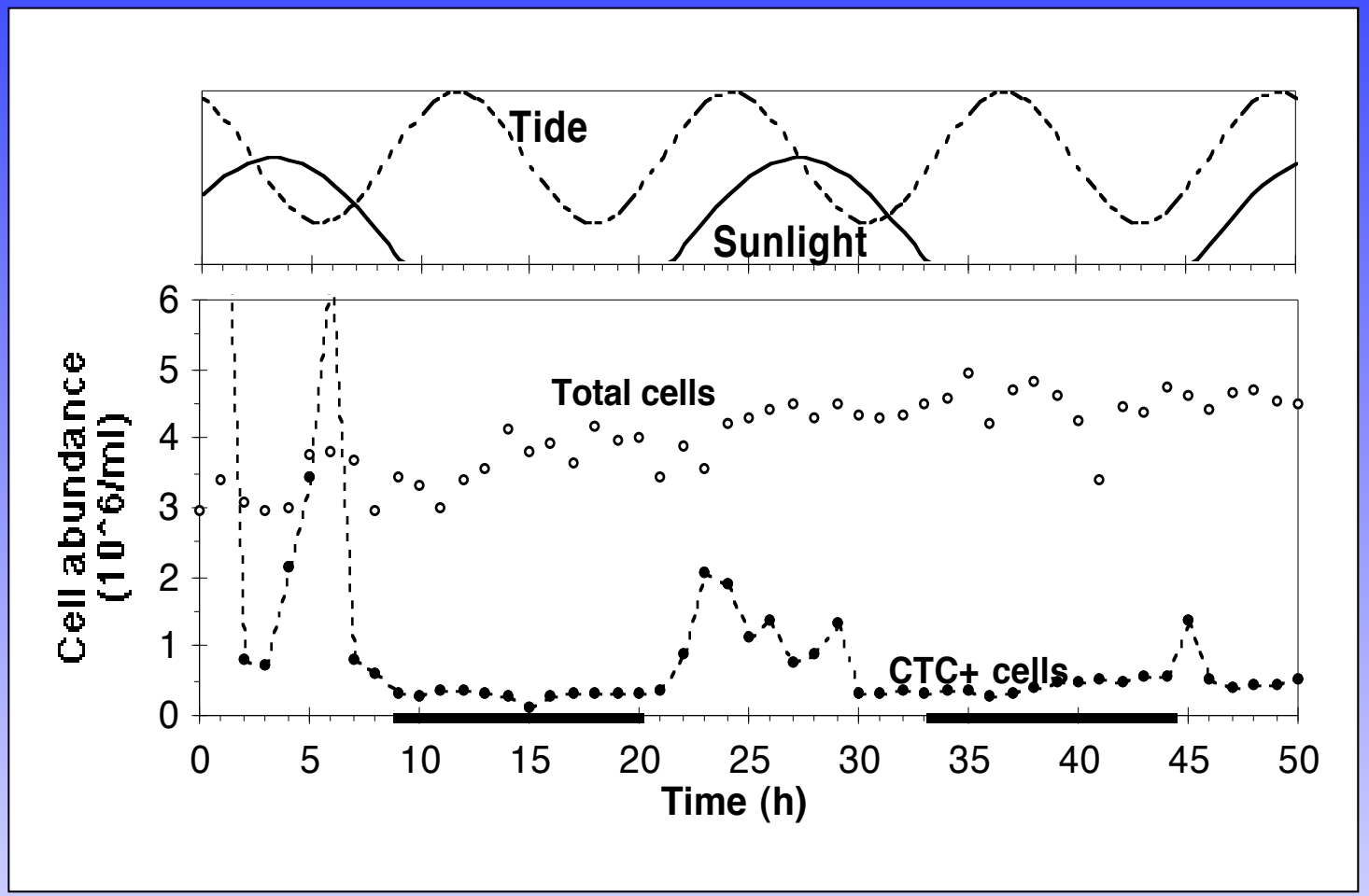


Range Factors

Total bact: 11 X

CTC+: 160 X

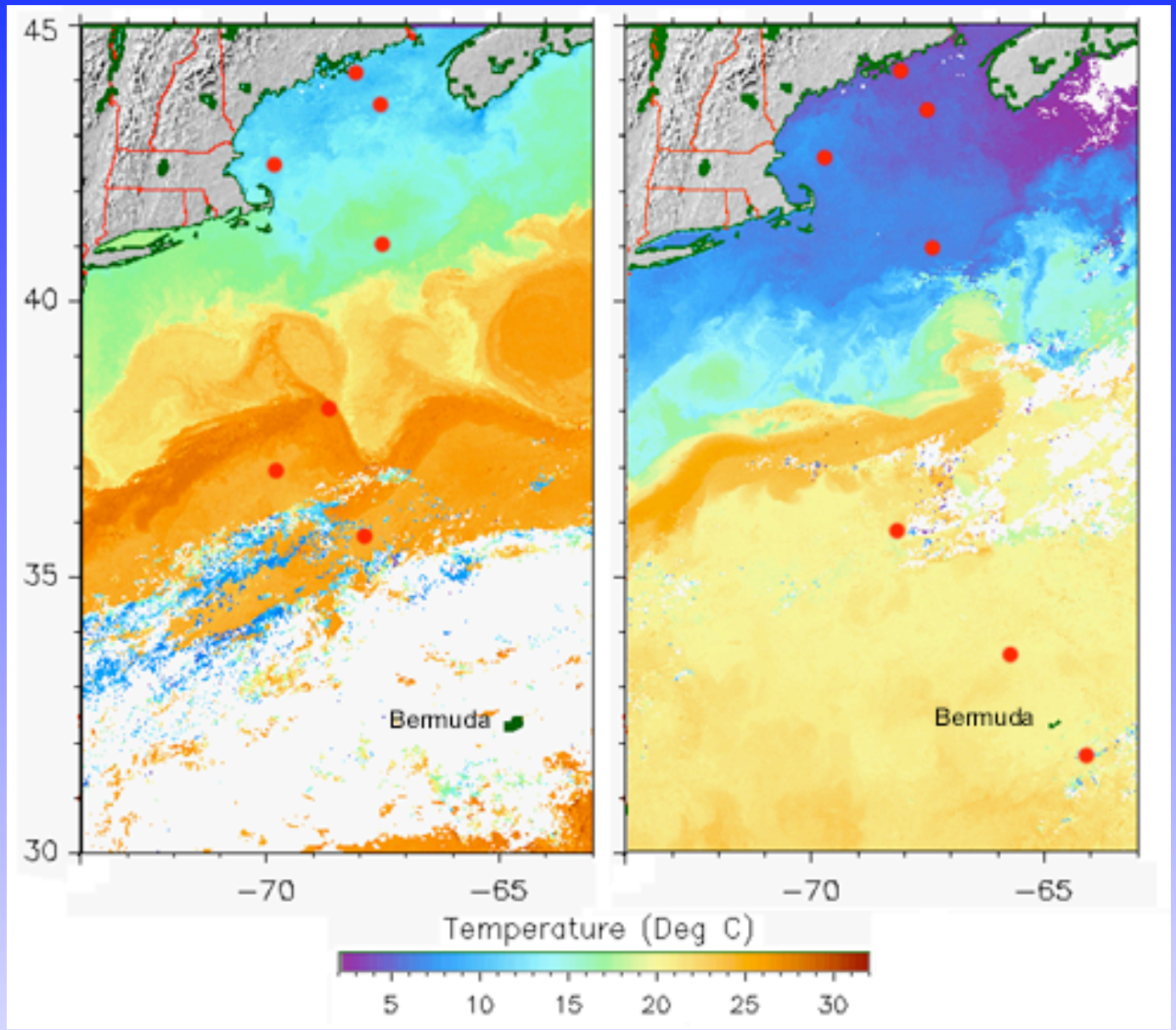
Diel Pattern in CTC+ Cells



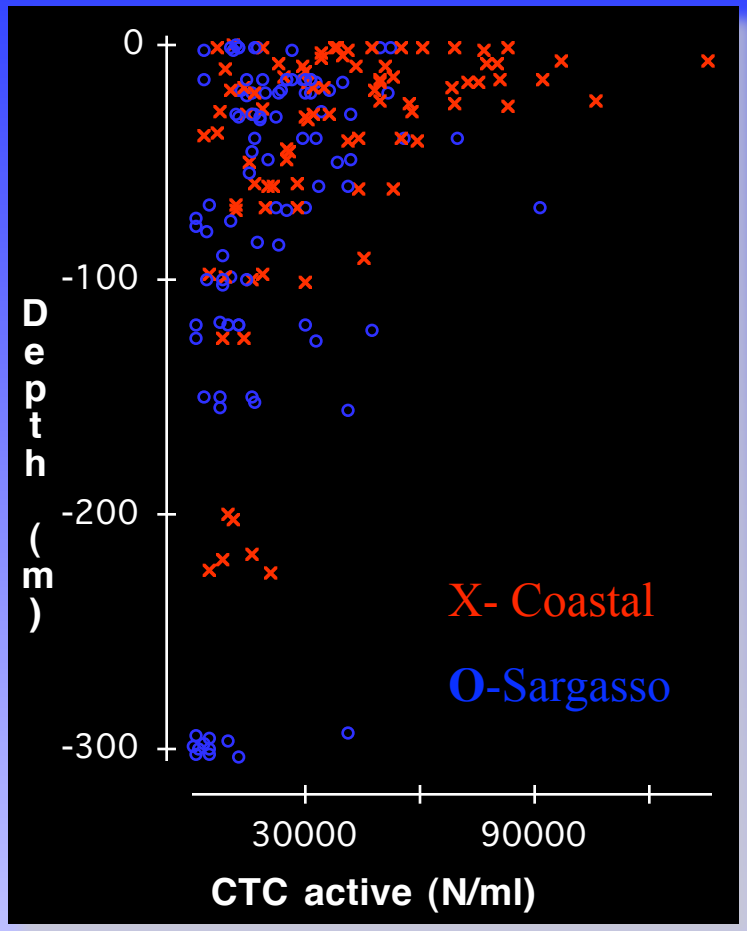
Stations sampled for bacteria

Wide trophic gradient

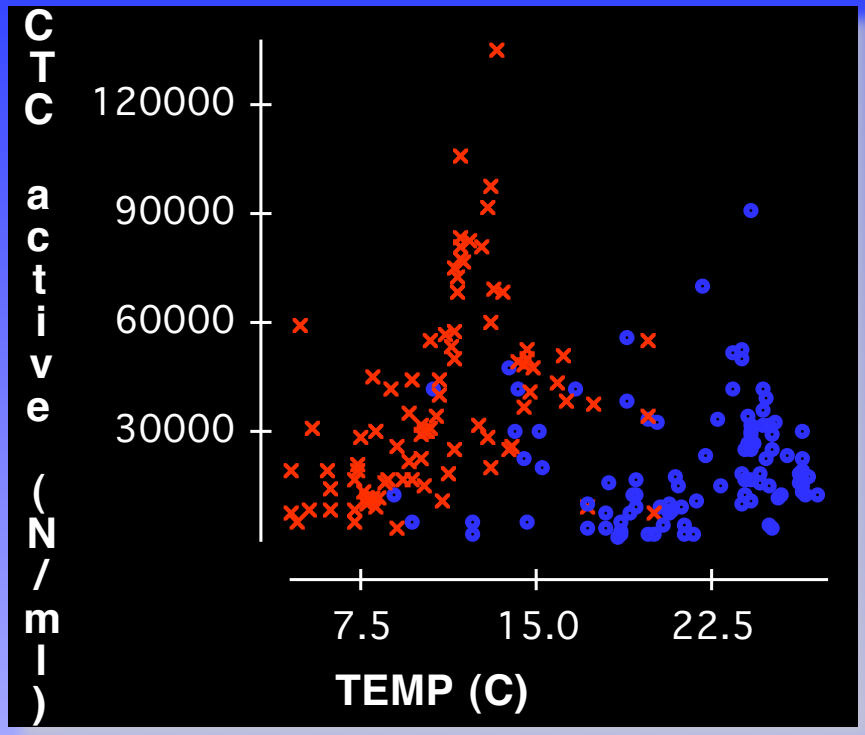
R/V Cape Hattaras



CTC-Active Bacteria - 2 cruises, N=185

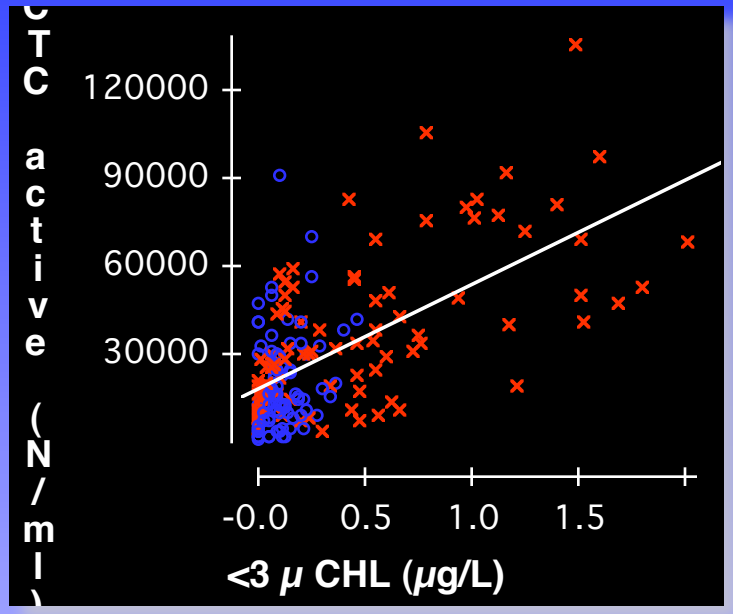


CTC-active bacteria higher in surface waters



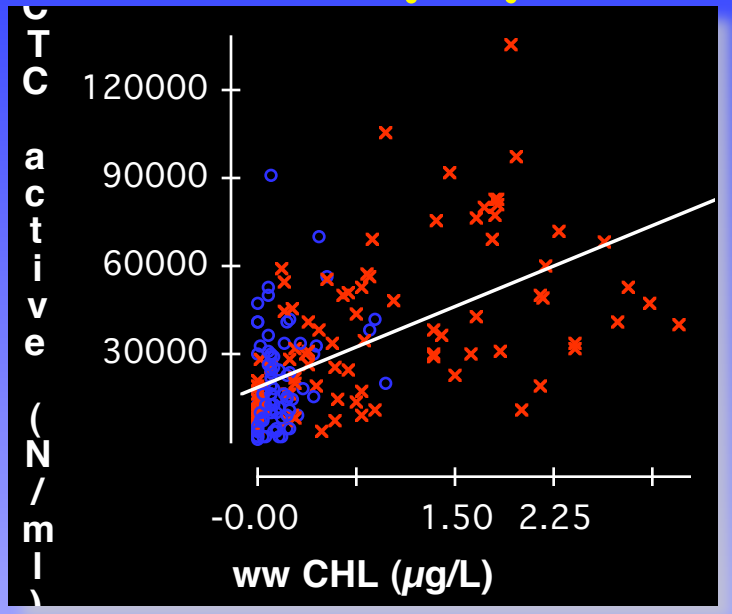
CTC-active bacteria are not correlated with temperature overall, but are **within** systems

< 3 um Chlorophyll



Active bacteria correlated with the smallest size fraction of chlorophyll (r^2=0.62)

Whole Water Chlorophyll

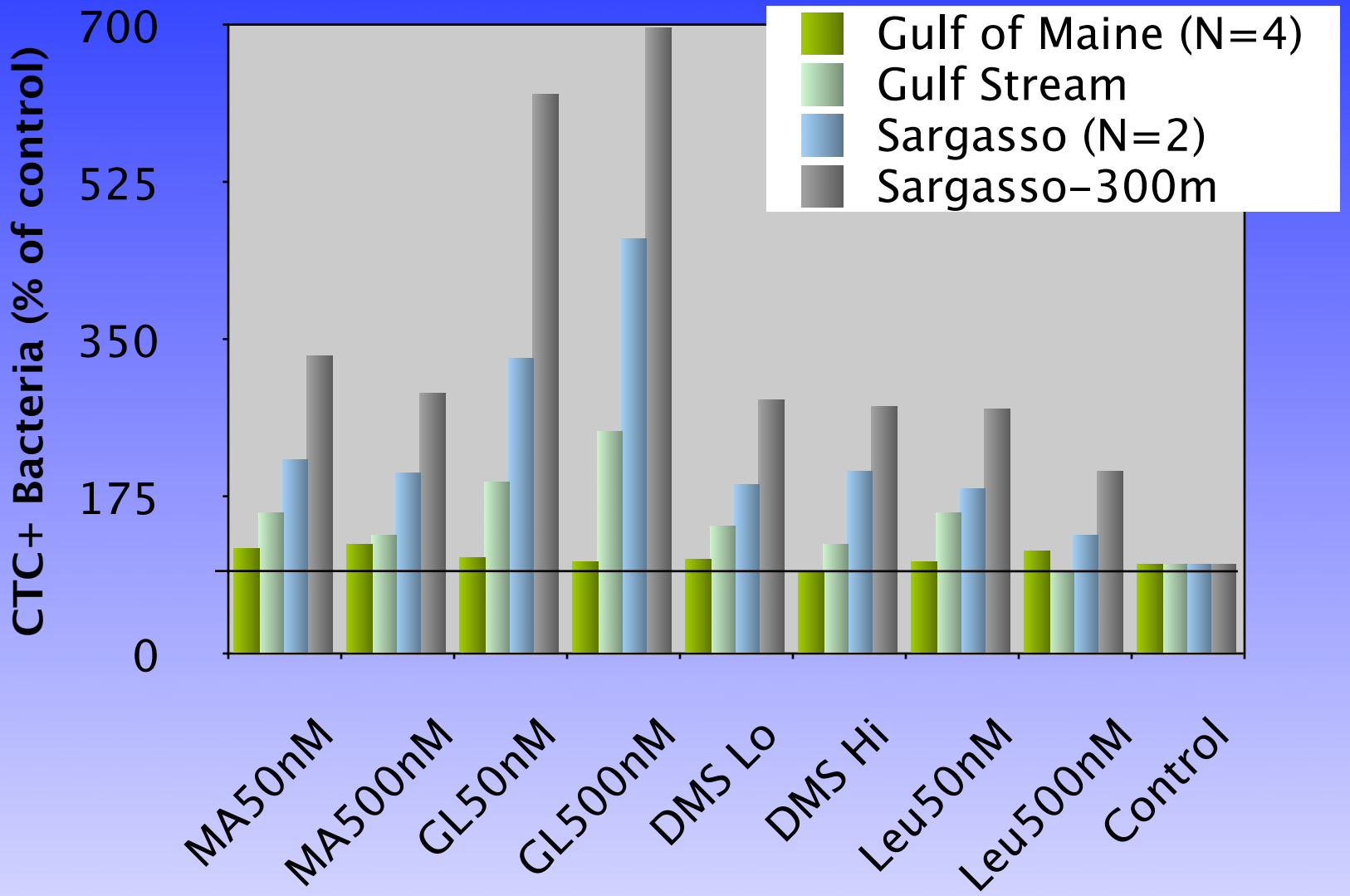


Also correlated with total chlorophyll (r^2=0.57)

Substrate-CTC Bioassays

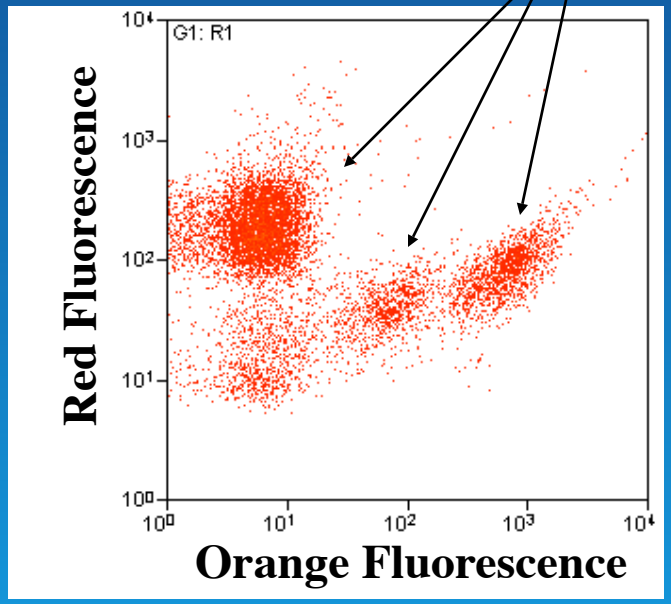
- 8 experiments on October cruise
- Low molecular wt. substrates added for 30min, then CTC assay (60min)
  - Methylamine, glucose, DMS, leucine
- Concentrations: 50 to 500 nM
- Results shown as number of active bacteria as % of un-amended control

Substrate-CTC Bioassays



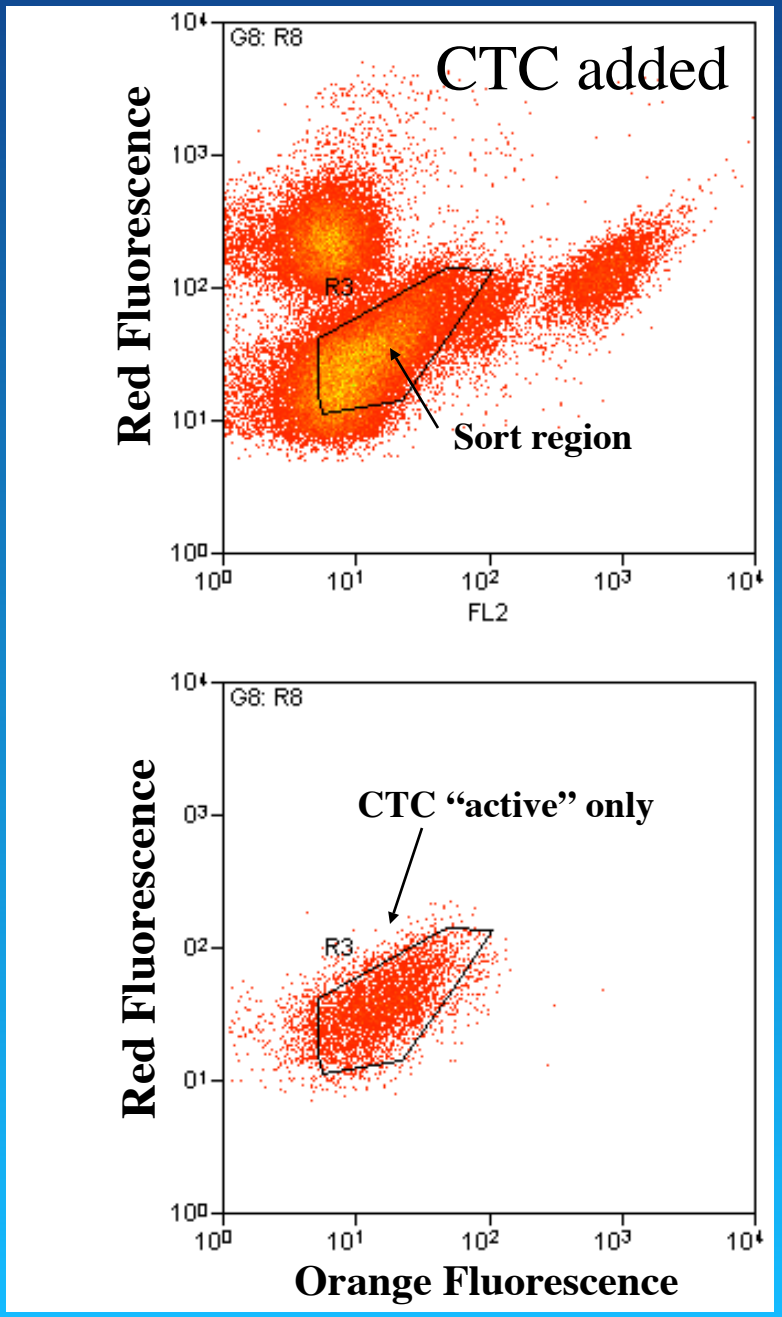
Analysis and isolation of CTC "active" bacteria from Boothbay Harbor Dock Water

Phytoplankton Populations



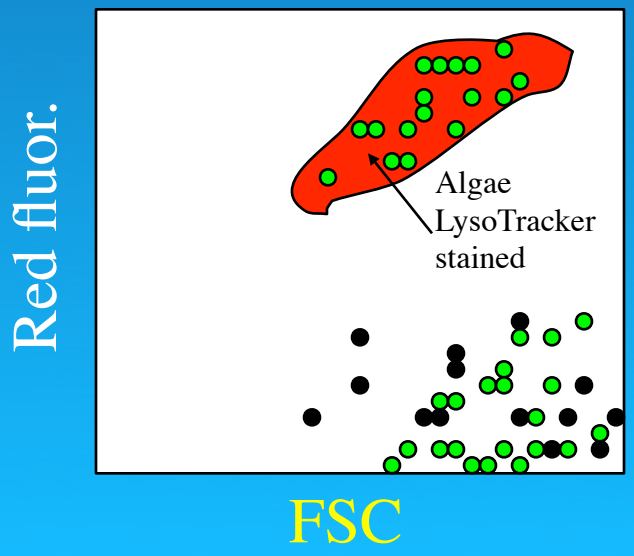
No CTC added

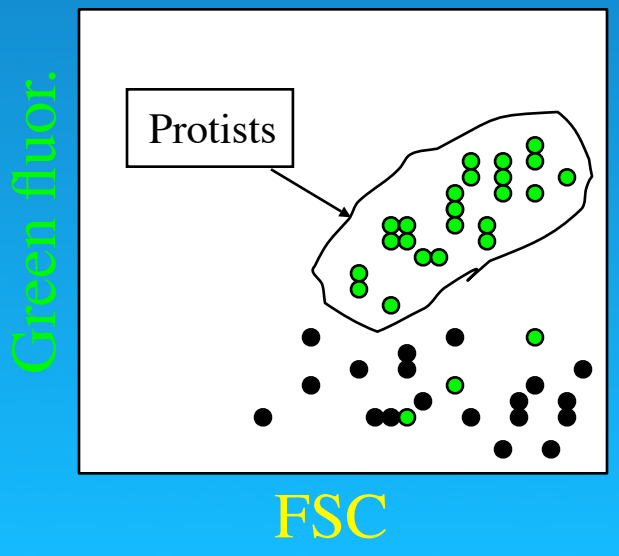
Post - Sorting



Protozoan Isolation using LysoTracker Detection of Flagellates and Ciliates

- LysoTracker is a fluorescent acidotropic probe that labels acidic organelles in live cells (eg. vacuoles and lysosomes)
- Specific for eukaryotes (must have internal organelles)
- allows for the detection & isolation of small heterotrophic protists that are not mixotrophs (both photosynthetic and heterotrophic).



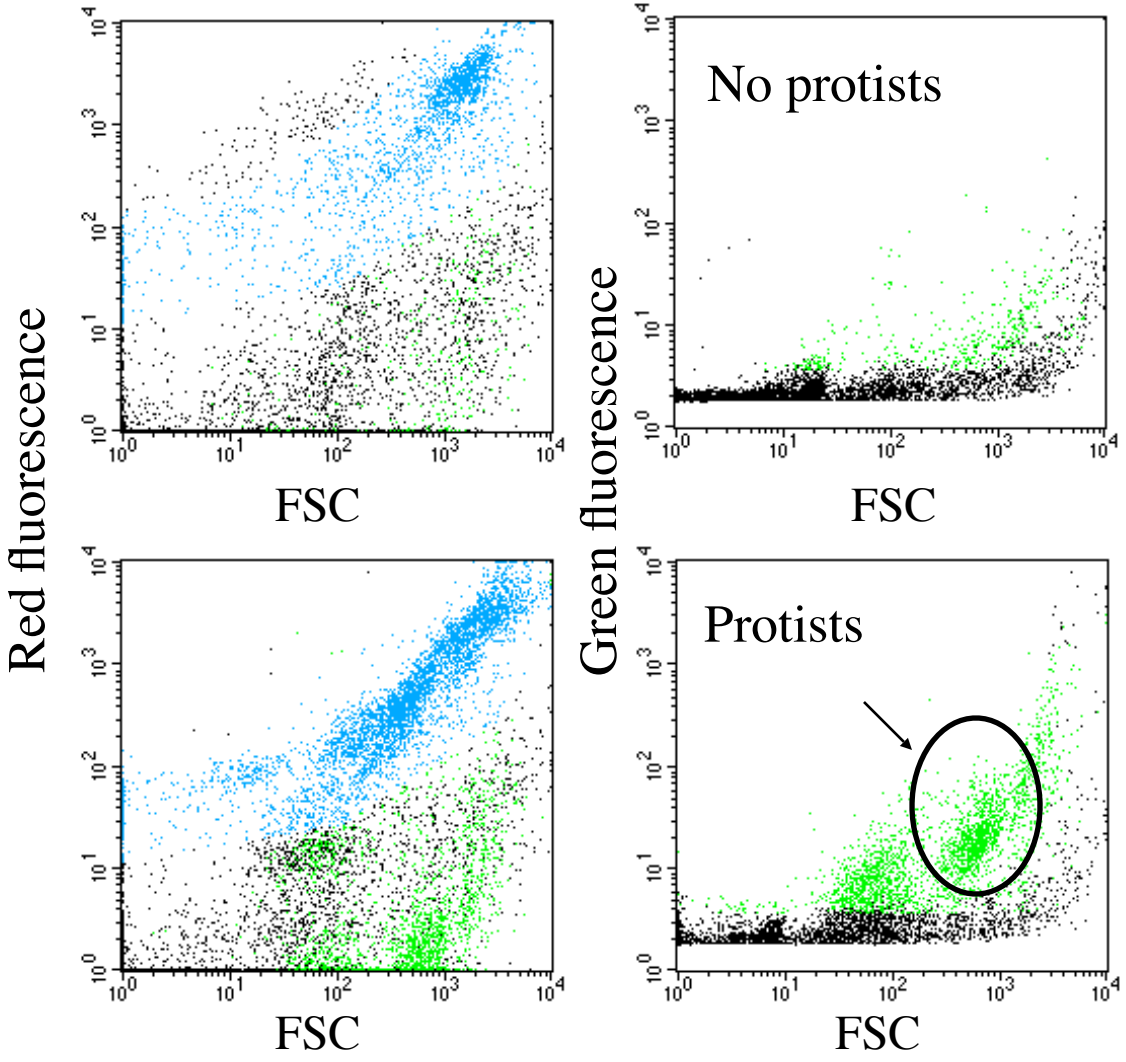


Green flu. trigger

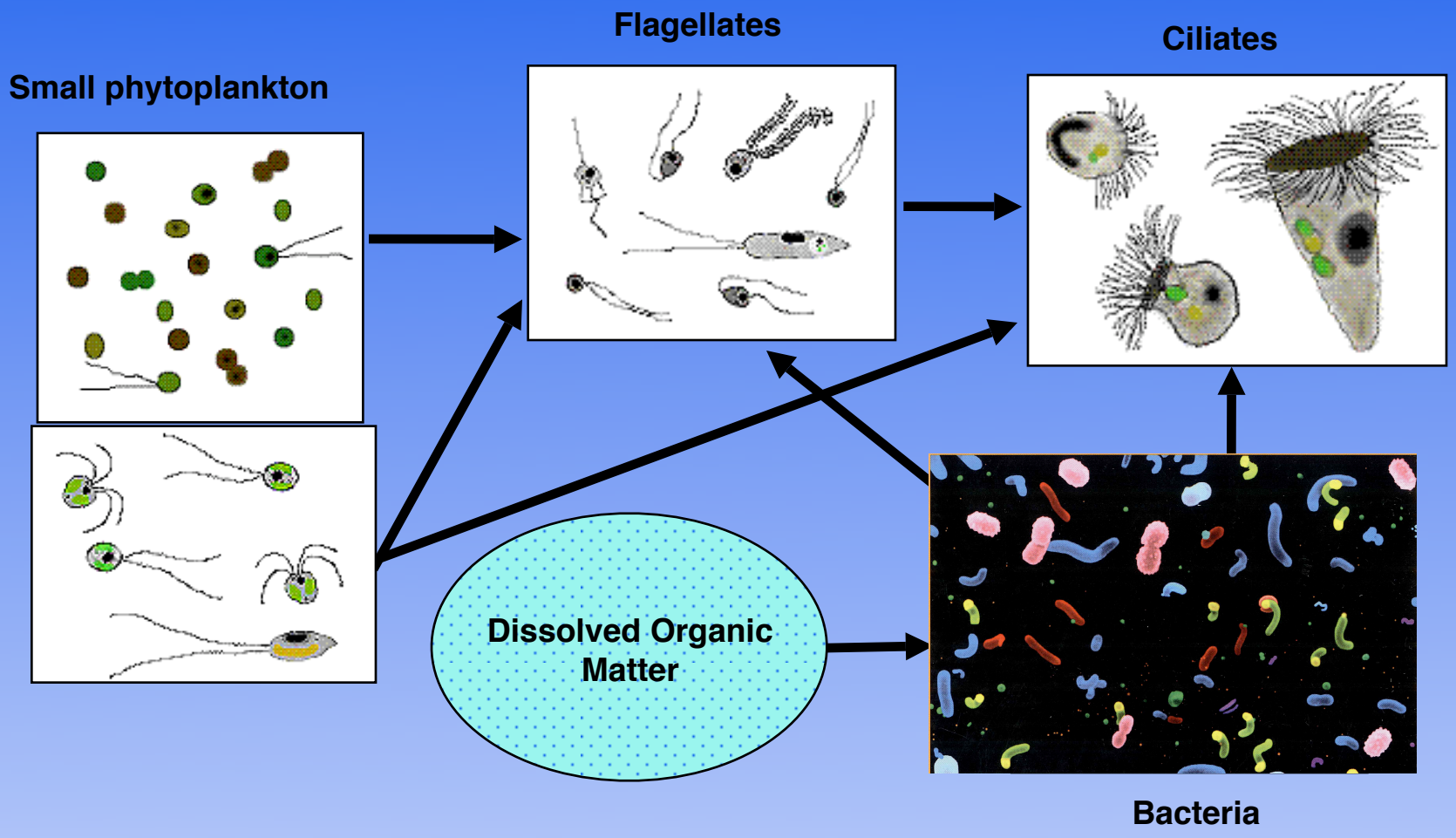
LysoTracker Green - Identification of Heterotrophic Protists

Negative control (preserved)

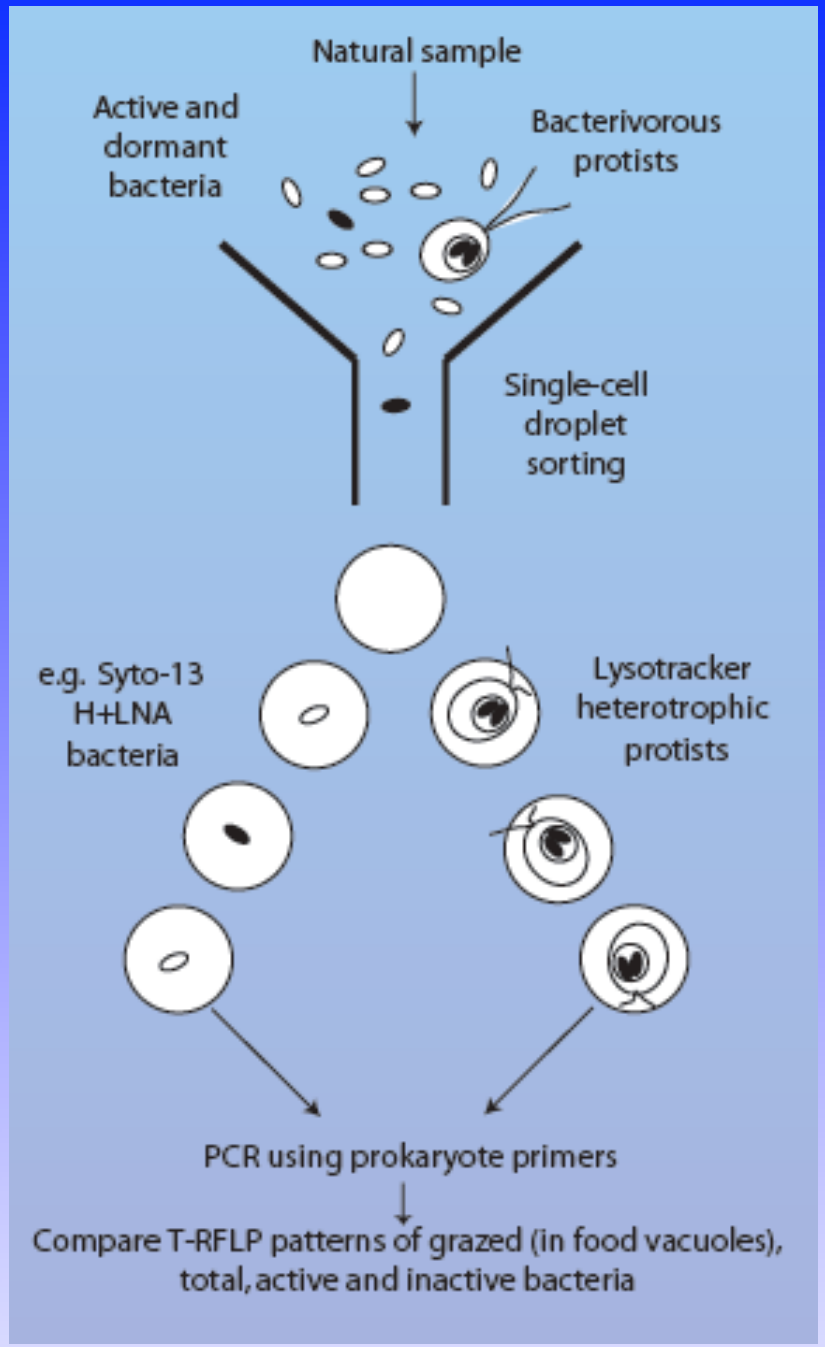
Positive Control (live)



Microbial Food Web



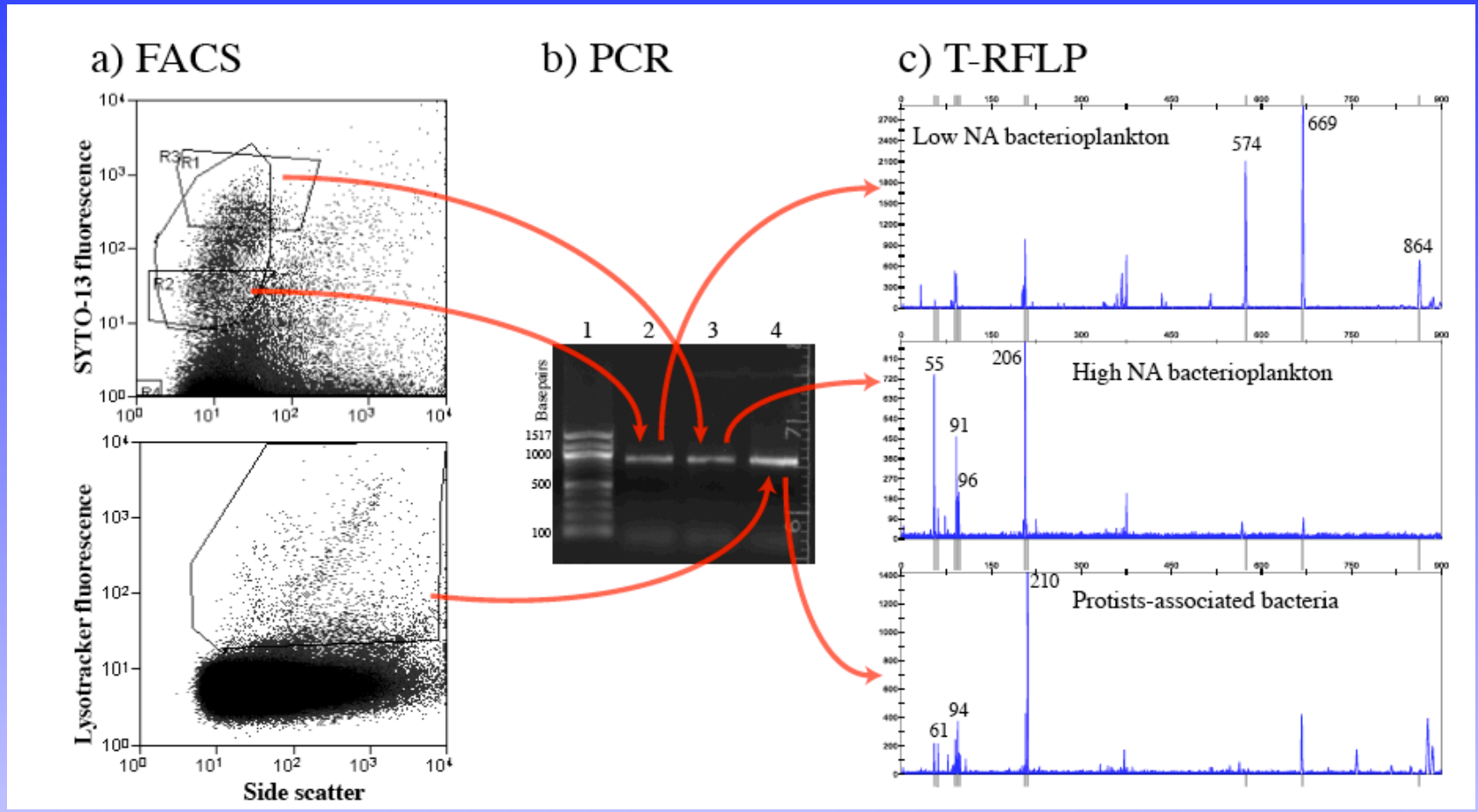
All forms produce dissolved organic matter



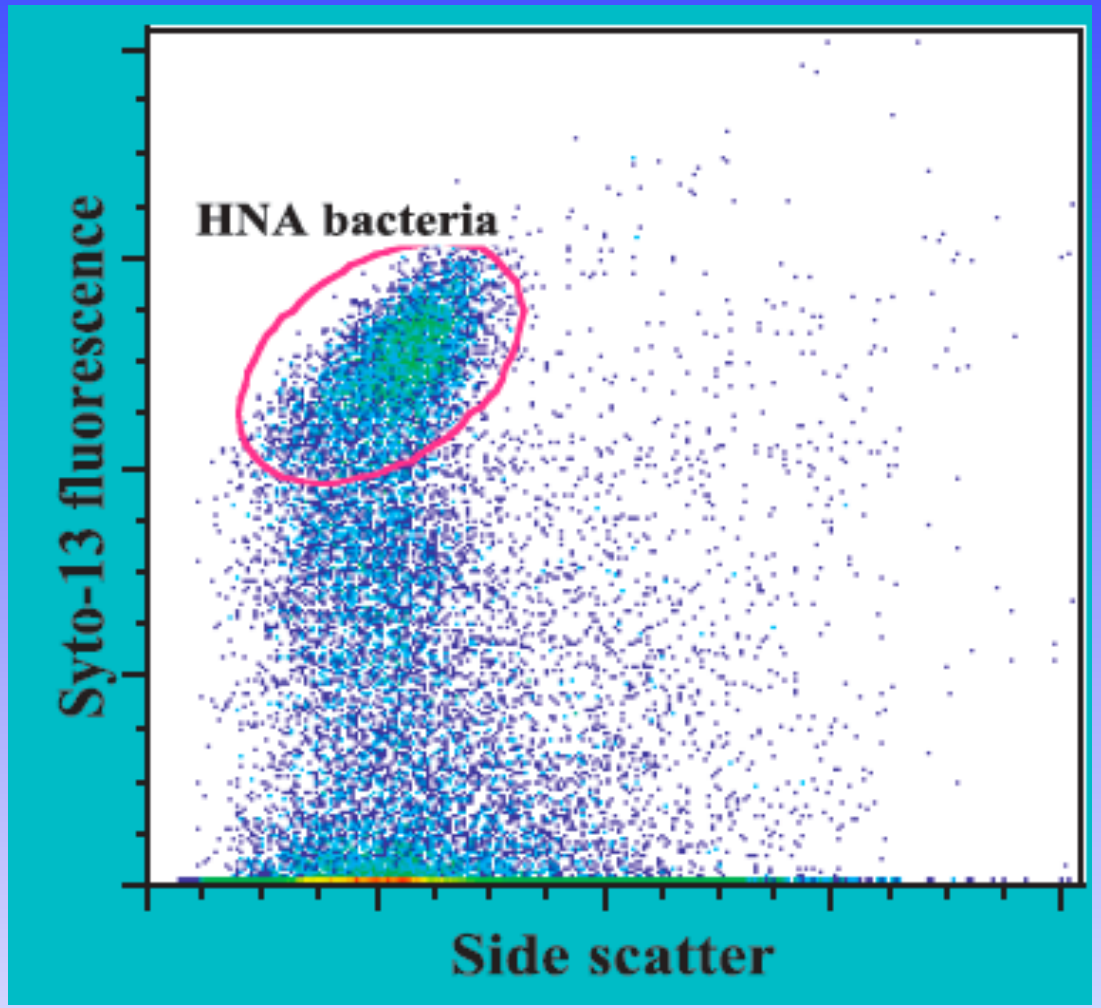
Using cell sorting to study grazer preferences for bacteria

- Stain subsamples with Lysotracker and bacteria activity indicator (e.g. Syto-13)
- Sort active and total bacteria
- Sort heterotrophic protists
- PCR using prokaryote primers
- Use DNA fingerprinting (e.g. T-RFLP) to compare sorted fractions

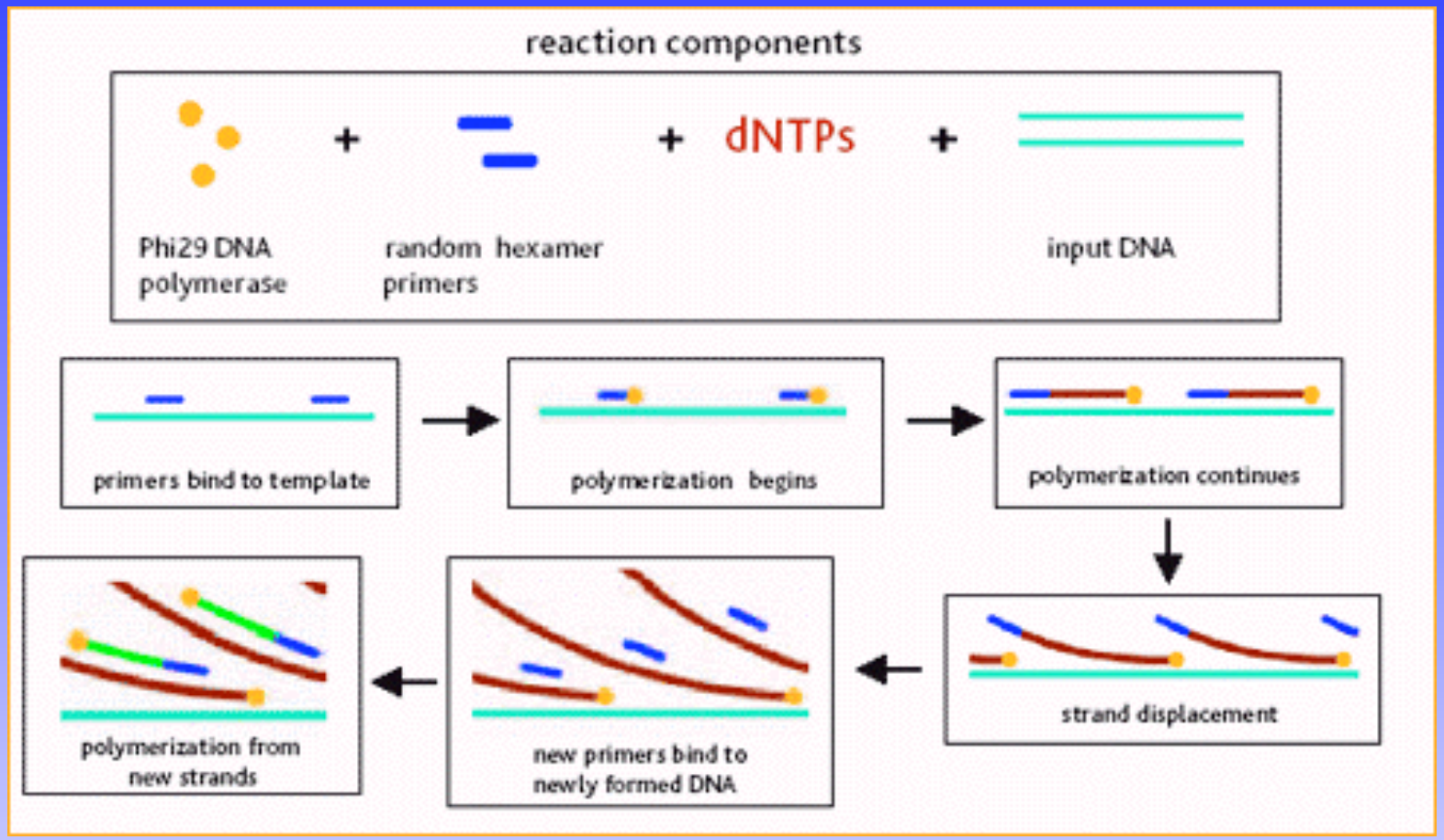
Sorting for activity and identification



Single-cell genomics
High nucleic acid single bacteria sorted

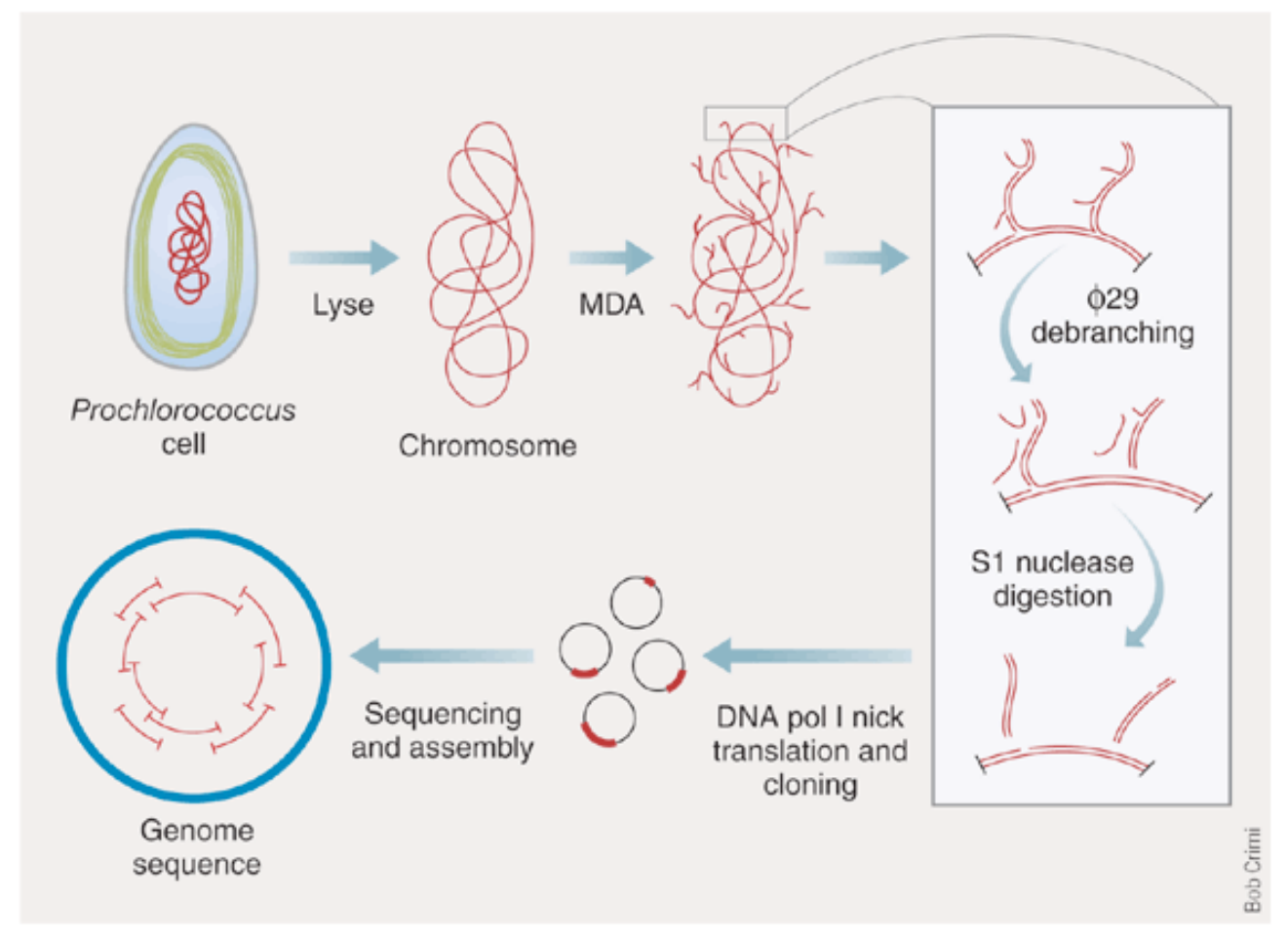


Multiple Displacement Amplification (MDA)



Phi-29 polymerase - just keeps on going….

MDA -> Whole genome sequencing



Hutchison CA, Venter JC (2006) Nat Biotechnol 24:657-658

Matching phylogeny and metabolism in the uncultured marine bacteria, one cell at a time

Ramunas Stepanauskas* and **Michael E. Sieracki**

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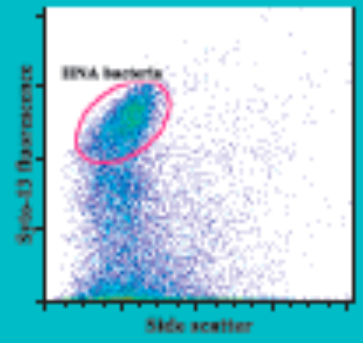
Sample collection

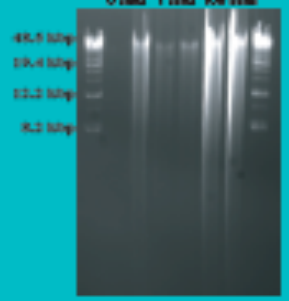
Coastal water sample was collected from Boothbay Harbor, Maine, on March 28, 2006.

Cell sorting



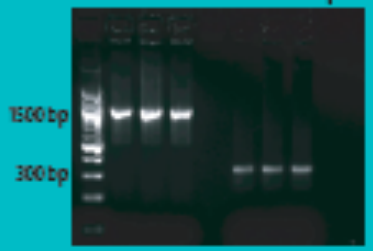
Following staining with SYTO-13, single cells of high nucleic acid (HNA) bacterioplankton were sorted into 96-well plates using a MoFlo™ (Cytomation) flow cytometer.

Whole genome amplification (WGA)



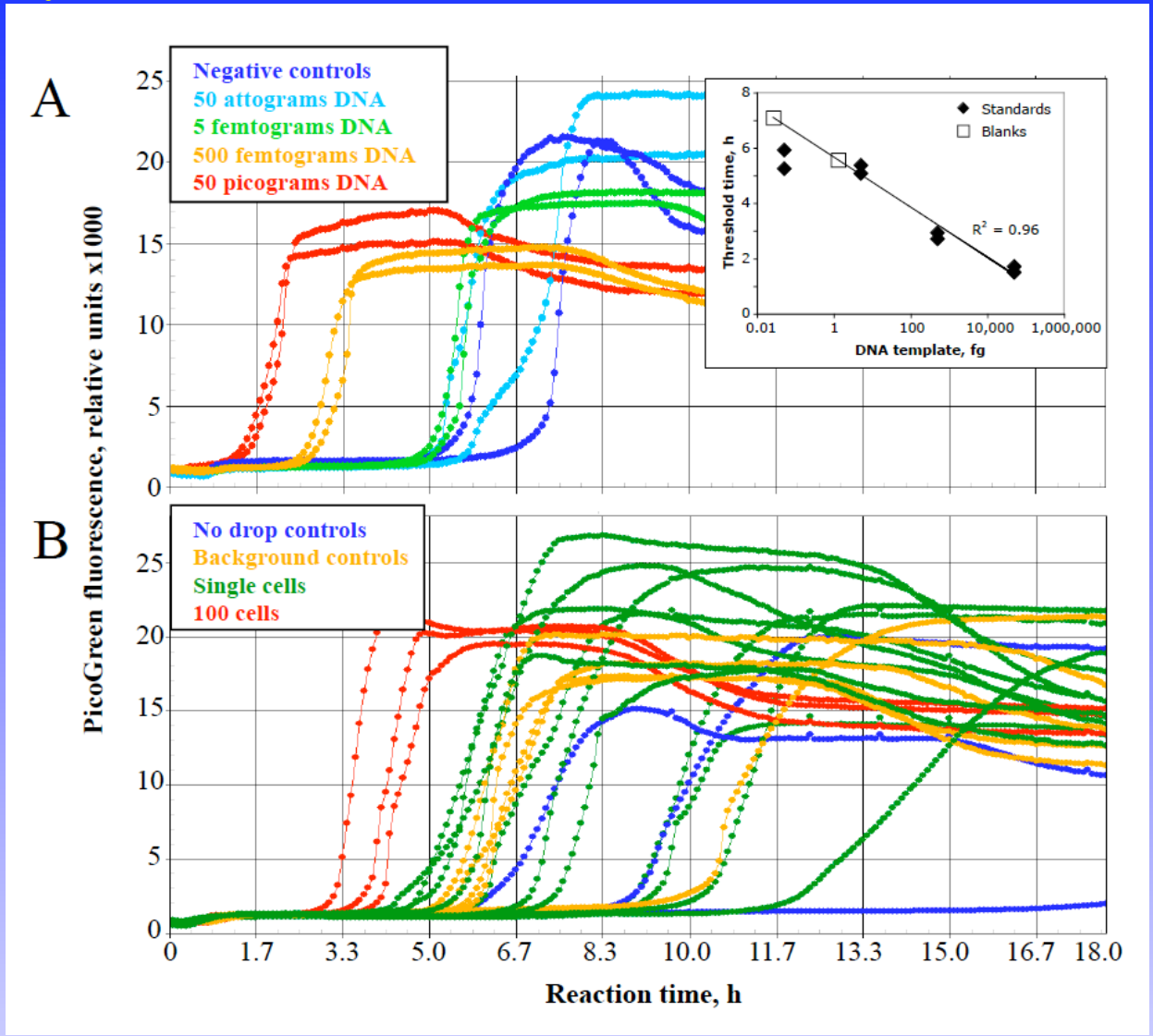
Qiagen REPLI-g Mini and Midi kits were used for cell lysis and phi29-based multiple displacement amplification (MDA) of the genomes.

PCR-screening and sequencing



Products of WGA were used as templates in PCR of ribosomal and protein-encoding genes. Sequencing and terminal restriction fragment length polymorphism (T-RFLP) analyses were performed on these PCR products.

qMDA: standards and controls



Stepanauskas R, Sieracki ME (2007) Matching phylogeny and metabolism in the uncultured marine bacteria; one cell at a time. PNAS 104:9052-9057

Our library of 11 bacterial Single Amplified Genomes (SAGs)

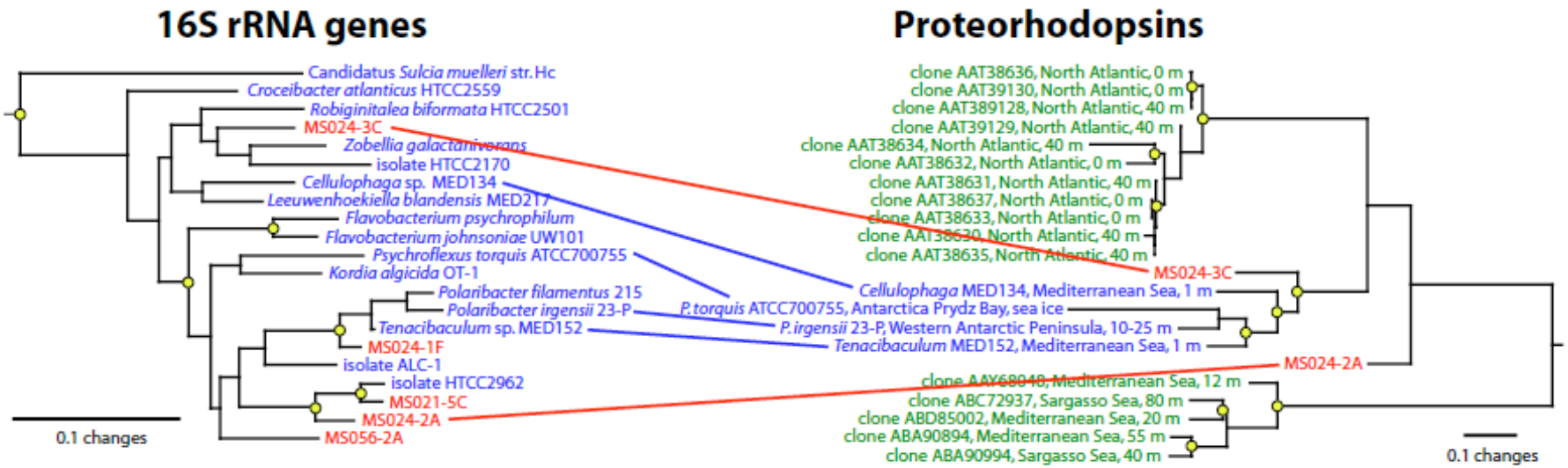
SAG ID	Closest isolate in RDP, % identity	Closest sequence in GenBank, % identity
Flavobacteria/Flavobacteriaceae		
MS021-5C	Flavobacterium sp. 3034, 91%	clone NorSea37, 96%
MS024-2A	Flavobacterium sp. 3034, 91%	clone NorSea43, 99%
MS024-3C	*Cellulophaga* sp. CC12, 93%	clone 1D10, 99%
MS024-1F	Sponge bacterium Zo9, 97%	clone WLB13-197 , 96%
MS056-2A	*Ulvibacter litoralis*, 95%	clone PB1.23, 99%
Sphingobacteria/Saprospiraceae		
MS190-1F	Saprospiraceae bact. MS-Wolf2-H, 88%	clone SanDiego3-A7, 100%
Alphaproteobacteria/Rhodobacteraceae		
MS056-3A	*Roseobacter* sp., 99%	clone F3C24, 100%
MS024-1C	*Ophiopholis aculeata* symbiont, 99%	clone EB080-L11F12, 100%
MS190-2A	*Ophiopholis aculeata* symbiont, 99%	clone EB080-L11F12, 100%
MS190-2F	*Octadecabacter orientus* KOPRI 13313, 97%	Rhodobacteraceae bact. 183, 99%
Gammaproteobacteria/Oceanospirillaceae		
MS024-3A	Isolate HTCC2120, 90%	clone Ant4D3, 99%
Gammaproteobacteria/Comamonadaceae		
MS024-2C	*Delftia acidovorans*, 99%	*Delftia acidovorans*, 99%

Bacterial 16S rRNA genes were succesfully PCR-amplified and sequenced from twelve SAGs. Of them, five were flavobacteria, one sphingobacterium, four alphaproteobacteria, and two gammaproteobacteria. Ribosomal genes of all representatives of Bacteroidetes and one gammaproteobacterium were distant from existing isolates. One of the gammaproteobacteria, *Delftia acidovorans,* was a likely contaminant. The success rate of single cell MDA-PCR ranged 10-50%, depending on cell lysis and MDA protocols.

PCR primers used for screening

Gene	Primers	Product, bp	References
Bacterial SSU rRNA	27F, 519F, 907R, 1492R	various	(41, 42)
Archaeal SSU rRNA	S-D-Arch-0344-a-S-20, 907R	550	(43, 44)
Eukaryote SSU rRNA	EUK328f, EUK329r	1500	(45, 46)
Proteorhodopsin	o-PR2, o-PR3	330	(32, 34)
Bacteriochlorophyll, *pufM*	pufM_228F, pufM_228R	228	(47)
Nitrogenase, *nifH*	nifUP, nifDN, NifH3, NifH4	450	(23, 48)
Assimilatory nitrate reductase, *nasA*	nas22, Nas1933, nas964, nasA1735	771	(24, 49)

Comparison of trees



Two Flavobacterium with proteorhodopsin genes.

These are being whole genome sequenced by JGI.

There may be a PCR bias against Flavobacteria (Kirchman, et al. 2000)

Workshop:
Single Cell Alternatives to Metagenomics in Environmental Microbiology

Financial support: the A.P. Sloan Foundation

Location: Spruce Point Inn, Boothbay Harbor, Maine

Time: 9 – 11 Sept 2007, hands-on 12-14 Sept

The workshop is limited to 40 participants.

The Hands-On Section is pending additional funding and is limited to 10 participants

Workshop Topics

Current environmental genomics – the context for single cell approach

Technical aspects of single cell genomics:

- Separation and lysis of single cells
- Single cell whole genome amplification
- Sequencing and assembly of single cell genomes
- Integration of single cell, isolate, and community genomic data

Science questions for the single cell genomics:

- Exploring global microbial diversity
- Examining ecological roles of the uncultured microorganisms
- Studying microbial evolution at organismal level
- Bio-prospecting and industrial applications of the uncultured microorganisms
- Environmental viral genomics

OPTIONAL HANDS-ON SECTION

- Fluorescence-activated cell sorting
- Cell lysis, whole genome amplification, and PCR-screening
- Bioinformatics