Microbes and flow cytometry:

from enumeration to community structure, diversity and ecosystem function

Josep M Gasol

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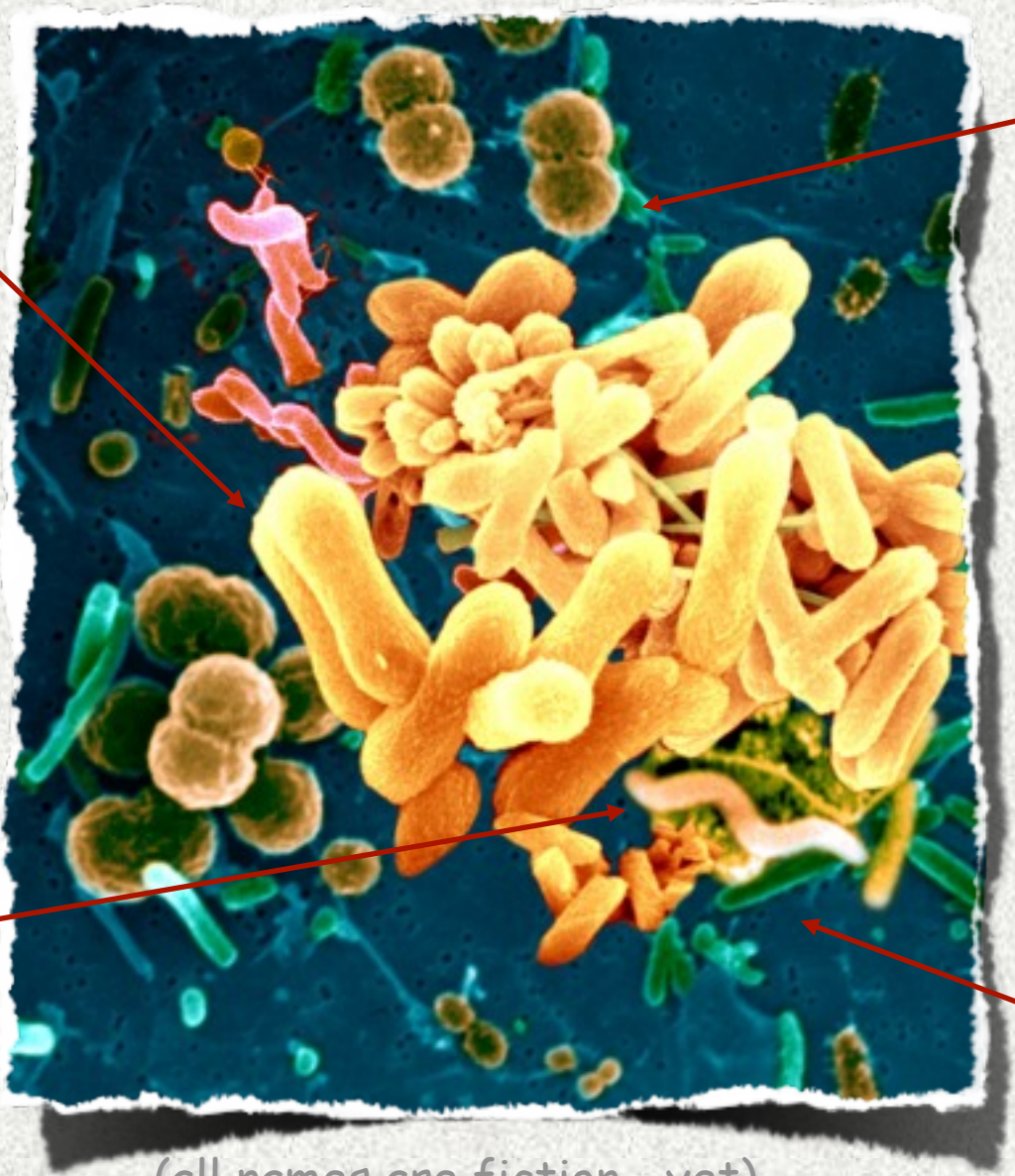
Our ultimate goal



Dalibacter banyuleus

preferentially grazed by HNF

very sensitive to viral attack

Spirovibrio kalmariensis

Roundicoccus plymouthii

In summer, 75% of BCD

dominates DMSP uptake

Tinymonas bremenensis

(all names are fiction… yet)

Microbes and flow cytometry (bias to heterotrophs)

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4) Measuring Bacterial activity and physiological status
5) Where are we? A personal view of our achievements (and lack of)
6) Going further: cytometric diversity
7) Going further: Probing ecosystem function
   Relating community structure to ecosystem functioning

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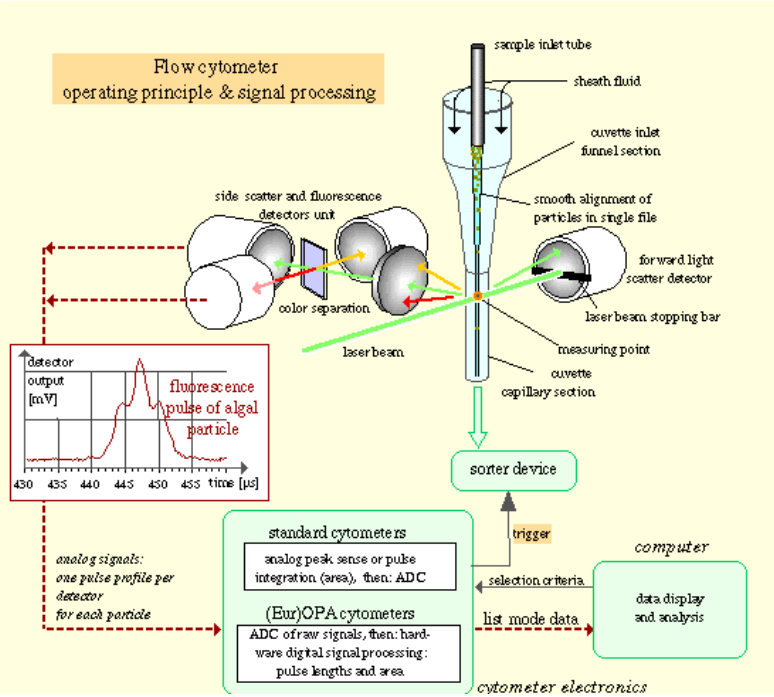
Flow Cytometry

- Measurement of individual cells
  (a fluidics system forces them to pass one at a time)
- It can measure:
  - Scattered light
    - FSC (FALS): light scattered at angles < 10°
    - SSC (RALS): light scattered at 90°
  - Fluorescence
    - after excitation by 350 nm (UV), 488 nm (Blue), 630 (red)...
- Up to 7/8 parameters in thousands of cells per second
- Enumeration /community structure / sorting
- Advantages
  - (Many) Individual cells
  - Better statistics
  - Supopulations can be identified
  - Cells can be sorted
- Disadvantages
  - Cells must be isolated
  - Limited information on structure
  - < 70 µm
  - ≤ 800 particles ml^{-1}



- High-speed cell sorters
  - Coulter EPICS, FACSVantage
  - FACSAria - MoFlo - Influx
- Medium-size cytometers
  - FacsCalibur, Coulter XL
  - FACSCanto, FACSVerse
- Portable cytometers
  - Guava, Apogee, Partec, Miltenyi
  - Accuri
- In situ - continuously monitoring FC
  - FlowCytobuoy, Flowcytobot
  - SeaFlow

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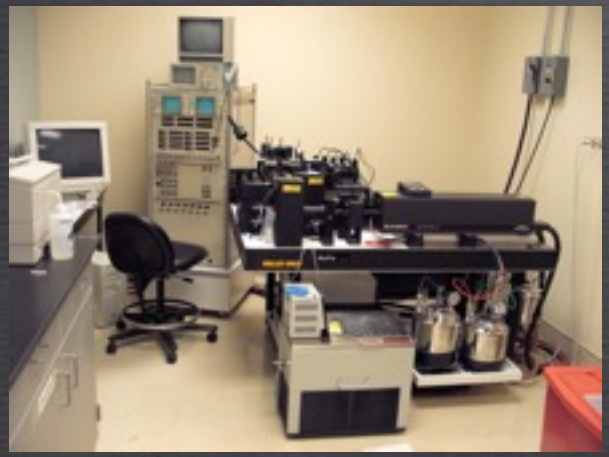

Thanks google for the images...

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  FacsCalibur, Coulter XL
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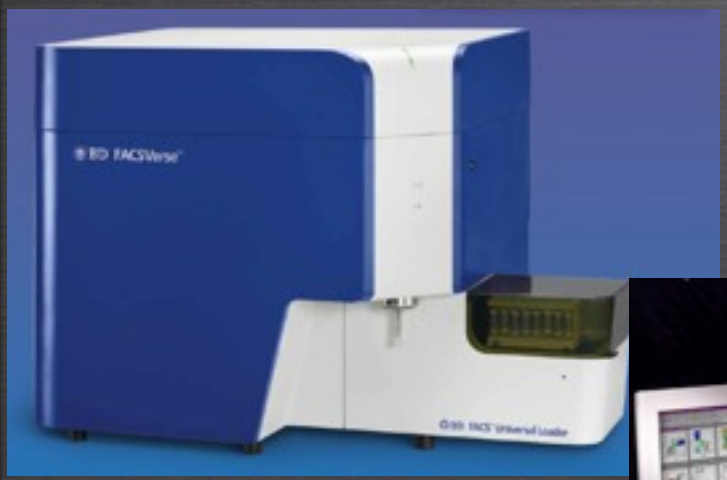

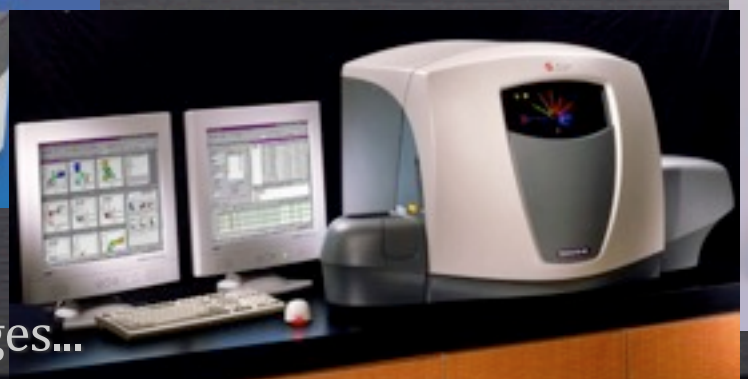

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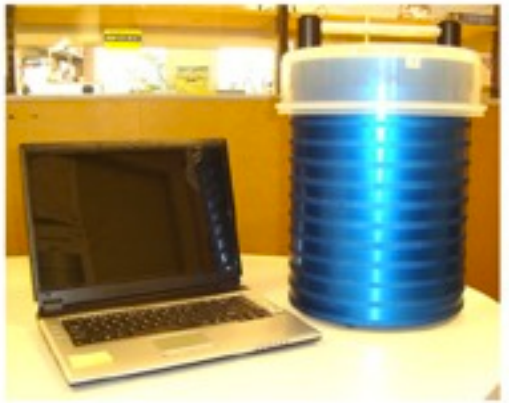
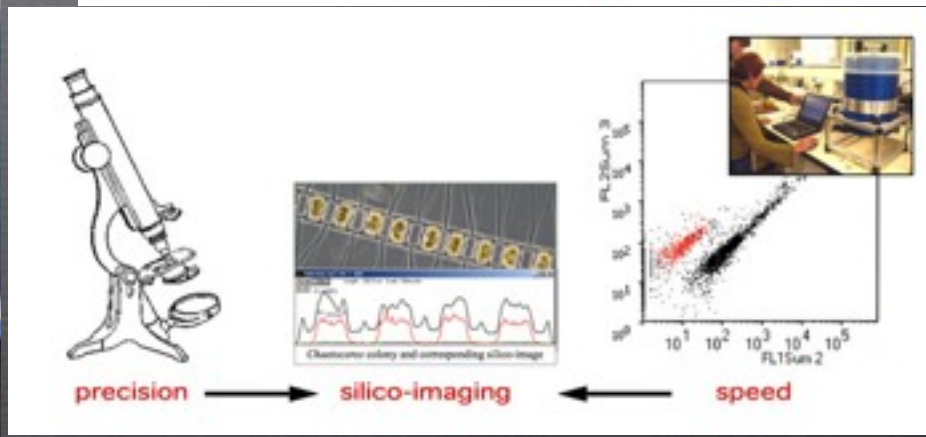




LIMNOLOGY
and
OCEANOGRAPH

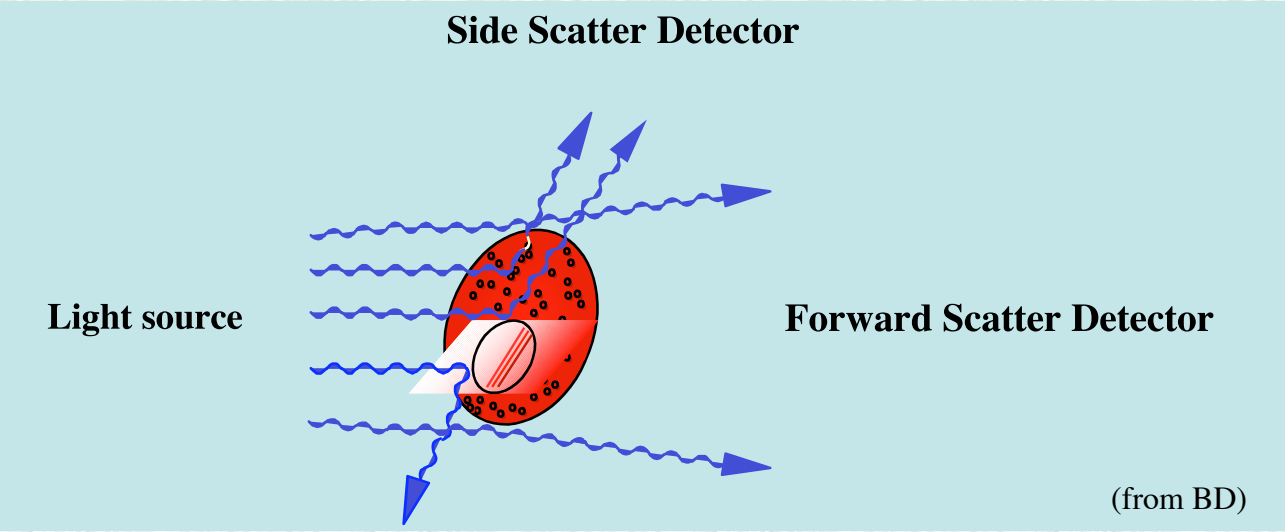
SeaFlow: A novel underway flow-cytometer for continuous observations of phytoplankton in the ocean

*Jarred E Swalwell, Francois Ribalet, E. Virginia Armbrust**
School of Oceanography, University of Washington, Box 357940, Seattle, Washington, 98195, USA

Thanks google for the images...

Flow Cytometry-1

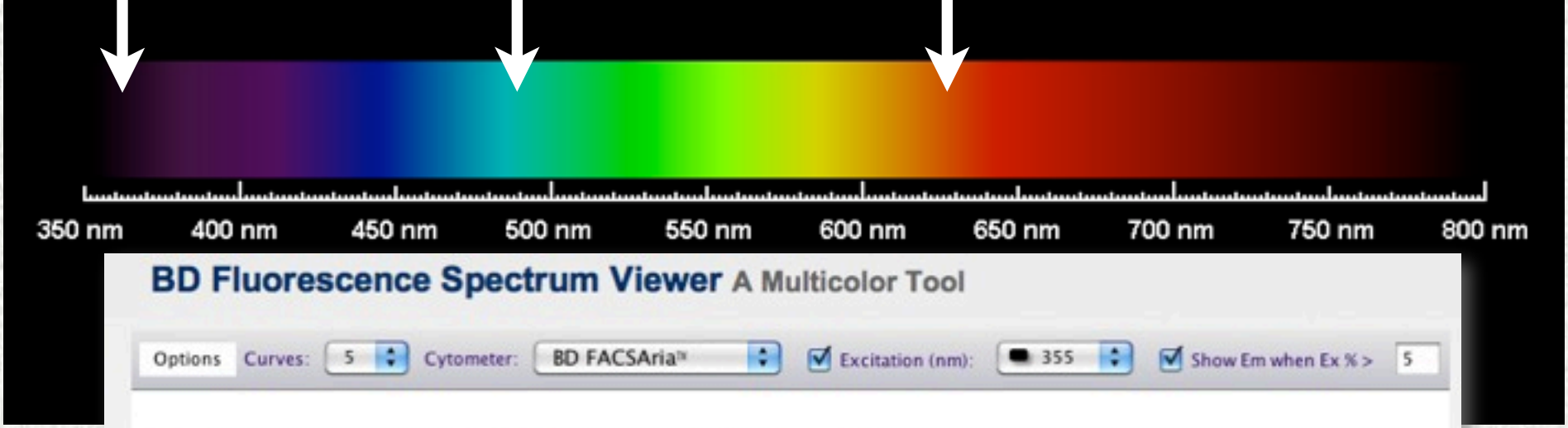
- Cytometry = Cyto (=cell) Metry (=measurement)
- Light scattered in two angles



images from BD

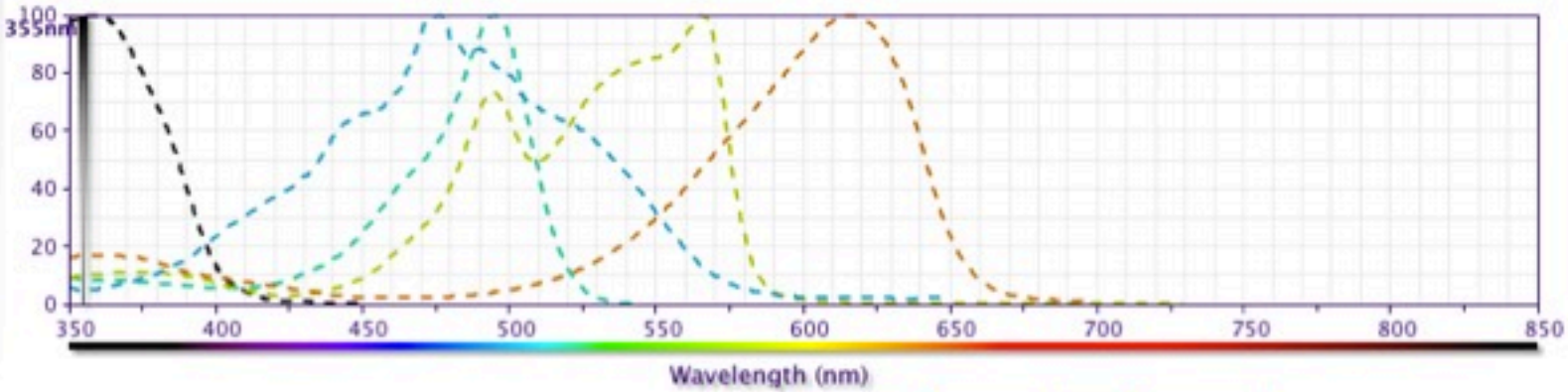
- Fluorescence: A fluorophore (fluorescent molecule) has the property of absorbing light energy (excitation) and to restore it quickly (< 1 ns) as fluorescent light (emission). The wavelength of the emission must be longer (less energetic) than that of the excitation light (this is called the Stoke's law)

- Characteristics of fluorochromes: affinity for target, excitation and emmision peaks, extinction coefficient, and photobleaching.



BD Fluorescence Spectrum Viewer A Multicolor Tool

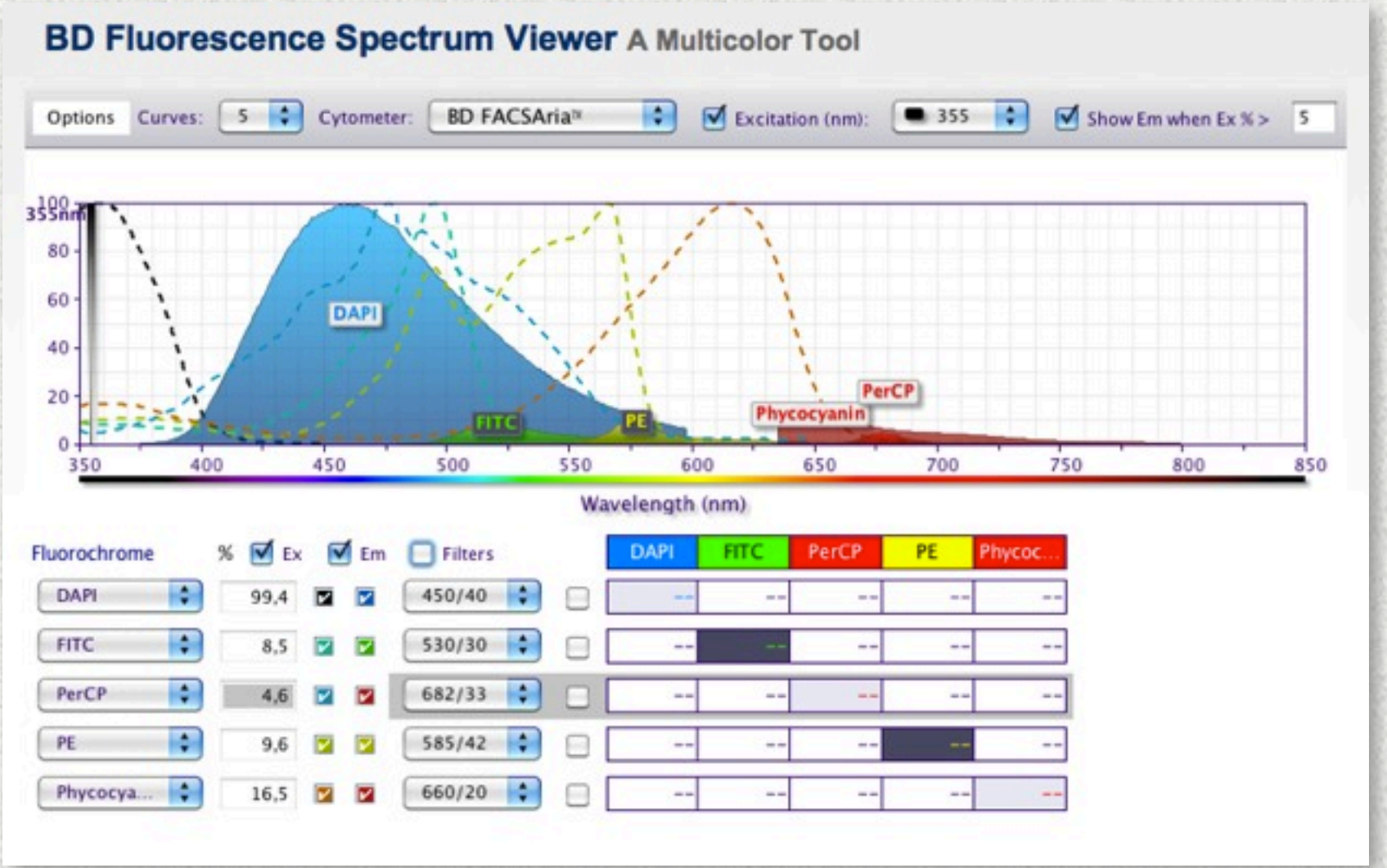
Options Curves: 5 Cytometer: BD FACSAria™ Excitation (nm): 355 Show Em when Ex % > 5



Fluorochrome	%	Ex	Em	Filters	DAPI	FITC	PerCP	PE	Phycoc...
DAPI	99,4	☑	■	450/40	--	--	--	--	--
FITC	8,5	☑	■	530/30	--	--	--	--	--
PerCP	4,6	☑	■	682/33	--	--	--	--	--
PE	9,6	☑	■	585/42	--	--	--	--	--
Phycocya...	16,5	☑	■	660/20	--	--	--	--	--

CHL

http://www.bdbiosciences.com/research/multicolor/spectrum_viewer/index.jsp



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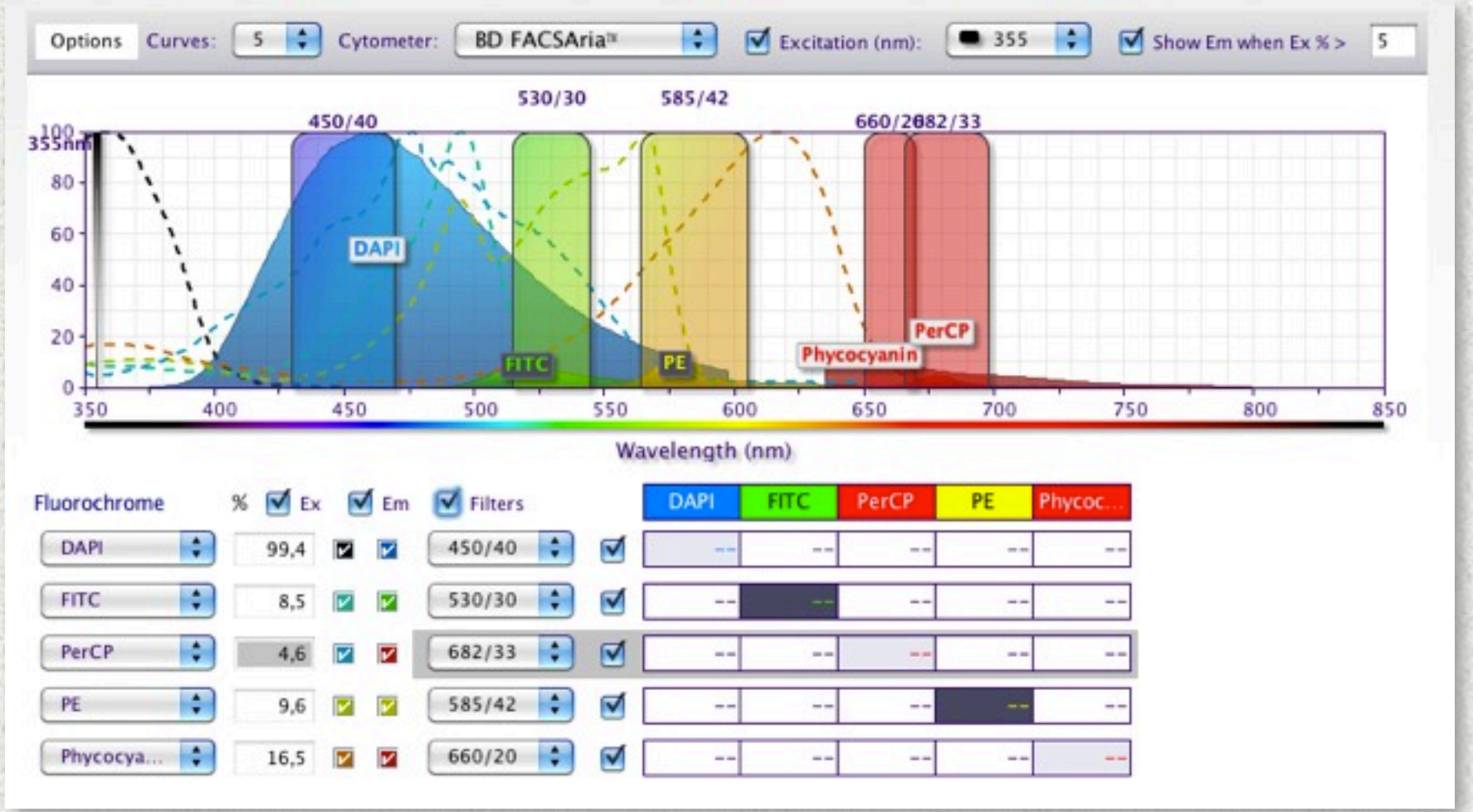

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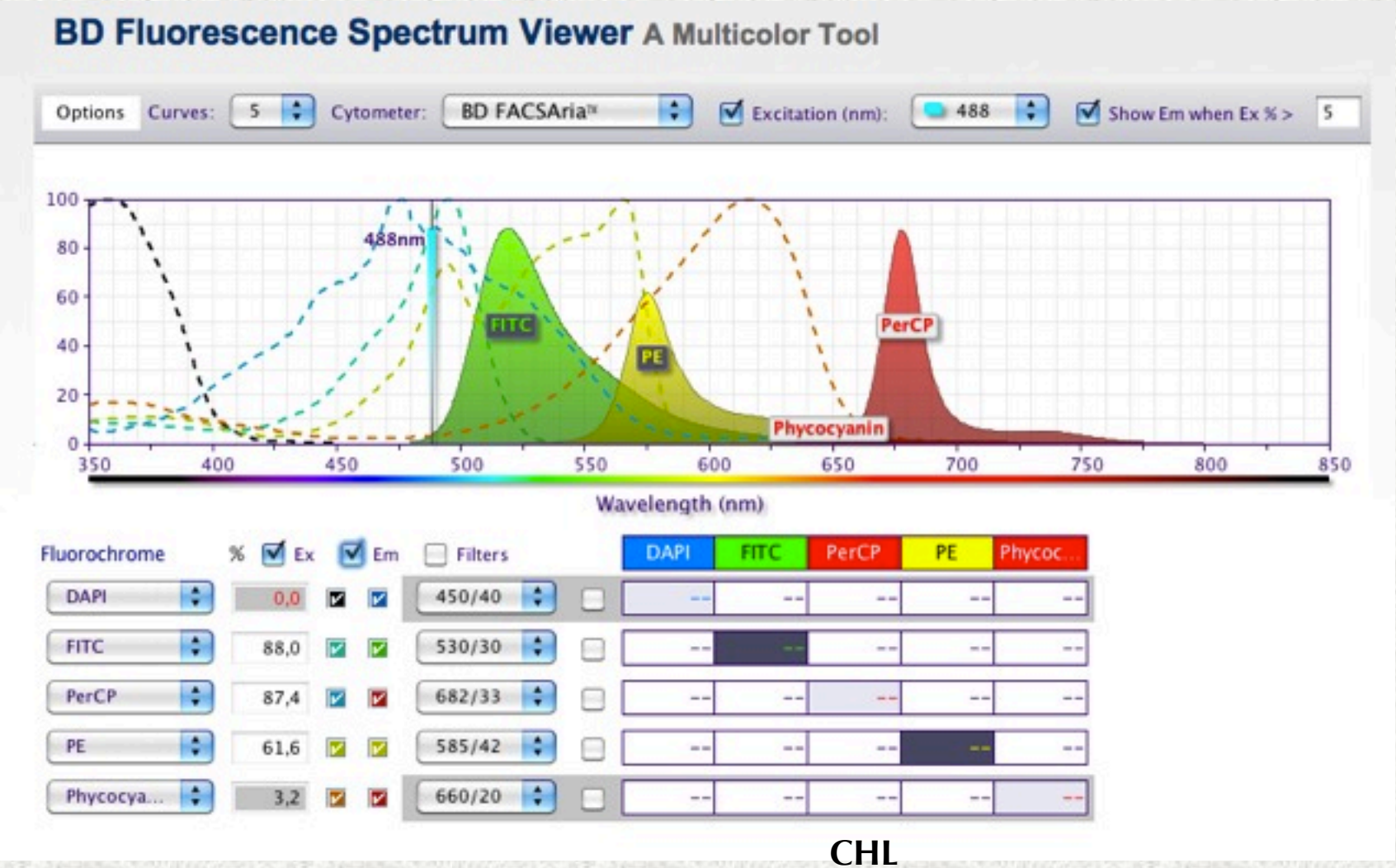

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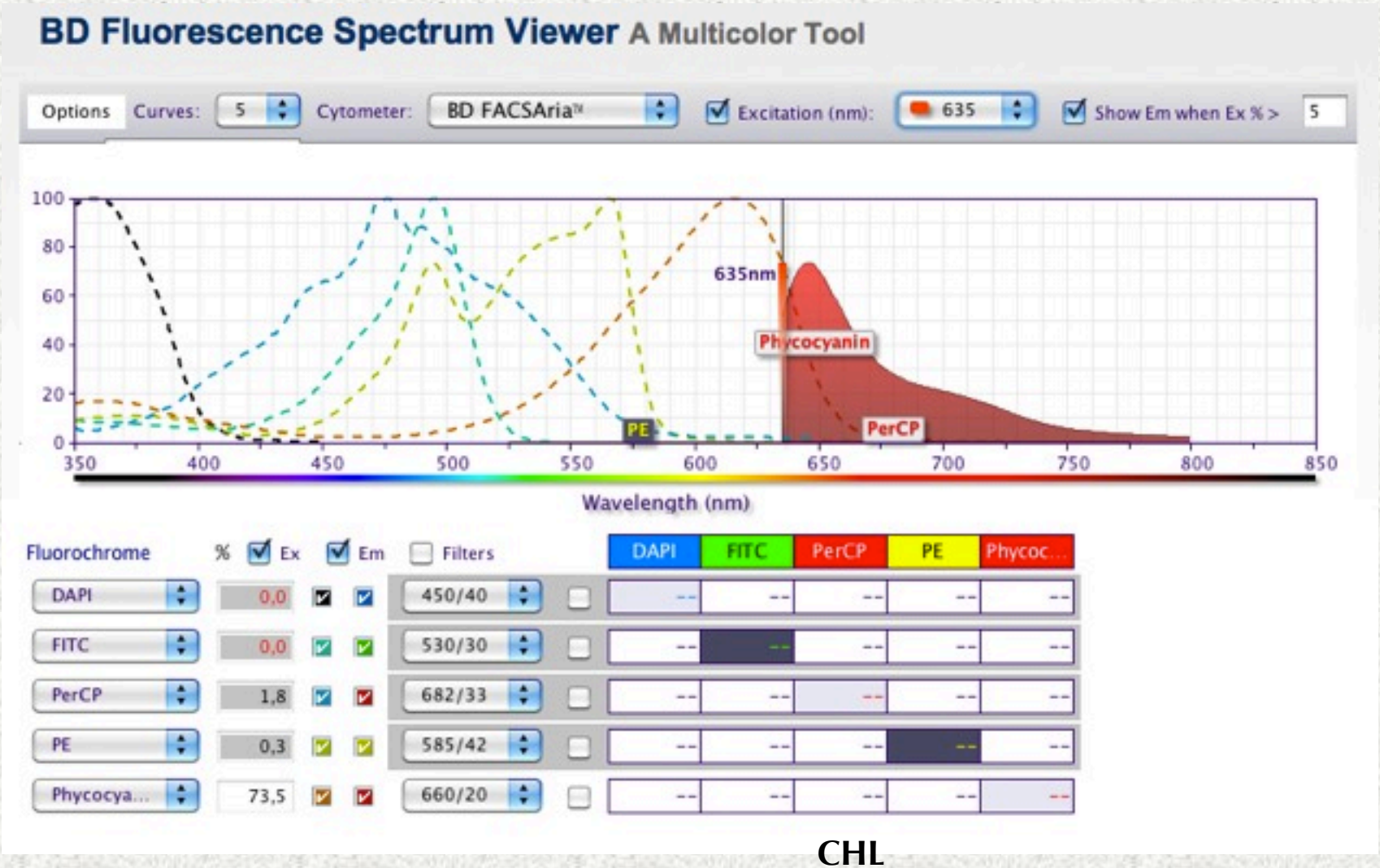

CHL

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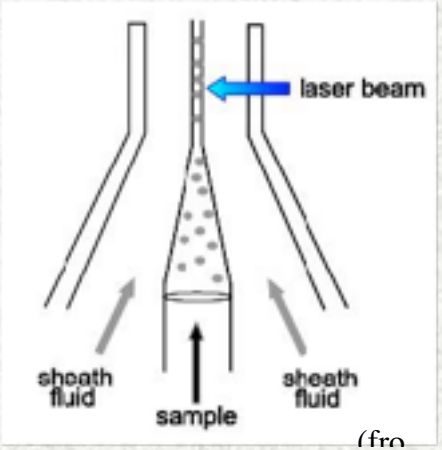
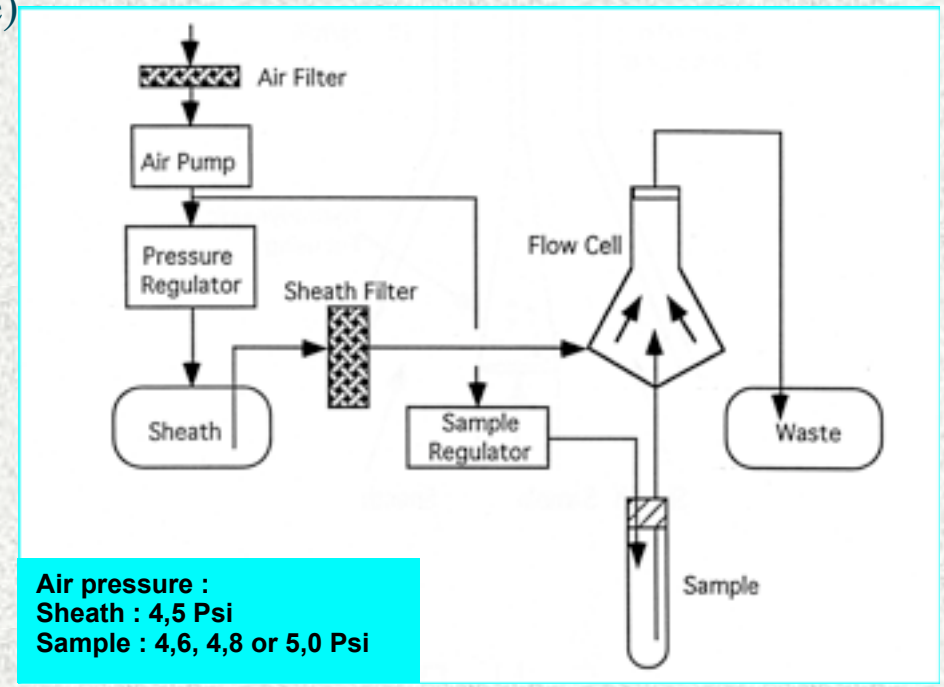
Flow Cytometry-2

- FC is based on three/four elements:
  - *Fluidics*: introduction and positioning of the cells
  - *Optics*: production of the signal and collection on PMT
  - *Electronics*: transformation of the photon signals into electronic signals proportional to the intensity of the light. Amplification and digitalization of signals
  - *Sorting*: After-processing cell separation

- FLUIDICS
- differential pressure (regulates flow rate)
- laminar flow
- hydrodynamic focusing
- sheath fluid





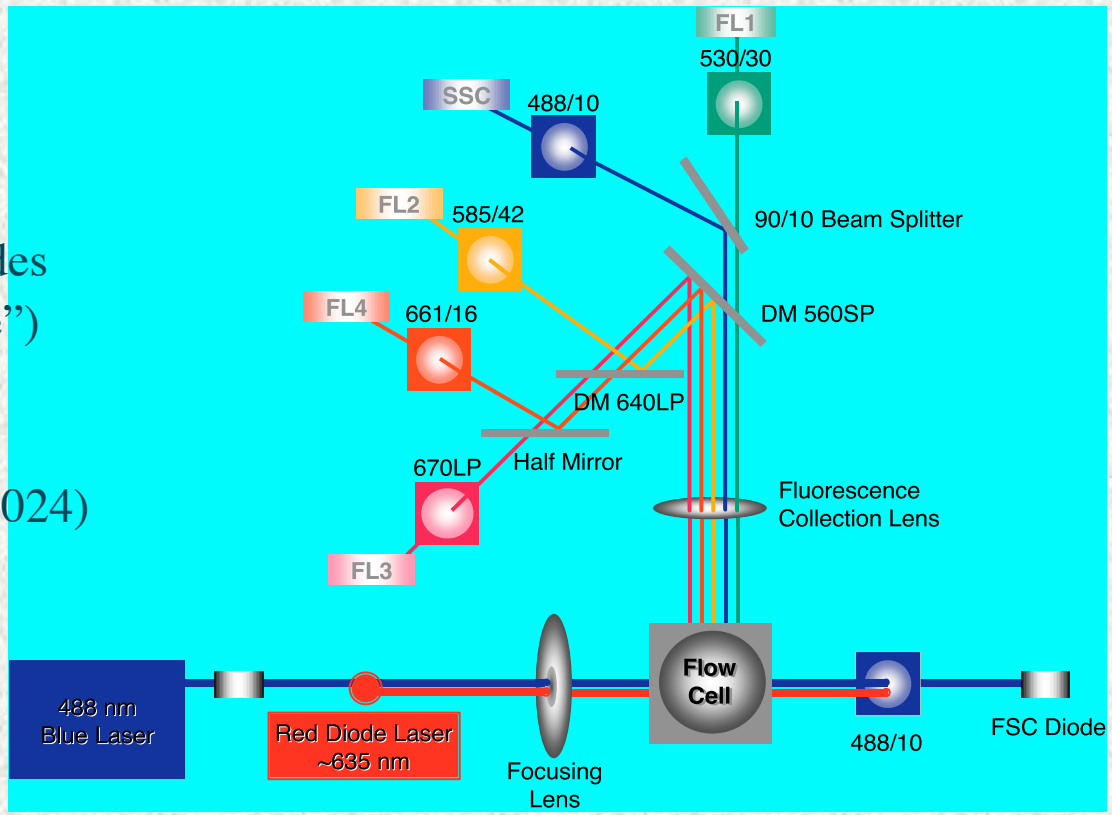
Air pressure :
Sheath : 4,5 Psi
Sample : 4,6, 4,8 or 5,0 Psi

images from BD

Flow Cytometry-3

- OPTICS
- laser(s): Argon, He-Ne, He-Cd, Kr // LEDs // Arc-Lamp (Mercury-Xenon)
  Typically, UV (355 nm), Blue (488 nm), Red (655 nm)
- filters: Longpass / Bandpass / Shortpass
- lenses/prisms/dichroic mirrors.

- ELECTRONICS
- photomultipliers or photodiodes
- signal amplification ("voltage")
- signal thresholding
- signal processing
- classification into channels (1024)
- computer-based processing



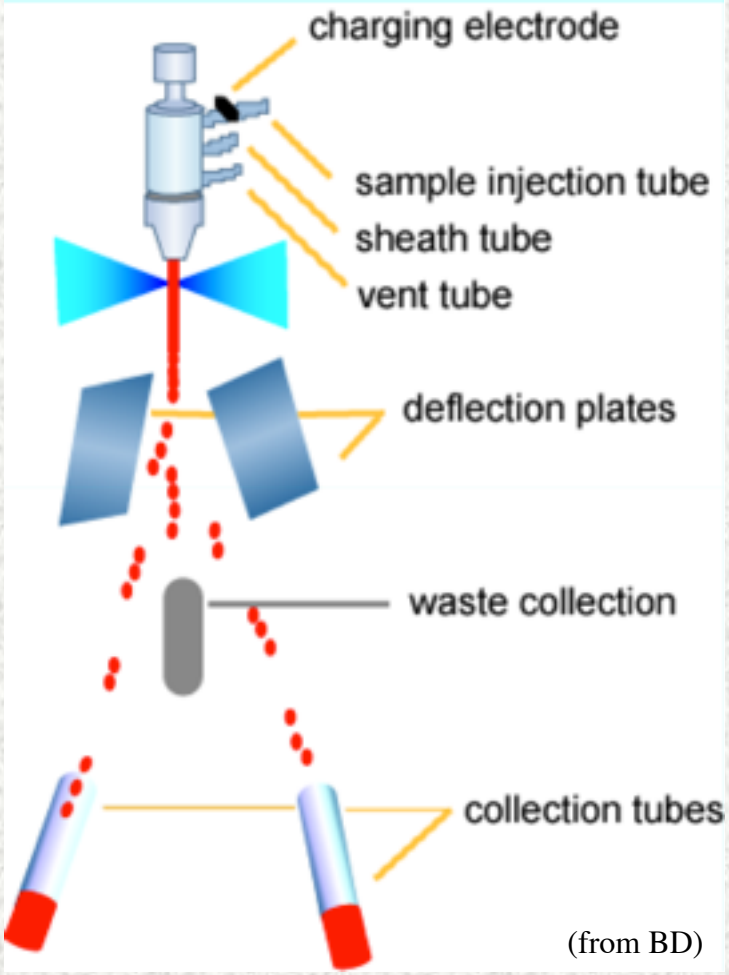
images from BD

Flow Cytometry-4

- SORTING

- Mechanical (in a flow cell) or electrostatic ("stream in air")
- stream in air: a vibrating nozzle creates spaced droplets which are then electrically charged

Mechanical	Electrostatical
Low Speed (300 s^{-1})	High-speed (>10000 s^{-1})
Flow cell	Stream in air (nozzle)
Laser fixed and aligned	Needs laser alignement
Mehanical sort	Electrostatic sort
Shaeth can vary	Saline sheath
Sort in one way	Sort in two ways
No aerosol	Creates aerosols
Highly-diluted sort	(almost) Undiluted sorting
Choice for radioact samples	Choice for molec. studies



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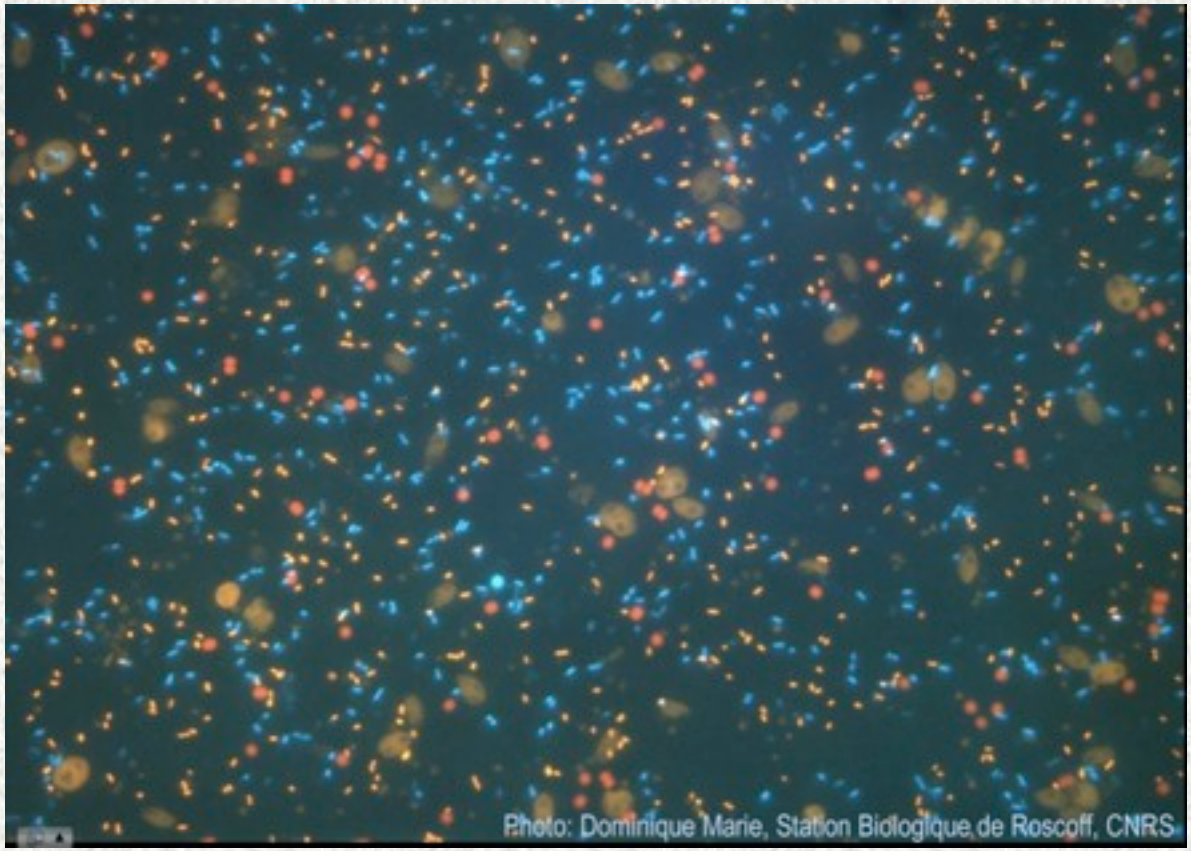
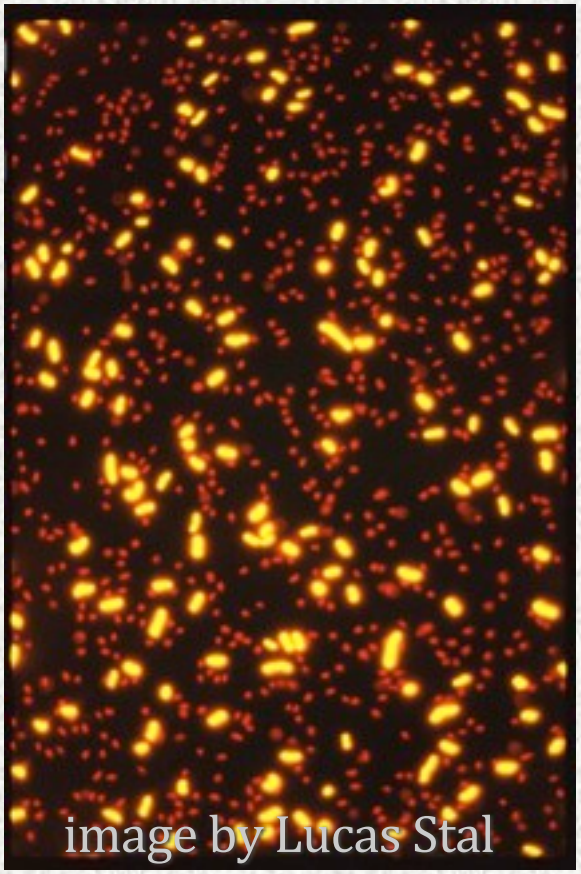
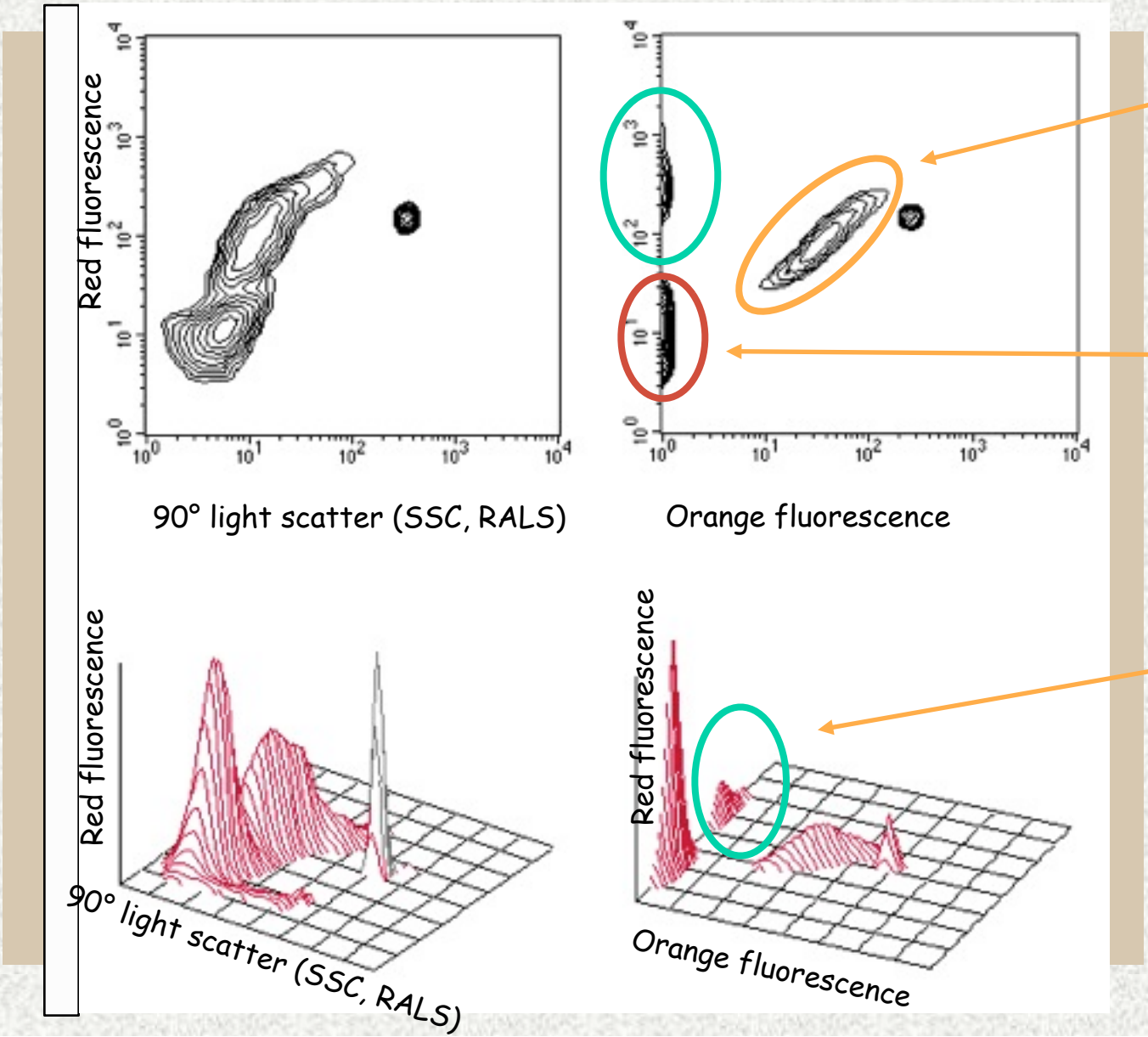
Size and (auto)fluorescence

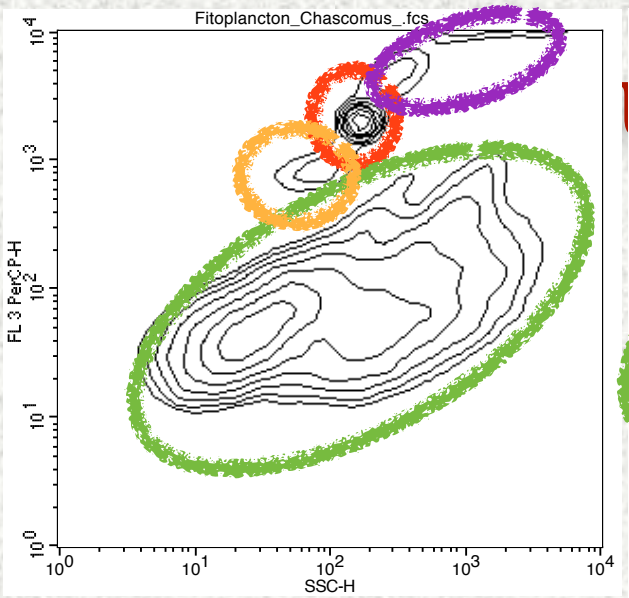
- Autofluorescence: natural emission of light by certain molecules after they have absorbed (excitation) light
- CHL, BCHLs, Phycobilins (Phycocyanin, phycoeritrin, allophycocyanine) have autofluorescence
- Carotenoids do not have it. SO not all pigments have fluorescence.



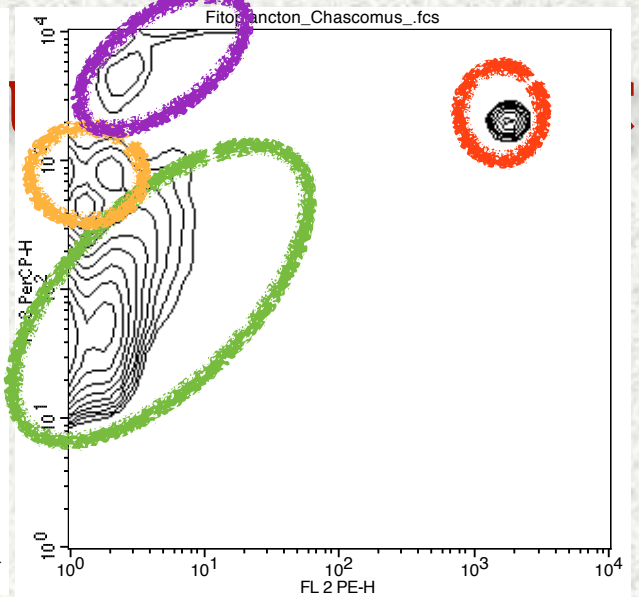


Size and (auto)fluorescence

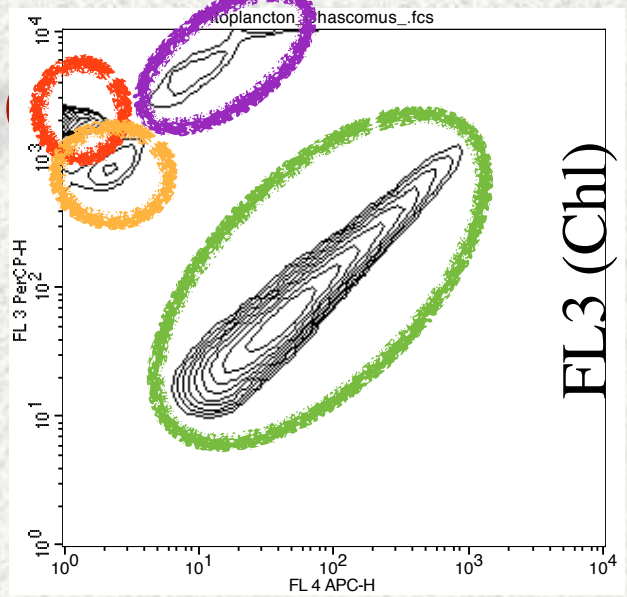




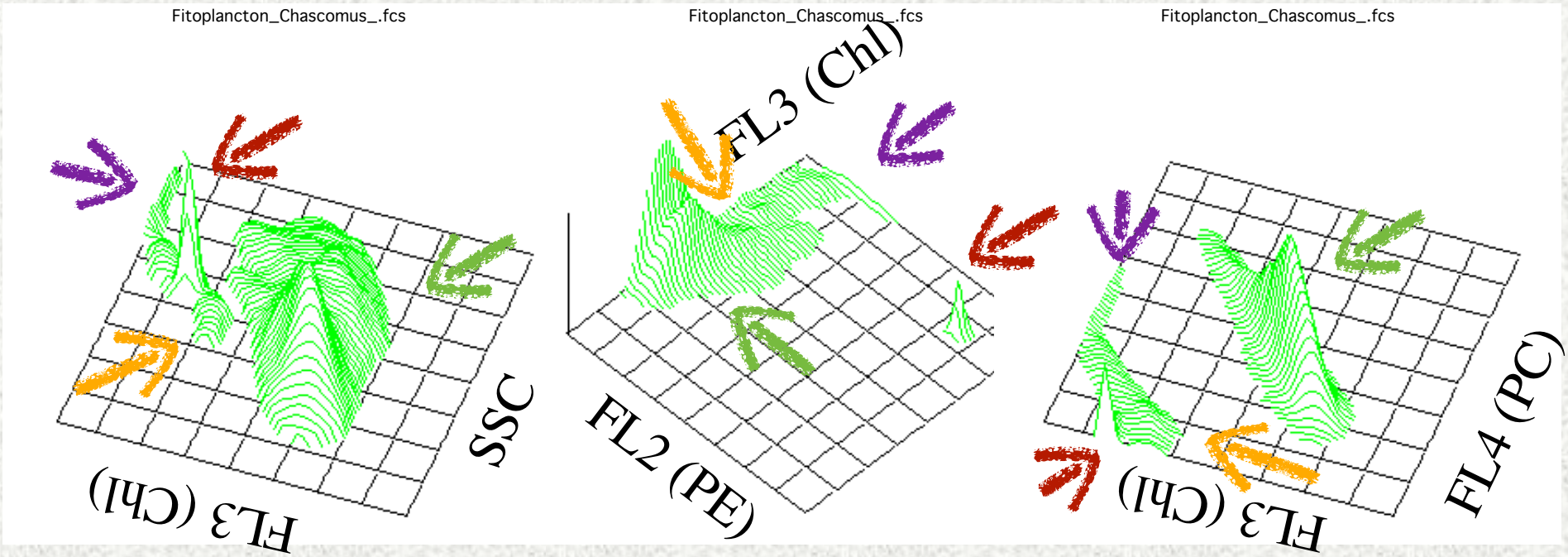
SSC



FL2 (PE)



FL4 (PC)



Size and (auto)fluorescence

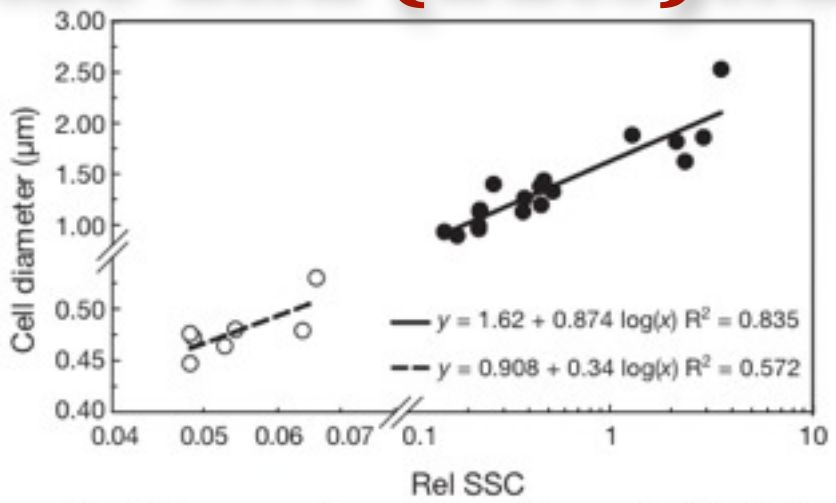
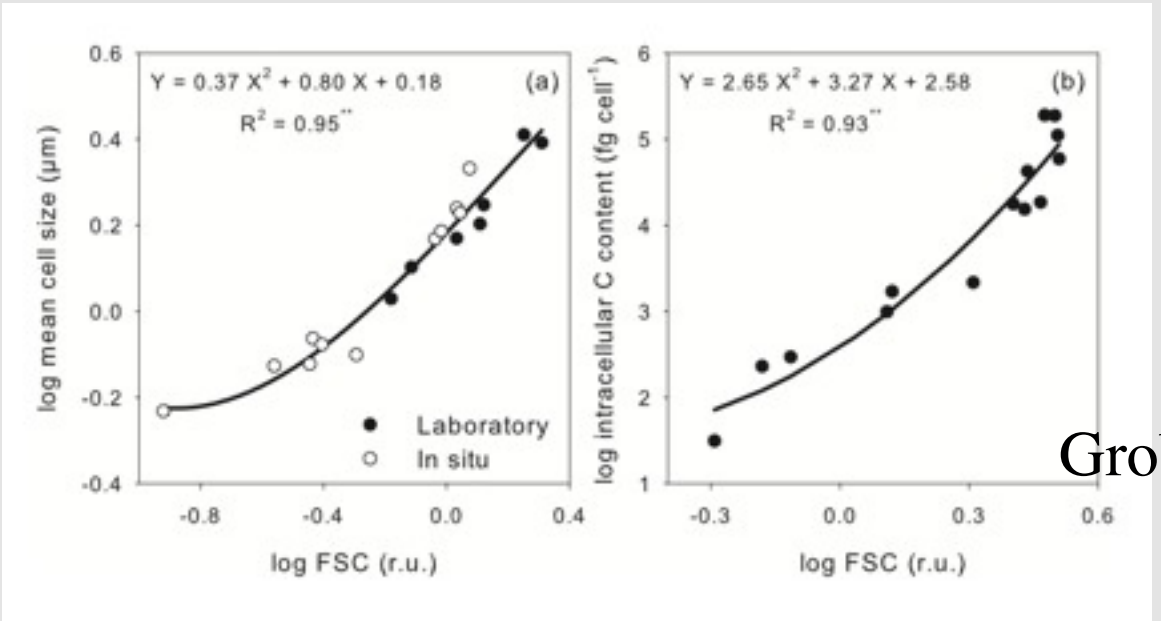


Fig. 1. Linear regression models used to convert relative light side scatter (SSC) data to cell diameter of picophytoplankton (●) and heterotrophic bacteria (○)

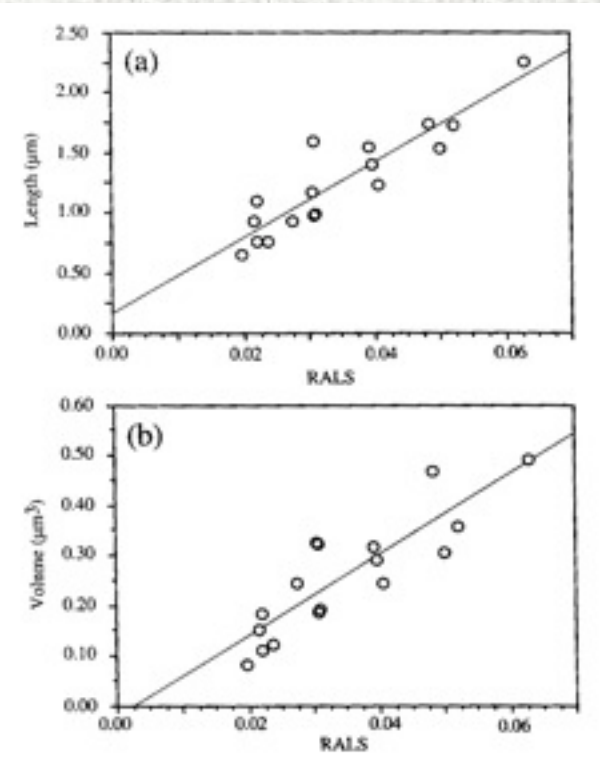
Calvo-Diaz & Morán 2006, AME

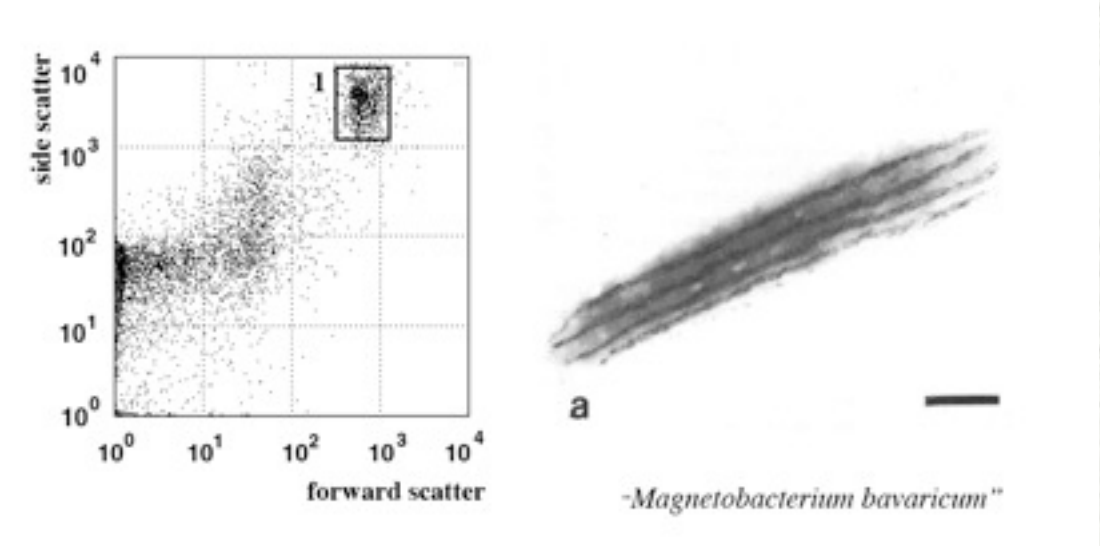


Grob et al. 2007, BGS

Size and (auto)fluorescence

- Sulfur — Casamayor et al. 2007-ENM
- PHB — Srienc et al. 1984-Biotecnhol. Bioeng.
- Magnetosomes — Wallner et al. 1997-AEM
- Vacuoles — Dubelaar et al. 1987-Cytometry
- Differentiate bacteria — Allman et al. 1993-in Lloyds' book
- Size bacteria — Troussellier et al. 1999-FEMS ME





Wallner et al. 1997-AEM

Troussellier et al. 1999-FEMS ME

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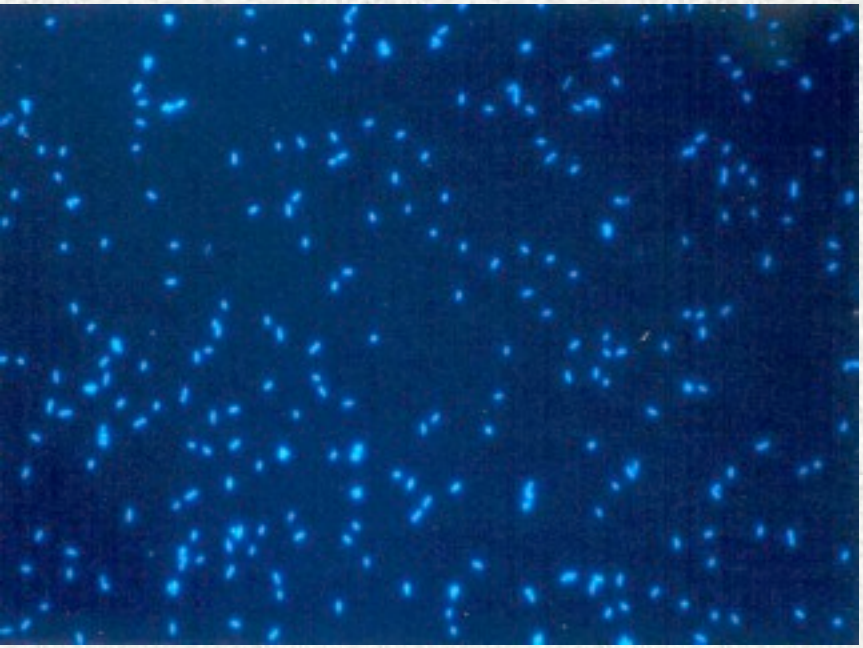
DNA (and RNA) fluorescence



The standard...
At least until 1995

Variability in DAPI counting

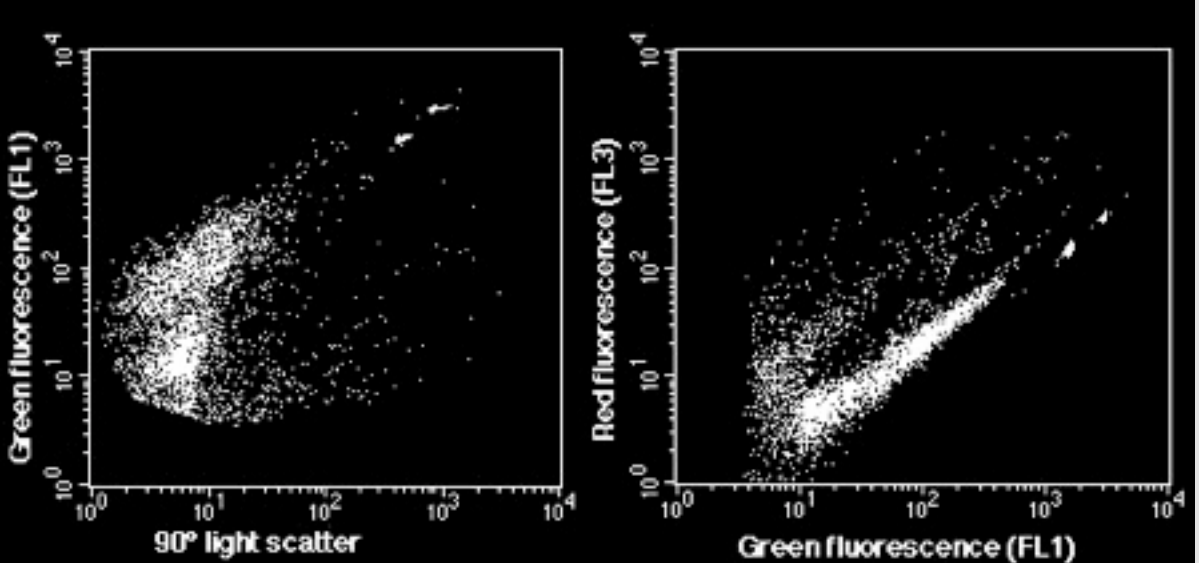
Site	BA (ml^{-1})	CV
Eutrophic reservoir	$1.68\ 10^7$	20 %
Med. coast-1	$3.63\ 10^5$	15 %
Med. coast-2	$2.56\ 10^5$	5.3 %
Mesocosm Exp.	$1.03\ 10^6$	8.2 %
Aged seawater	$1.02\ 10^5$	17 %

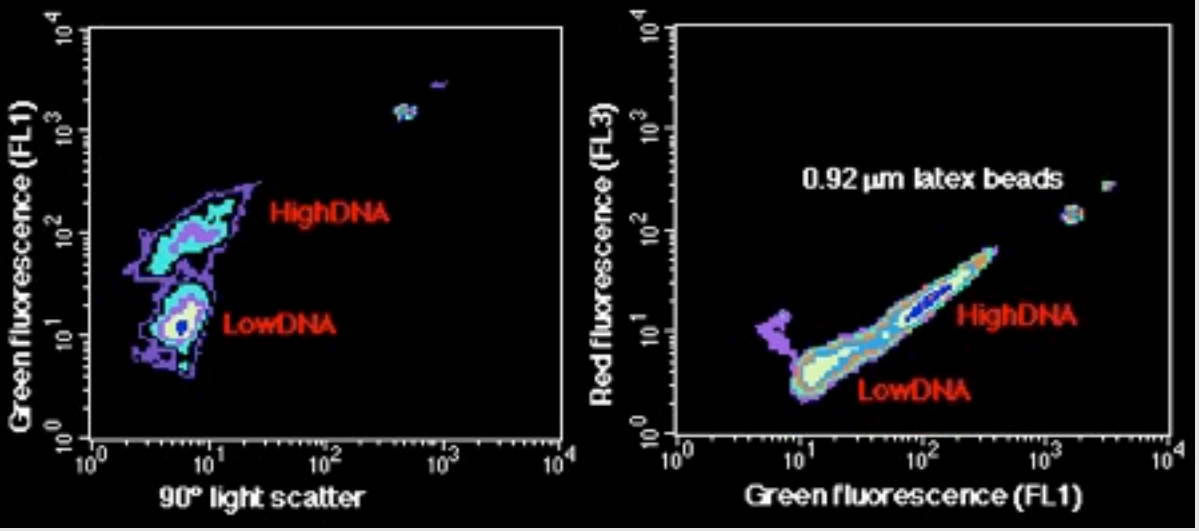
TABLE 1. – Some characteristics of the stains that have been employed to detect bacteria with flow cytometry (dye characteristics adapted from Haugland 1996 and Davey and Kell 1996).

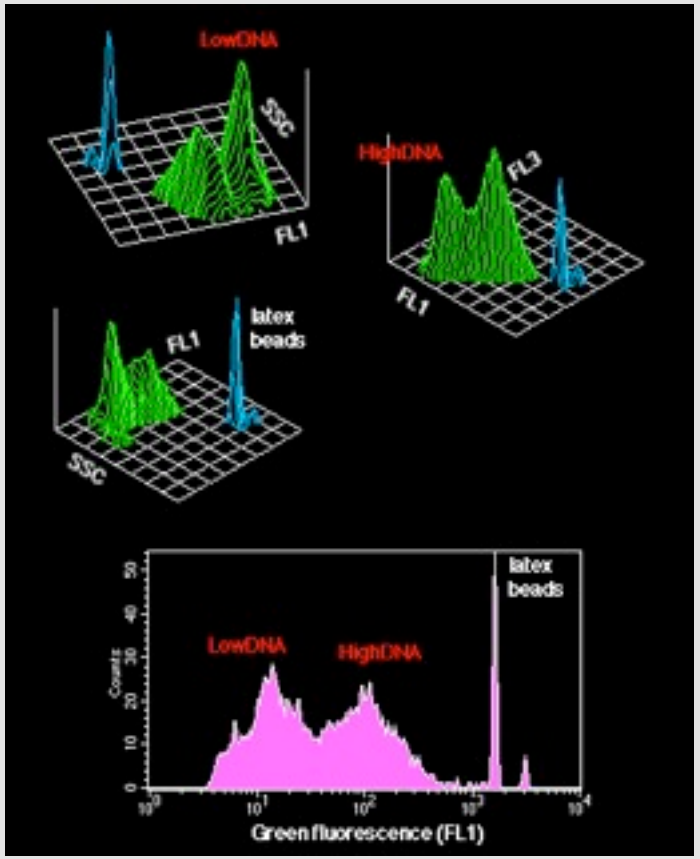
Stain	Binds to:	Exc. / Em. (nm)	Type of sample	References
Ethidium bromide (EthBr)	DNA and RNA	518 / 605	cultures	Paau *et al.* 1977, Pinder *et al.* 1990
Propidium iodide (PI)	DNA and RNA	535 / 617	cultures	Bailey *et al.* 1977, Hutter and Eipel 1979, Miller and Quarles 1990
			cooling towers	Tyndall *et al.* 1985
Fluorescein isothiocyanate (FITC)	protein	495 / 520	cultures	Bailey *et al.* 1977, Miller and Quarles 1990, Allman *et al.* 1990
Chromomycin A3	DNA (GC)	340 / 470	cultures	Boye *et al.* 1983, van Dilla *et al.* 1983
Acridine orange (AO)	RNA*	460 / 650*	cultures	Nishimura *et al.* 1995
			seawater	Nishimura *et al.* 1995
Mithramycin and EthBr	DNA and RNA	425 / 550	cultures	Boye *et al.* 1983, Thorsen *et al.* 1992, Allman *et al.* 1992, Steen *et al.* 1994
DAPI	DNA	358 / 461	cultures	van Dilla *et al.* 1983
			marine and freshwater	Robertson and Button 1989
HOECHST 33342	DNA (AT)	350 / 461	cultures	van Dilla *et al.* 1983
			marine	Monger and Landry 1993
Benzoxazinone-kanamycin (BVC kanamycin)	cell surfaces	495 / 616	cultures	Depierreux *et al.* 1990
TO-PRO-1	DNA and RNA**	515 / 531	marine	Li *et al.* 1995
TOTO-1	DNA and RNA**	514 / 533	marine	Li *et al.* 1995, Zubkov *et al.* 1998
SYTO-13	DNA and RNA**	488 / 514	freshwater	del Giorgio *et al.* 1996
			marine	Guindulain *et al.* 1997, Lebaron *et al.* 1998
YOYO-1	DNA and RNA	491 / 509	cultures	Marie *et al.* 1996
YO-PRO-1	DNA and RNA	491 / 509	cultures	Marie *et al.* 1996
PicoGreen	dsDNA	480 / 520	cultures	Marie *et al.* 1996, Veldhuis *et al.* 1997
			marine	Sieracki *et al.* 1999
SYBRGreen I	DNA and RNA	494 / 521	marine	Marie *et al.* 1997
			freshwater	Lebaron *et al.* 1998
SYTOX	dsDNA	504 / 523	cultures	Veldhuis *et al.* 1997
SYTO-9, 11, BC	DNA (and RNA)	480-510 / 500-520	freshwater and marine	Lebaron *et al.* 1998
SYBRGreen II	RNA (and DNA)	492 / 521	freshwater and marine	Lebaron *et al.* 1998
SYTO-17	DNA and RNA	633 / 675	cultures	Comas and Vives-Rego 1997
SYTO-16	DNA and RNA	488 / 518	cultures	Ibrahim *et al.* 1997
SYPRO	Protein	550 / 630	cultures	Zubkov *et al.* 1999

* AO also stains DNA with excitation / emission maxima at 500 and 526 nm
** Only DNA in plankton samples (see Li *et al.* 1995 and Guindulain *et al.* 1997)

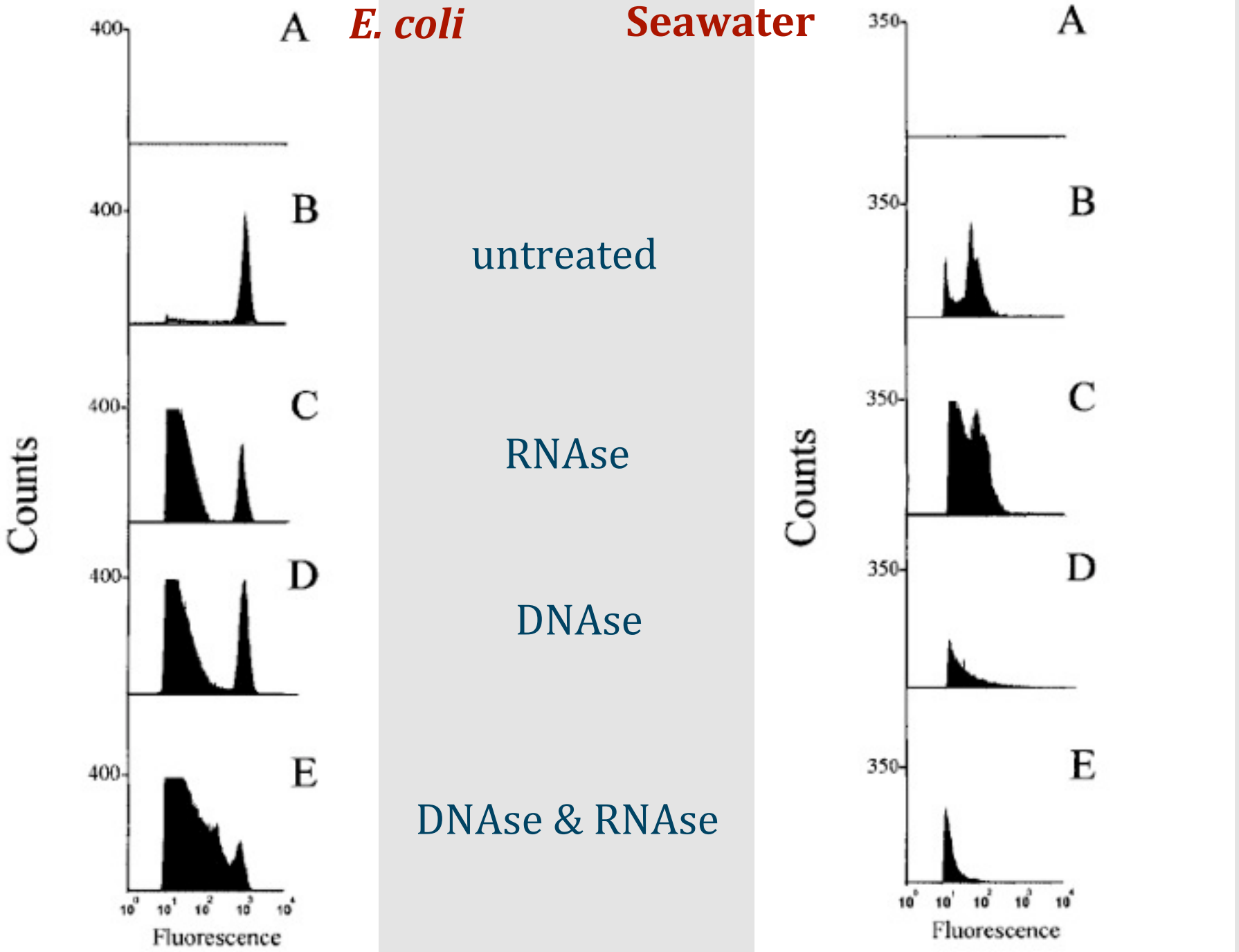
Gasol & del Giorgio 2000-Sci.Mar.







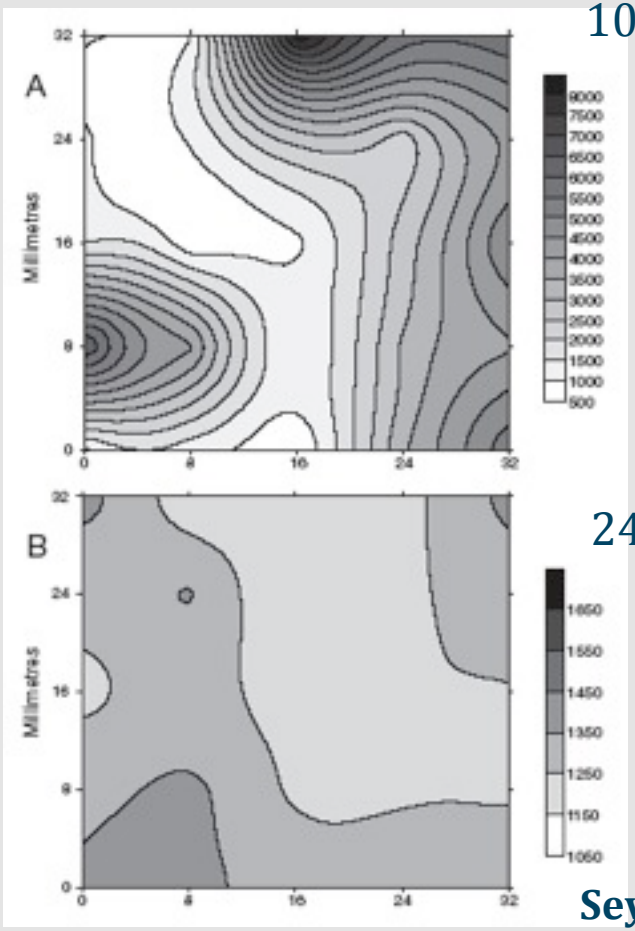
Gasol et al. 1999-AEM


E. coli
Seawater
A
B
C
D
E
400
350
Counts
Fluorescence
untreated
RNAse
DNAse
DNAse & RNAse
Guindulain et al'97-AEM

Advantages of counting bacteria with a FC

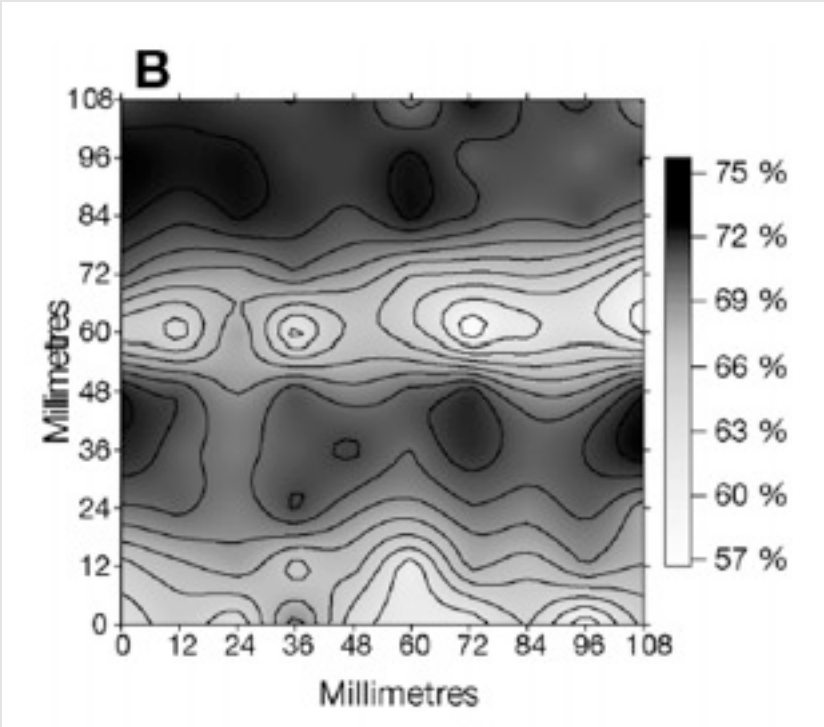
- Fast ! (> 100 samples a day ?)
- Very small volumes (1 µl !)
- Allows to know more about “bacteria”
- Processing can be automated
- It’s 50% cheaper

Abundance



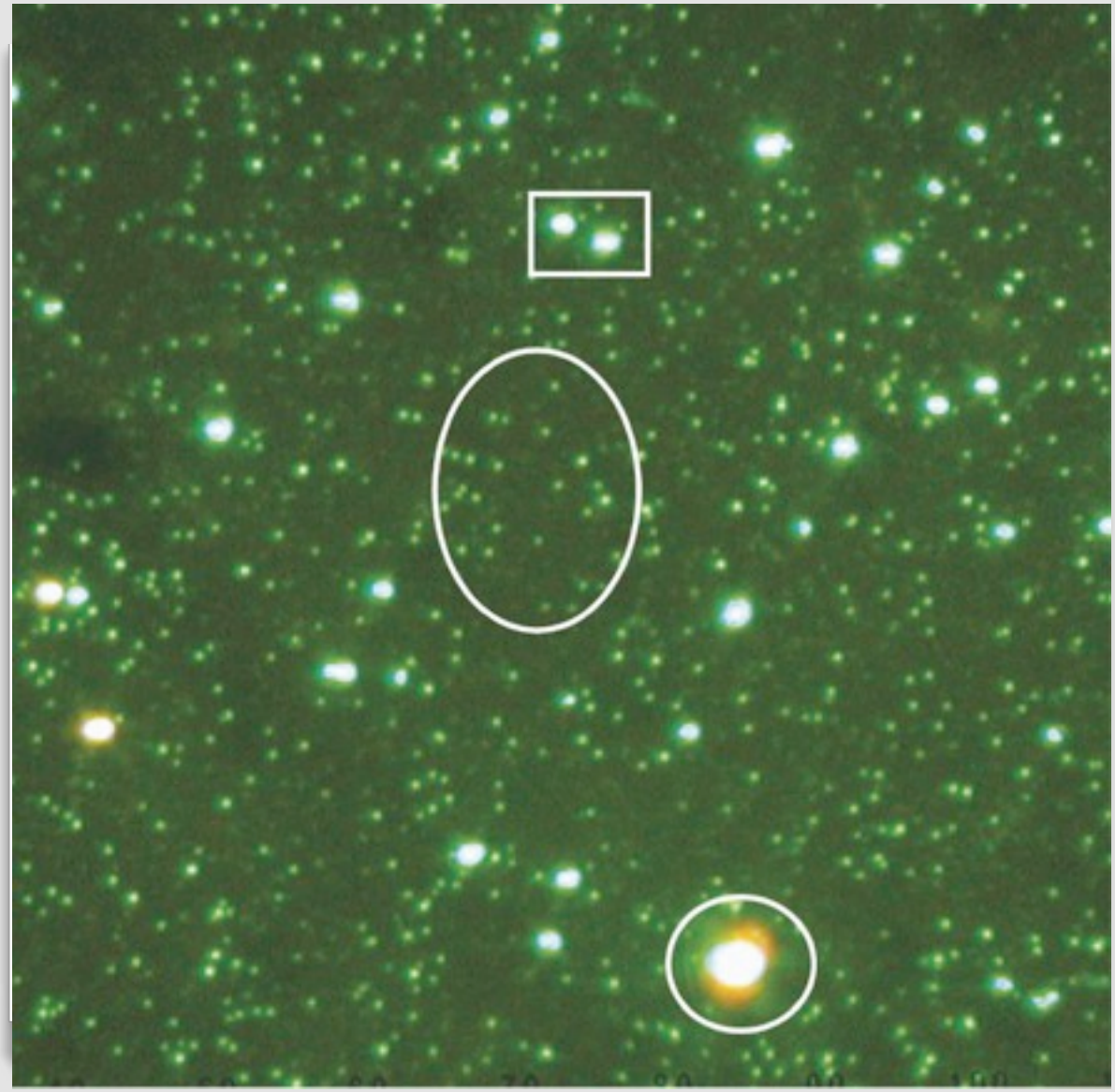
Seymour et al’00-AME

%HNA



Seymour et al’04-AME

Viruses and all protists get also stained



Patel et al. 2007-Nat.Prot.

Zubkov et al'04-JMBAUK

Microbes and flow cytometry (bias to heterotrophs)

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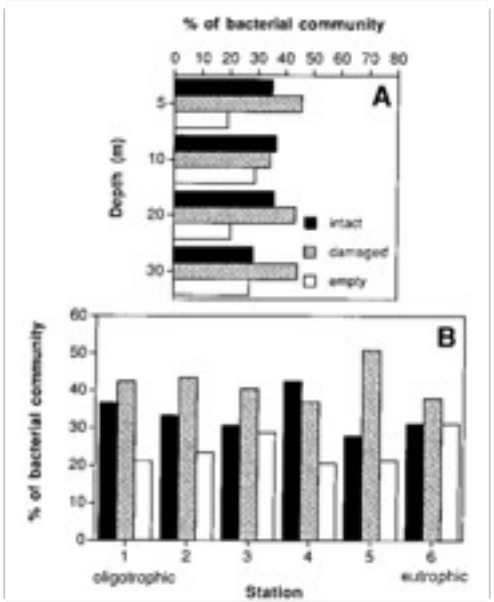
7) Going further: Probing ecosystem function
   Relating community structure to ecosystem functioning

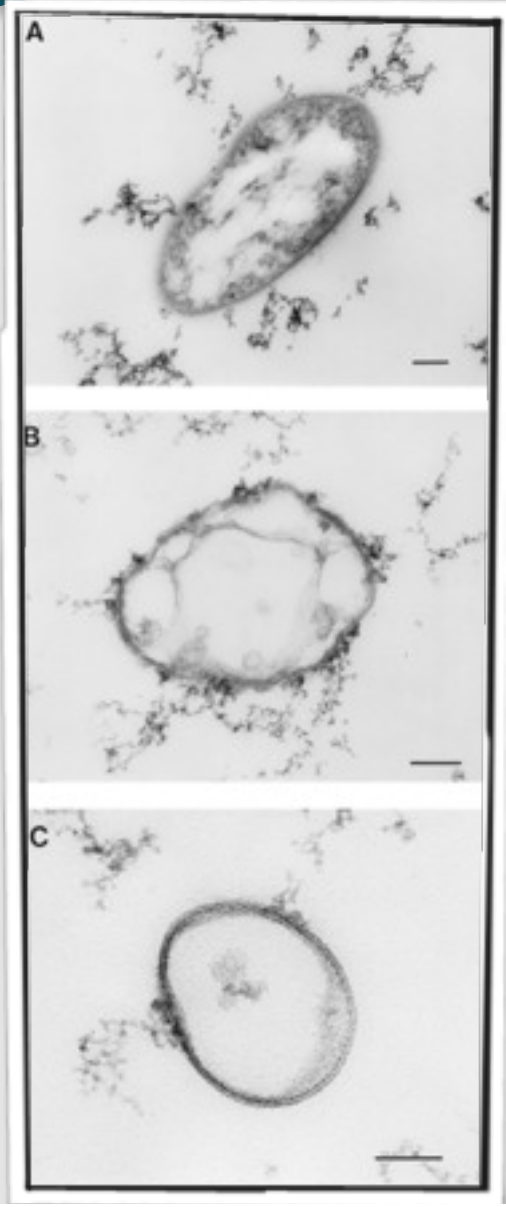
Renewed interest into the live/dead/ inactive/active bacteria

TABLE 1. Measurements of bacterial abundance by various enumeration techniques in depth profiles

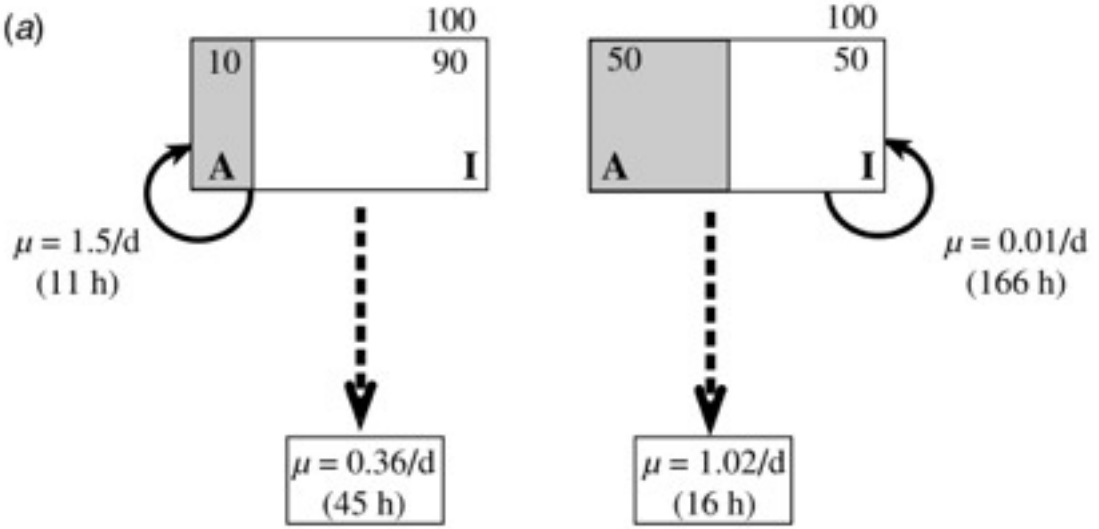
Sea, sampling site, date (mo/day/yr) and depth (m)	Temp (°C) (salinity [‰])	Cell count (ml^{-1})				% Total count	
		Total count (10^9)	NUCC (10^9)	MPN (10^9)	CFU (10^5)	NUCC	MPN
Baltic Sea, NB1, 10/18/93, 0–30	7–8 (4)	2.5–3.2	1.1–2.3	0.4–1.0	1.8–7.8	4 6	0.1 0.3
Baltic Sea, SR5, 5/6/94, 0–100	0.8–2.5 (5–7)	0.7–1.2	1.5–2.8	4.5–15	2.5–160	17 27	7 14
Baltic Sea, US5b, 5/5/94, 0–200	0.9–3.2 (5–7)	0.6–2.7	1.4–3.5	4.7–19	0.7–21	12 27	6 15
North Sea, Skagerrak, 8/10/94, 0–25	11–20 (31)	1.1–1.4	0.3–0.7	0.7–0.8	1–44	2 5	0.5 0.6
North Sea, Sakgerrak, 8/10/94, 25–340	5–11 (35)	0.2–0.8	0.1–0.5	0.1–0.2	0.1–4	4 32	0.2 0.8
Mediterranean Sea, Point B,* 10/15/94	19 (35)	0.5	1.4	7.5	10	20	16

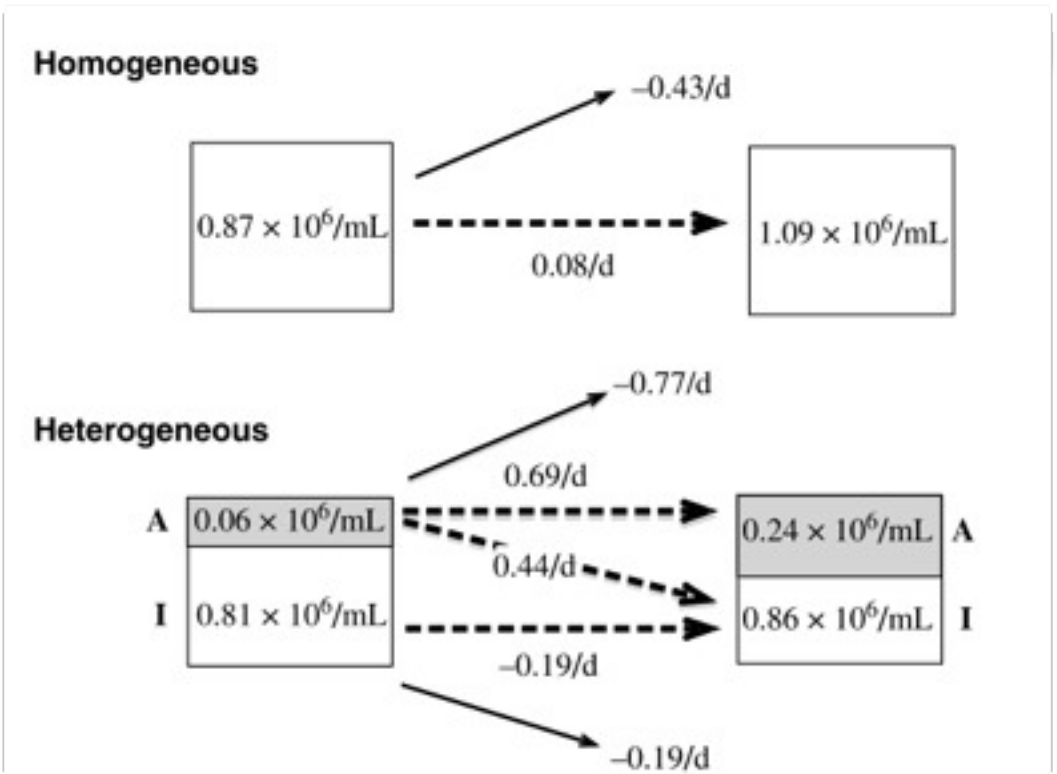
* Villefranche-sur-Mer, France.



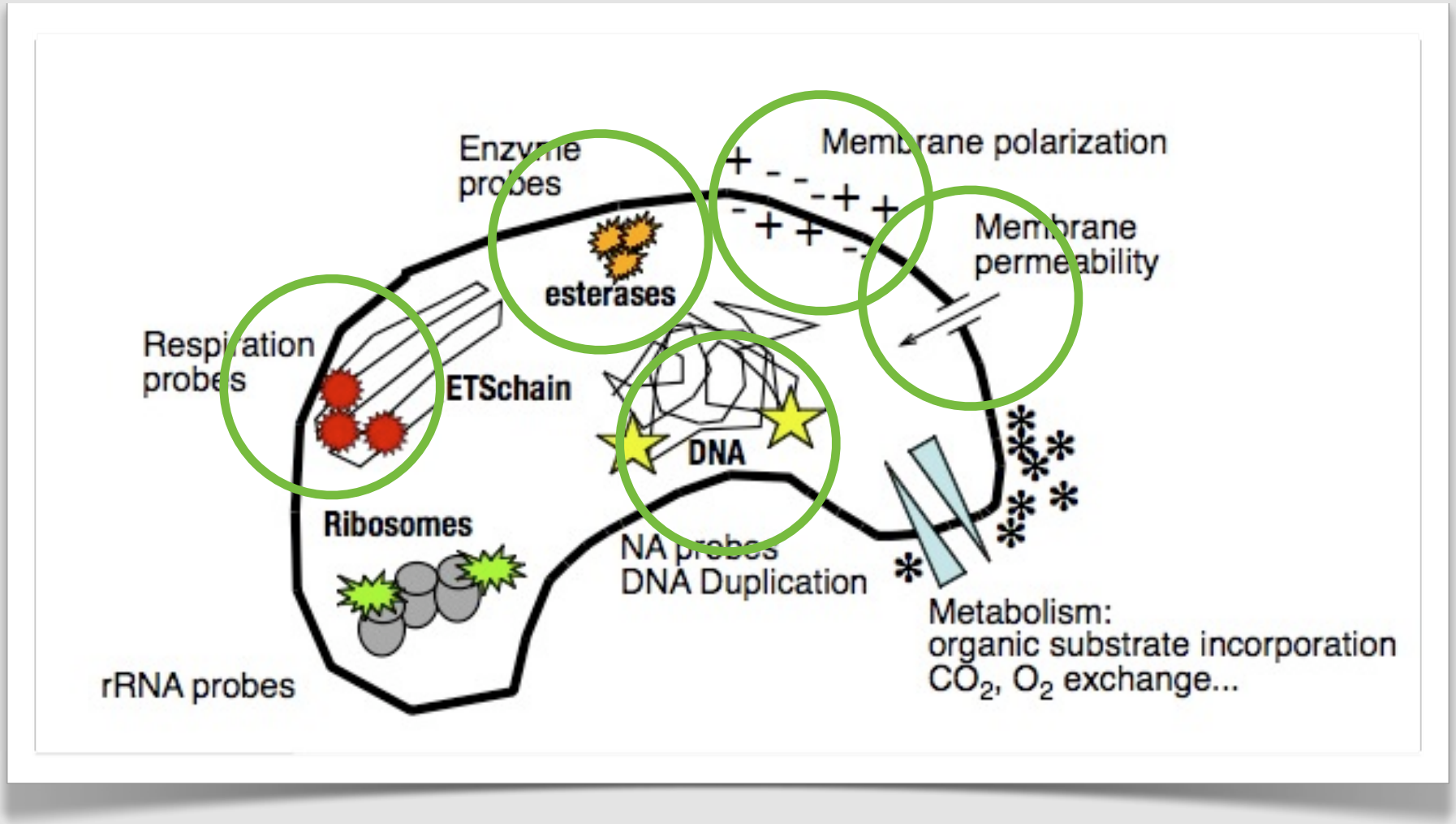


Zweifel & Hagström, 1995-AEM
Heissenberger et al. 1996-AEM





del Giorgio & Gasol, 2008-Kirchman's book



del Giorgio & Gasol, 2008-Kirchman's book

TABLE 3. – Dyes used for monitoring bacterial viability by flow cytometry

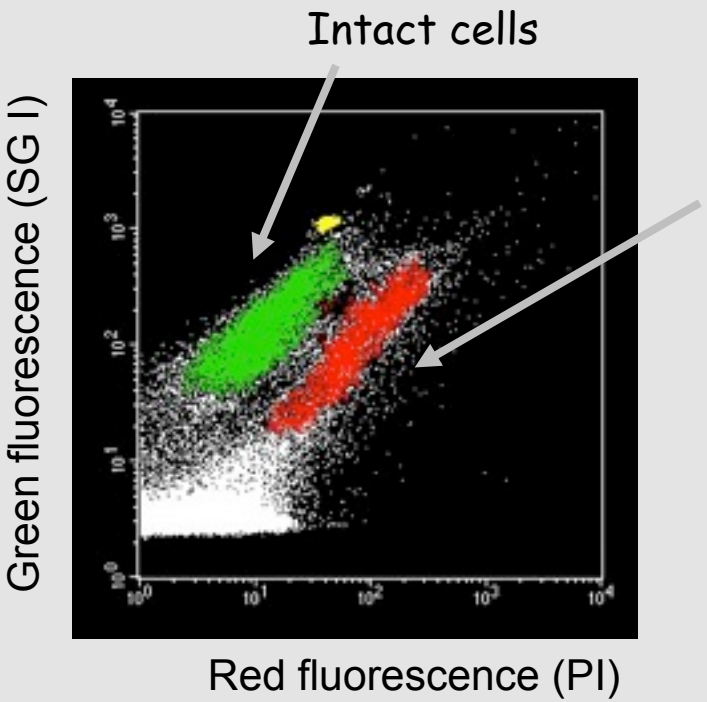
Stain	Mode of action	Exc/Em	applied in	References
AO (acridine orange)	Different color when linked to DNA or other things	460 / 650	cultures	Nishimura *et al.* 1995 Darzynkiewicz and Kapuscinski 1990, McFeters *et al.* 1991
PI (propidium iodide)	excluded by living cells	536 / 623	cultures	Jepras *et al.* 1995
			cultured bact in seawater	López-Amorós *et al.* 1995b
EthBr (ethidium bromide)	excluded by living cells	510 / 595	cultures	Paau *et al.* 1977, Pinder *et al.* 1990
Oxonols (JC-1, oxonol VI, $DiBaC_4(3)$)	accumulate when energy-defficient	488 / 525	cultures	Mason *et al.* 1995, Deere *et al.* 1995, Jepras *et al.* 1995, López-Amorós *et al.* 1995a Comas and Vives-rego 1997, Beck and Huber 1997
FDG (fluorescein-galactopyranose)	Activity of the enzime ß-galactosidase	494 / 518	cultures	Nir *et al.* 1990, Miao *et al.* 1993
Fluorescein diacetate (FDA)	cleaved by intracellular enzims	492 / 517	cultures	Diaper *et al.* 1992
CFDA, Chemchrome B, calcein blue AM, SFDA...	cleaved by intracellular enzims	(488-520/560)	cultures	Diaper and Edwards 1994a, Jepras *et al.* 1995, Jacobsen *et al.* 1997, Beck and Huber 1997
			compost	Diaper and Edwards 1994b
			freshwater	Porter *et al.* 1995a
CTC	indicator of respiratory-chain activity	480 / >585	cultures	Kaprelyants and Kell 1993a, López-Amorós *et al.* 1997
			freshwater	del Giorgio *et al.* 1997b, Yamaguchi and Nasu 1997
			marine	López-Amorós *et al.* 1998, Sieracki *et al.* 1999
Rh123 (rhodamine 123)	accumulated in live cells	510 / 580	cultures	Diaper *et al.* 1992, Davey *et al.* 1993, Kaprelyants and Kell 1992
			cult bact in SW	López-Amorós *et al.* 1995b
Cyanine dyes ($DiOC_5(3)$, $DiOC_2(3)$...)	accumulated in live cells	488/ 520-560	cultures	Mason *et al.* 1995, Monfort and Baleaux 1996
		488 / > 600	cultures	Novo *et al.* 1999
c-SNARF-1 AM	intracellular pH	~488 / ~610	cultures	Leyval *et al.* 1997
Calcafluor white, Tinopal CBS-X...	excluded by living cells	347 / 436	cultures	Mason *et al.* 1995
		325 / 430	cultures	Davey and kell 1997
SYTOX Green	excluded by living cells	504 / 523	cultures	Roth *et al.* 1997, Veldhuis *et al.* 1997
TOPRO-1	excluded by living cells	515 / 531	freshwater	del Giorgio *et al.*, in press
TOPRO-3	excluded by living cells	642 / 661	cultures	Davey *et al.* 1999
16S rRNA probes	attach to ribosomes	-	cultures	Amann *et al.* 1990; Wallner *et al.* 1993
DVC*	live cells elongate when in presence of ABs	-	cultures	Thorsen *et al.* 1992, Joux *et al.* 1997
			seawater	Nishimura *et al.* 1995
BacLight Live / Dead	(Syto9 / PI)	-	cultures	Joux *et al.* 1997, Jacobsen *et al.* 1997

* DVC: Direct viable count. Samples are incubated with added organics and antibiotics that stop cell division. Active cells elongate without division and can be detected by changes in light scatter.

Gasol & del Giorgio 2000-Sci.Mar.

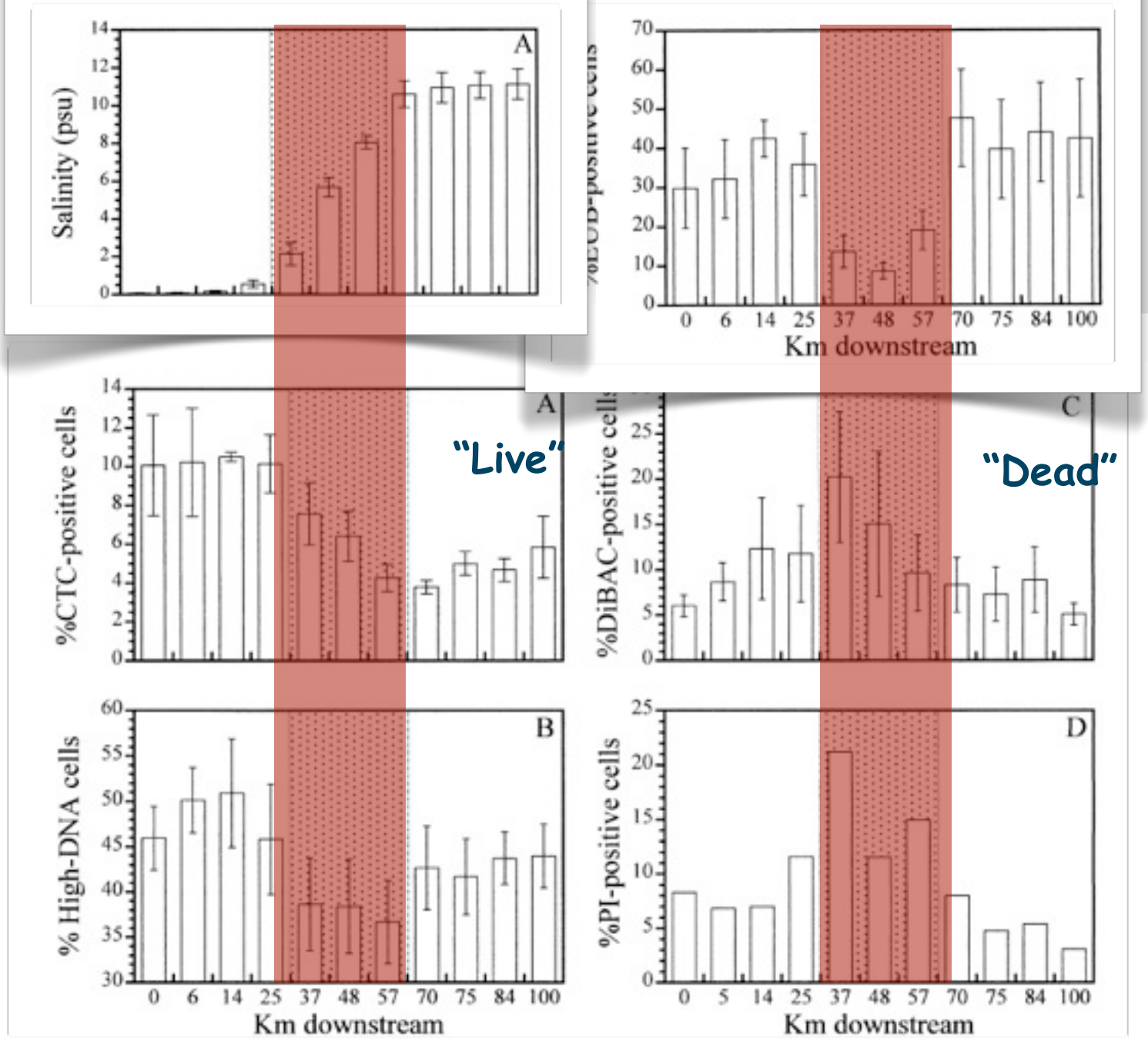
What is bacterial death?

- **NADS (nucleic acid double staining) protocol**
  - PI and SybrGreen 1/2 (equiv. to MP Live&Dead)
  - Live cells stain in GREEN, dead cells stain in RED
  - Cultures (Barbesti et al.00) , Field samples (Gregori et al.01)
  - Death-generating controls?

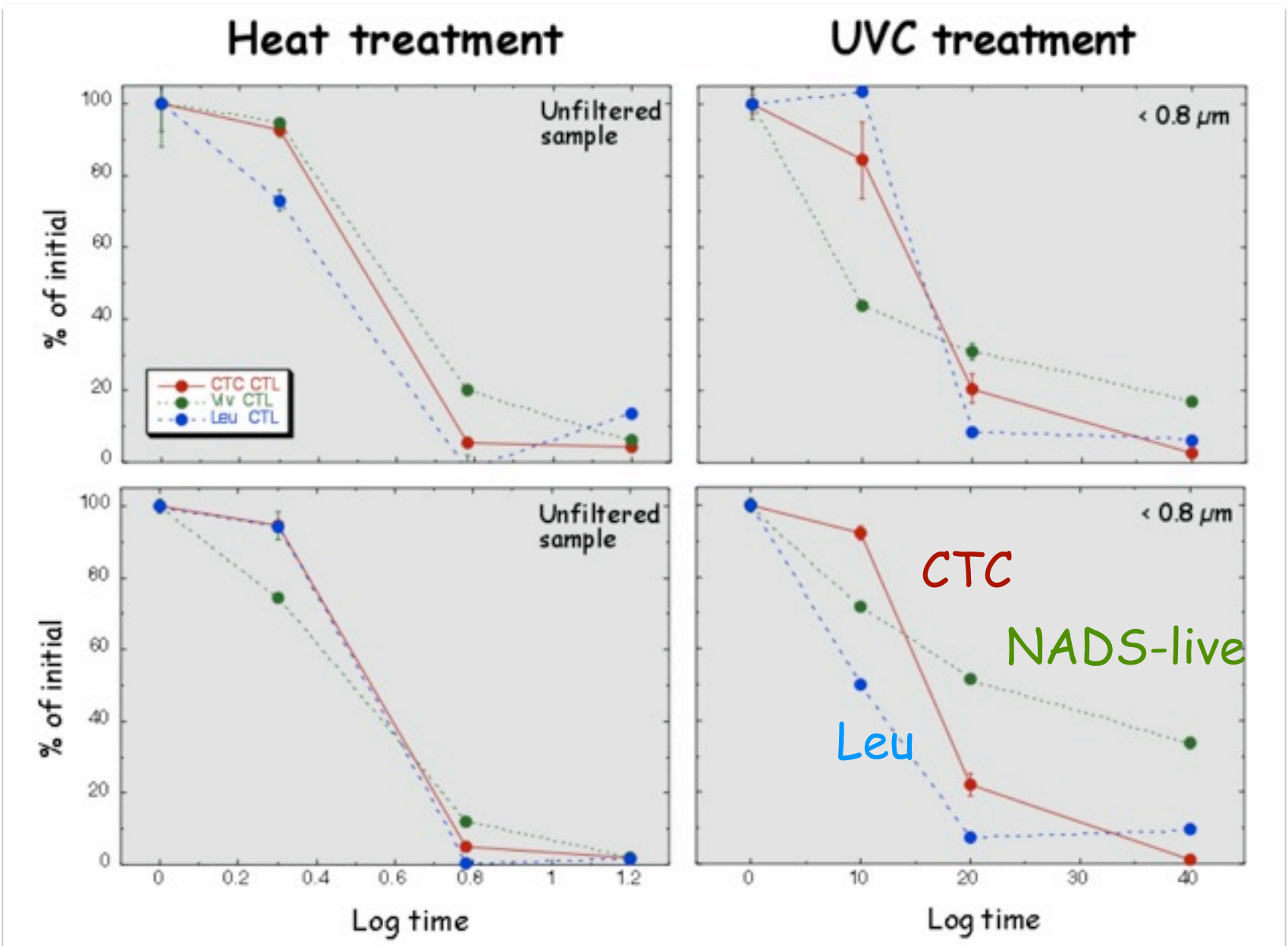


BUT

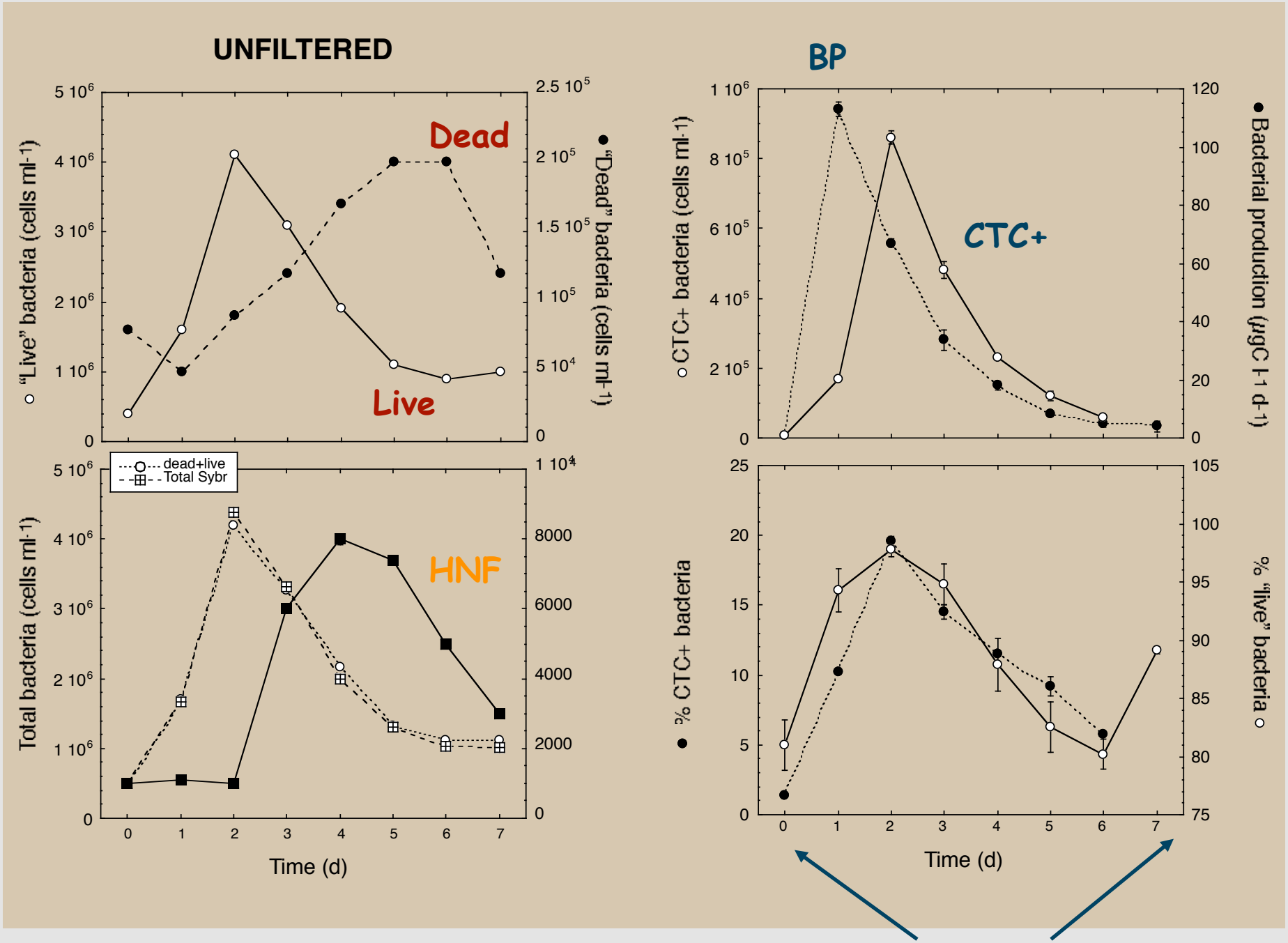
- PI also labels Leucine-incorporating cells… (Pirker et al. 2005)



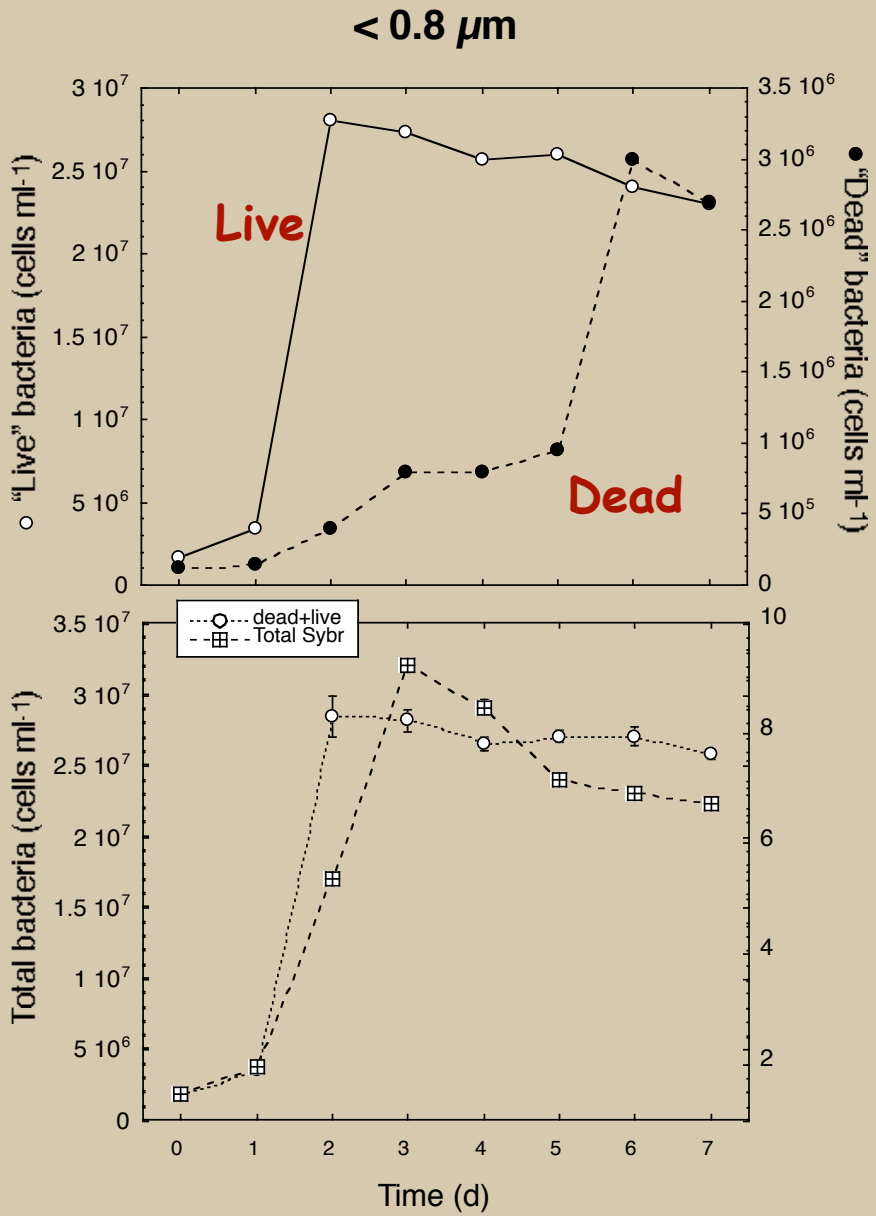
del Giorgio & Bouvier'02-L&O

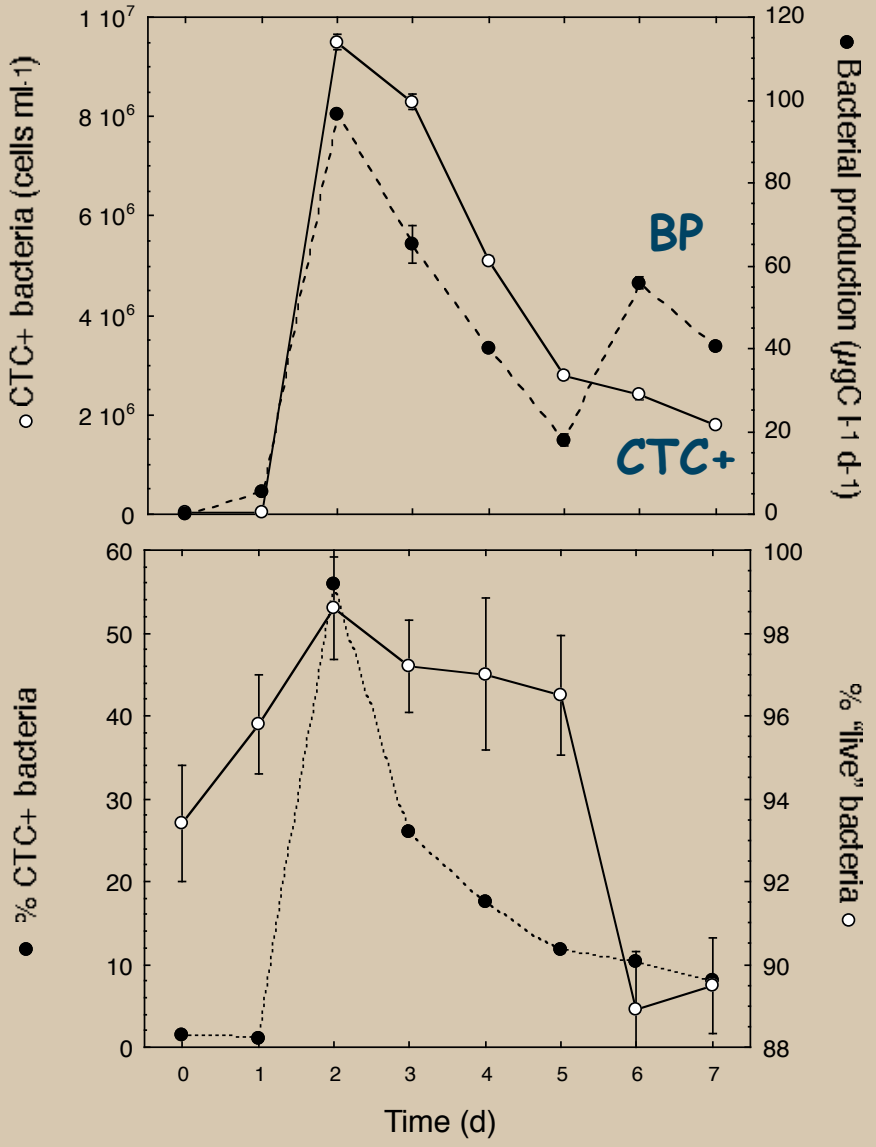


Falcione et al. 2008-AEM.



Falcionei et al. 2008-AEM.





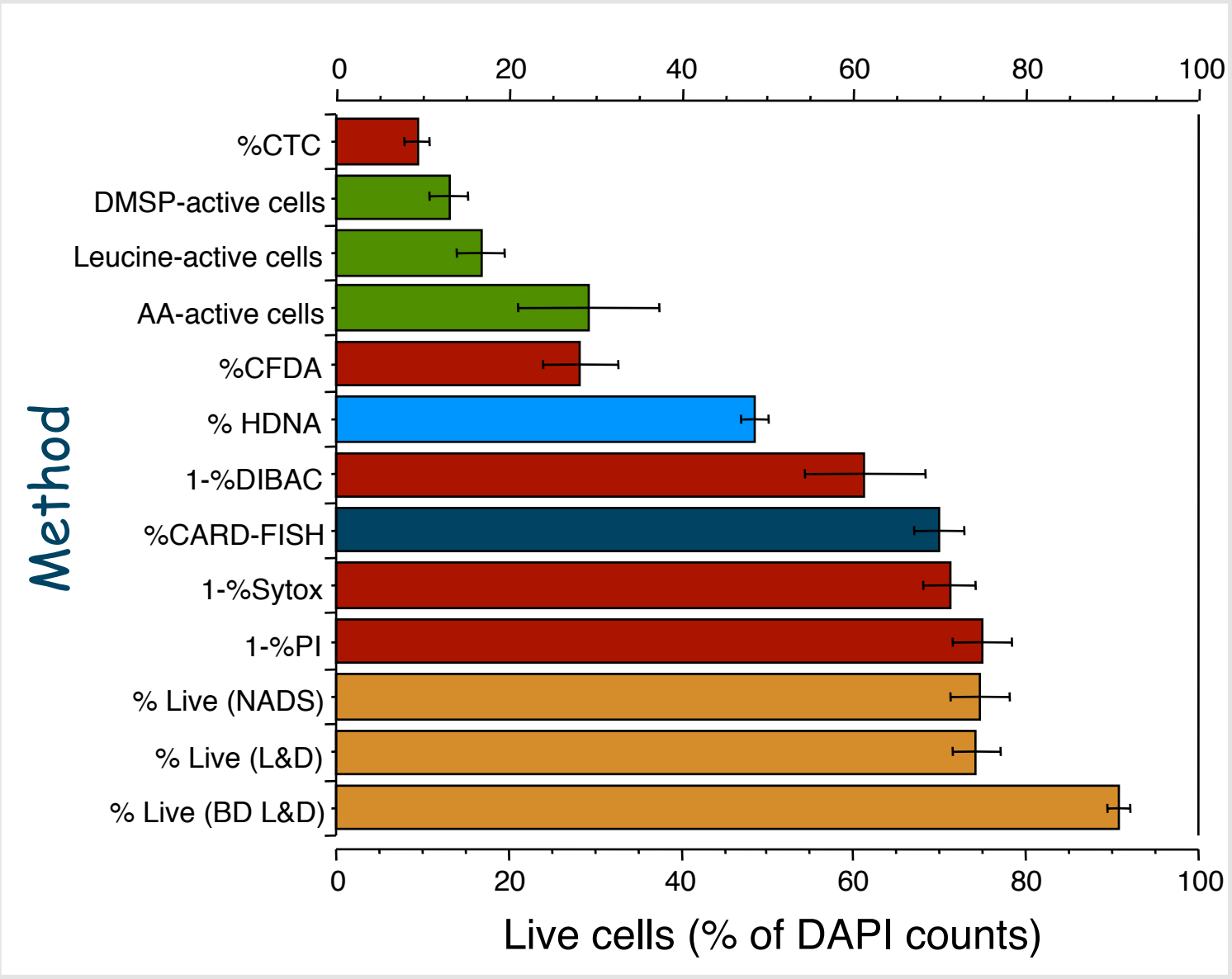
Falcioni et al. 2008-AEM.

Activity probes (seasonal study@Blanes)

% HNA	Active (?)
*CTC+	Very active (respiration)
*PI	damaged membrane
*Sytox	damaged membrane
*CFDA/SE	Intracellular esterases
*Dibac	Membrane w/o polarity
MP Live & Dead	PI + Syto9
NADS	PI + SybrGreen I
BD Live & Dead	PI + Thiazol Orange

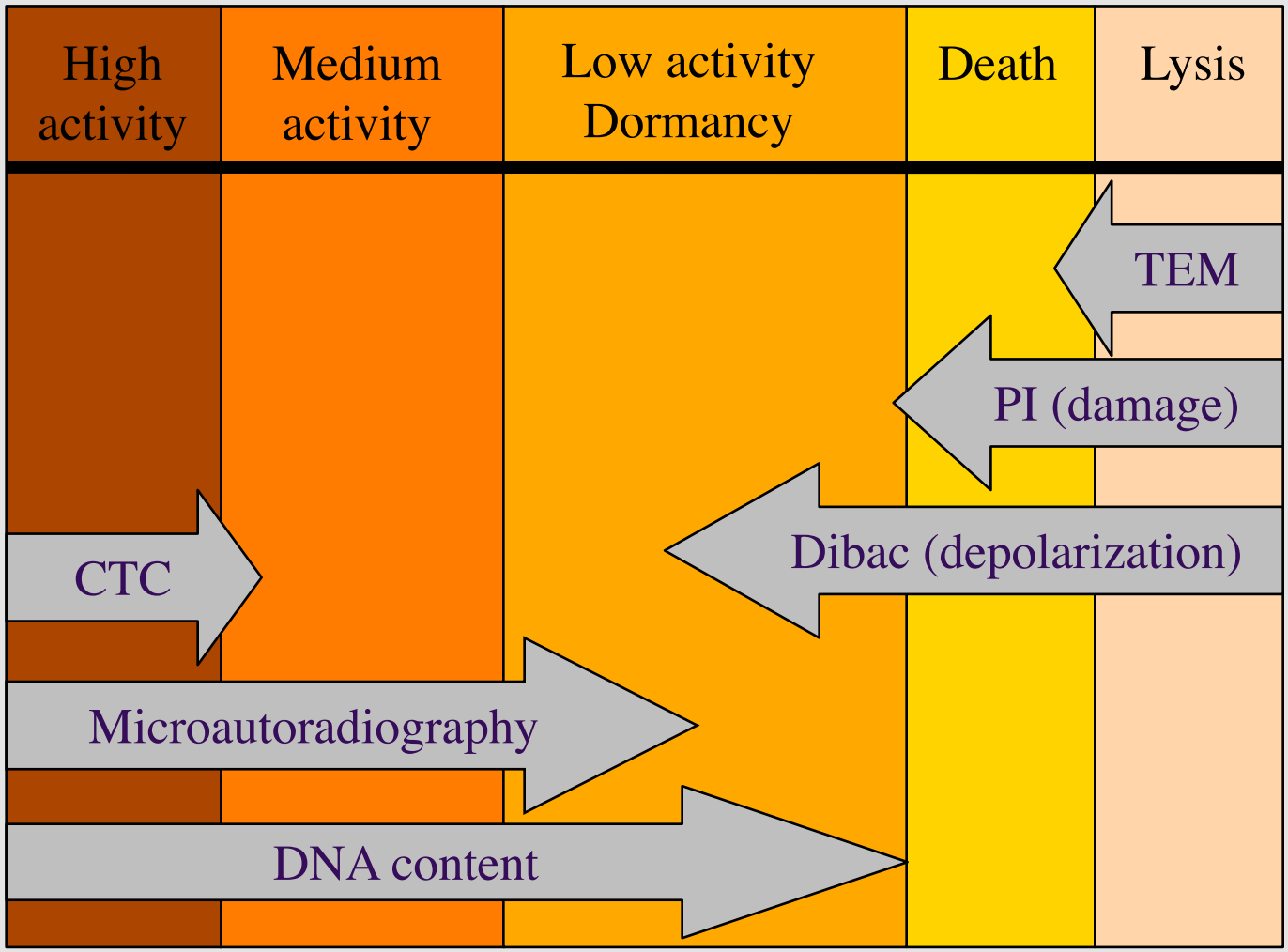
* Microautoradiography (Leu, Gluc, AA, ATP, DMSP)
* 16 rRNA content (FISH & CARD-FISH)

VSP (rRNA + PI + DAPI)

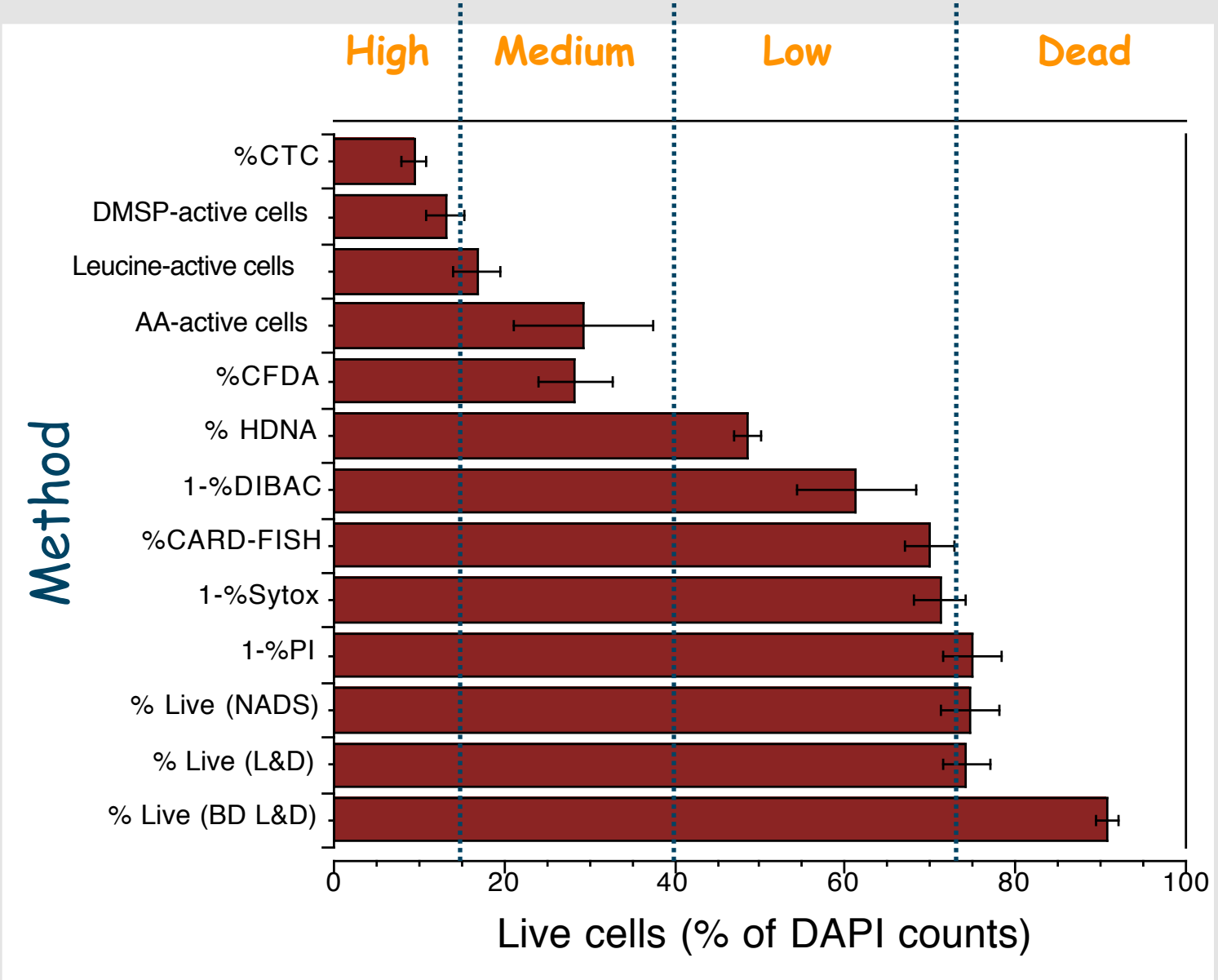


Blanes Bay, seasonal survey

The physiological-state continuum



del Giorgio & Gasol, 2008



Blanes Bay, seasonal survey

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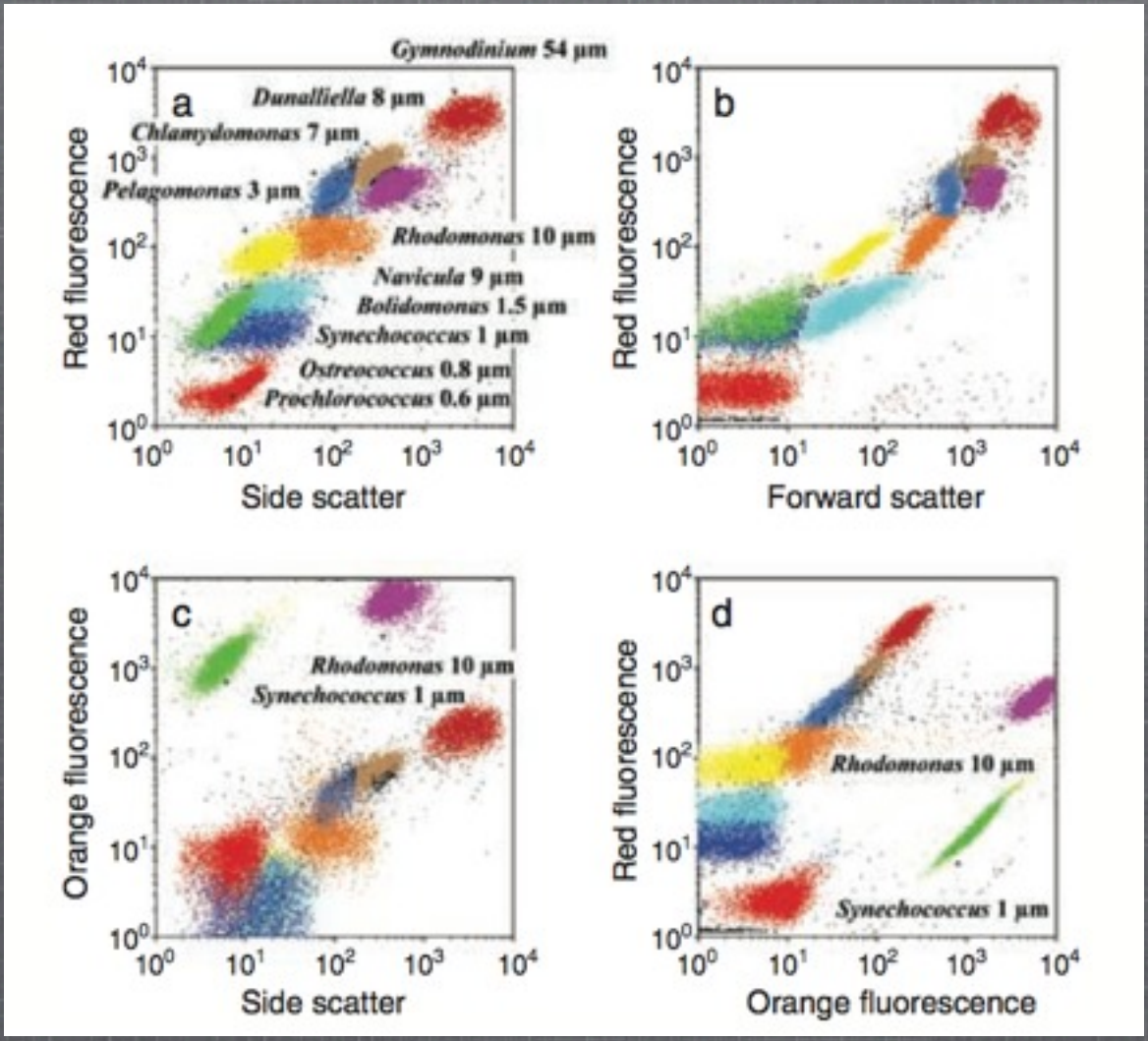
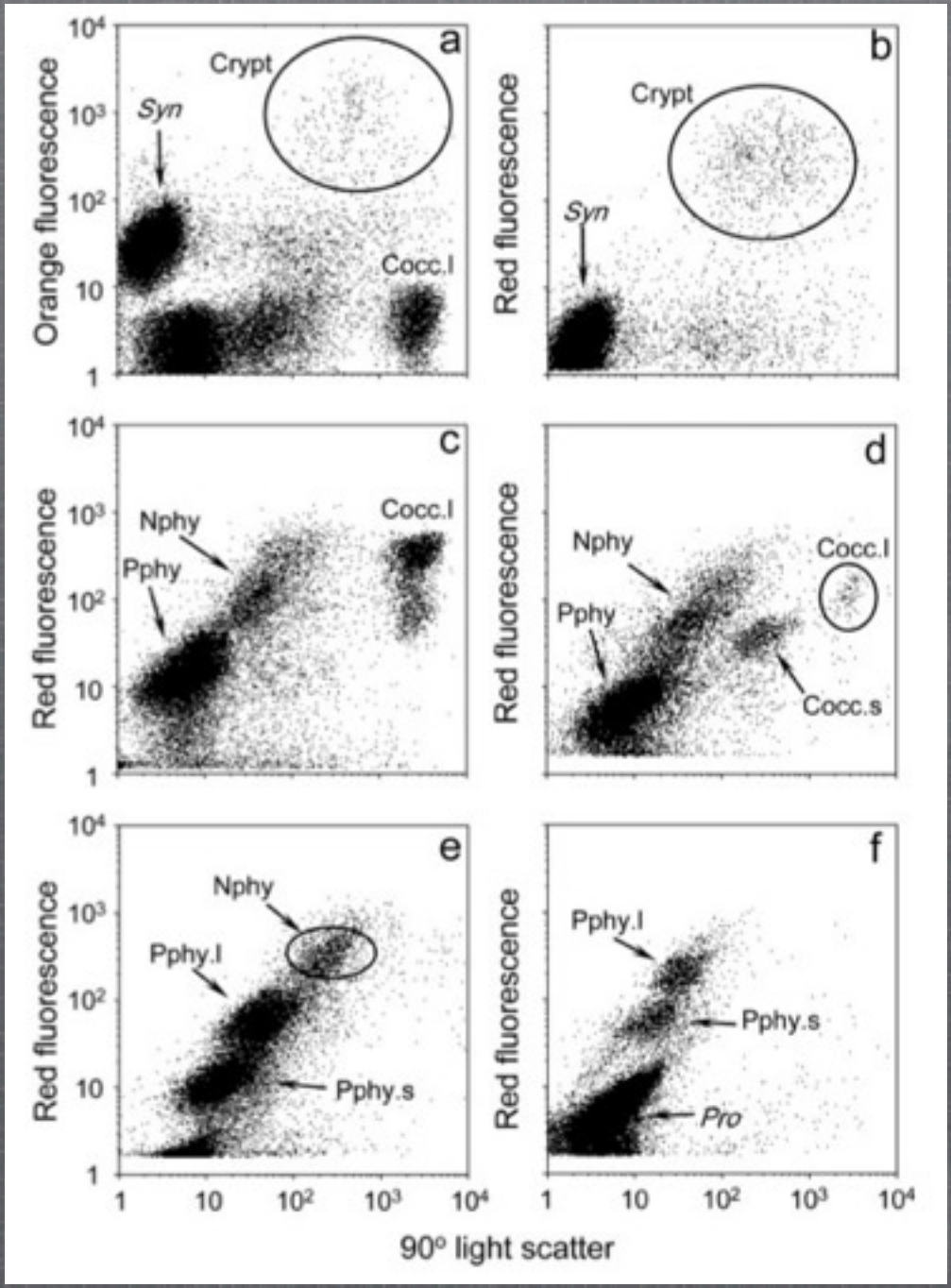
A personal view of the last 20 yr. highlights

- Routine enumeration of pico- and nanoalgae (>80's)



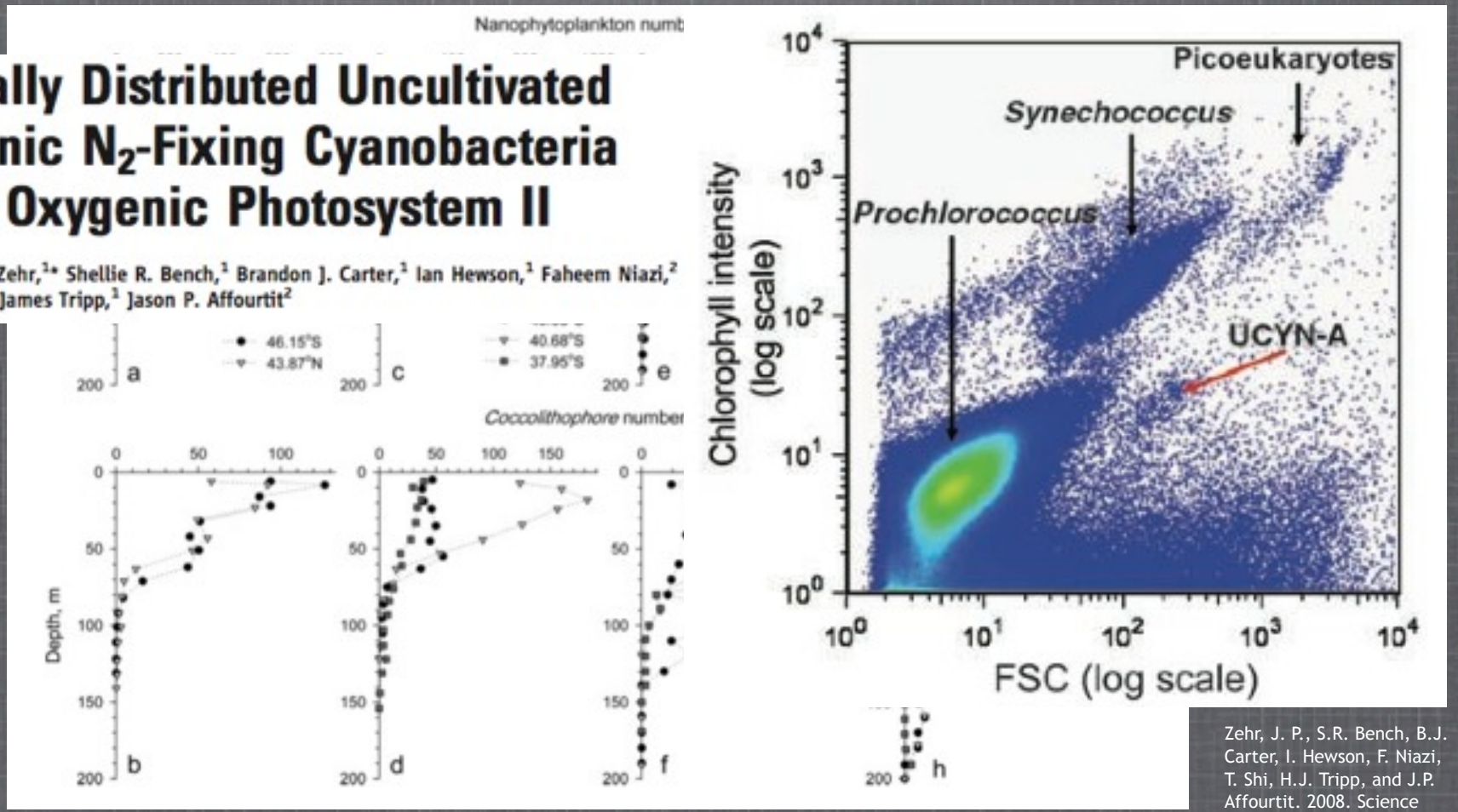
Marie, D., N. Simon, and D. Vaulot. 2005. *In* R.A. Andersen [eds.], Algal Culturing Techniques. Academic Press.



Zubkov, M. V., and P.H. Burkill. 2006. Cytometry A **69**: 1010-1019.

A personal view of the last 20 yr. highlights

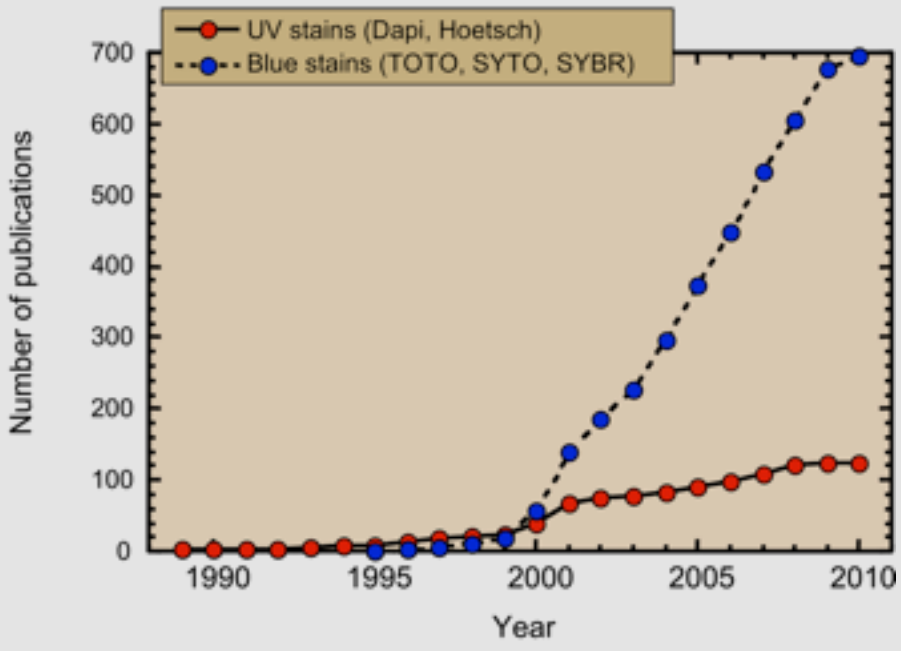
- Routine enumeration of pico- and nanoalgae (>80's)
- Not yet: enumeration of large and "rare" cells



Zehr, J. P., S.R. Bench, B.J. Carter, I. Hewson, F. Niazi, T. Shi, H.J. Tripp, and J.P. Affourtit. 2008. Science **322**: 1110-1112.

A personal view of the last 20 yr. highlights

- Routine enumeration of pico- and nanoalgae (>80's)
- Not yet: enumeration of large and "rare" cells
- Routine enumeration of heterotrophic bacteria (90's)

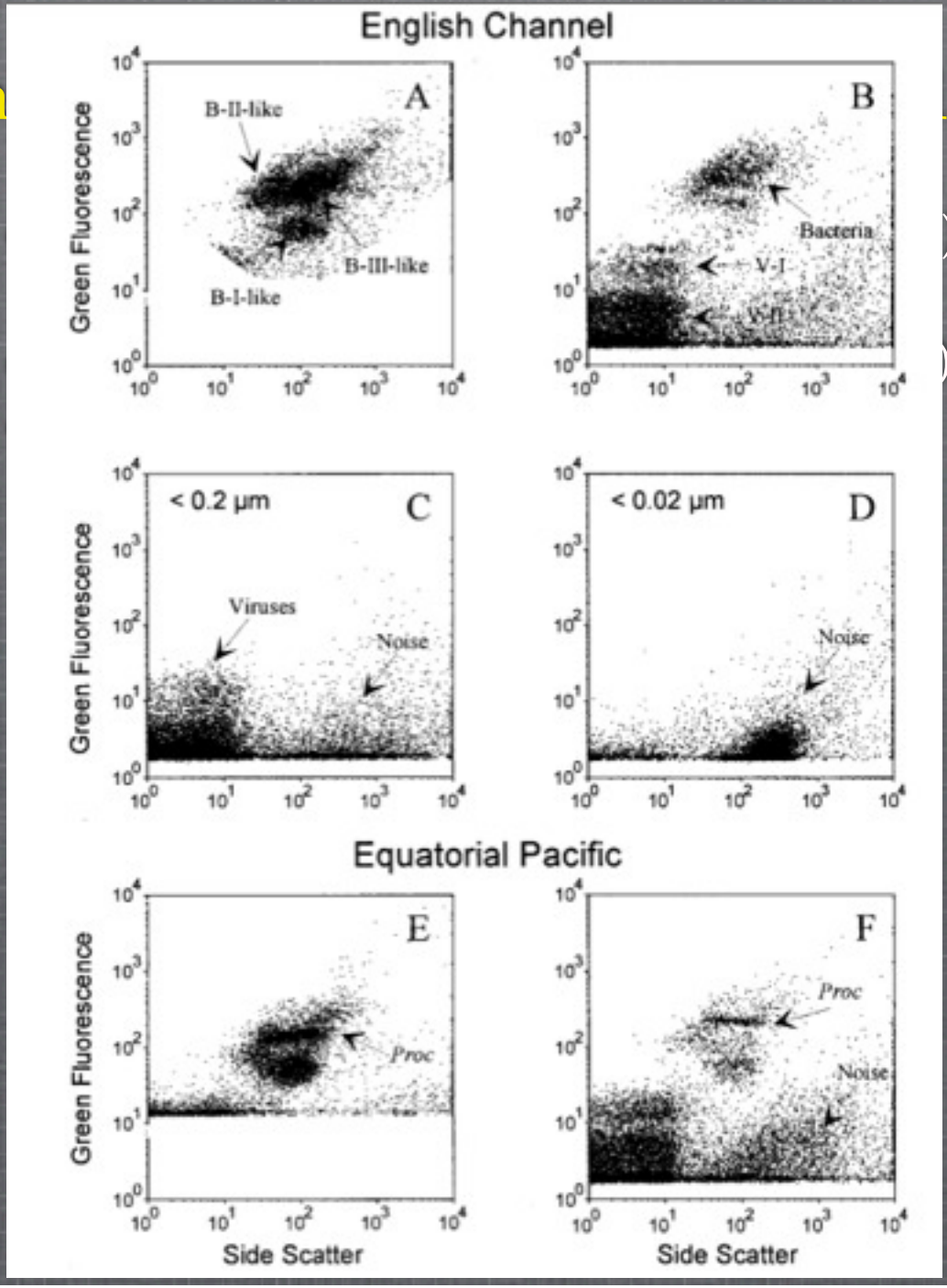


Robertson & Button 1989
Monfort & Baleux 1992
Troussellier et al. 1993
Monger & Landry 1993

Li et al. 1995
del Giorgio et al. 1996
Marie et al. 1997

A person ights

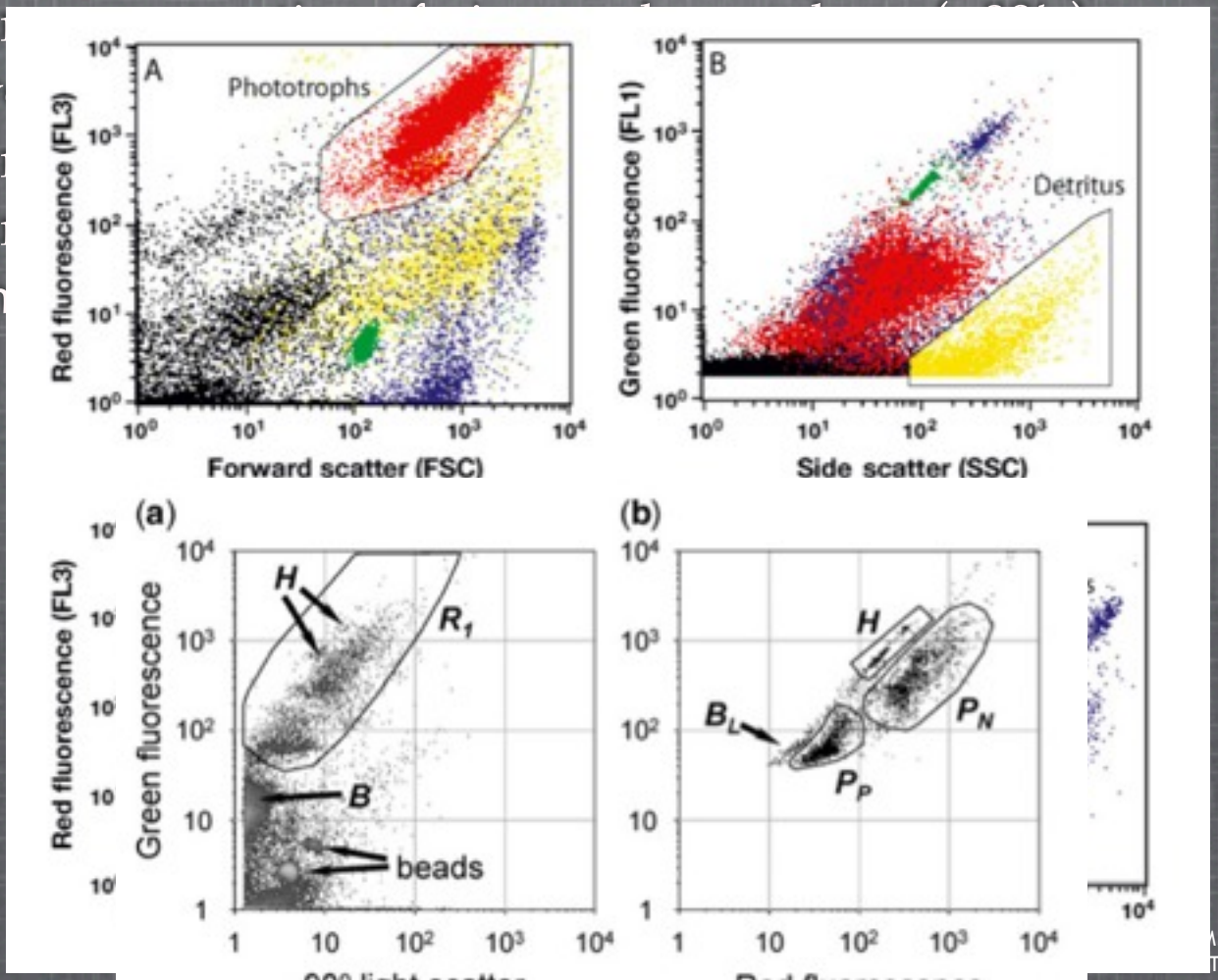
- Routine)
- Not yet:
- Routine)
- Routine



Marie, D., C.P.D. Brussaard, R. Thyrhaug, G. Bratbak, and D. Vaulot. 1999. Appl Environ Microbiol **65**: 45-52.

A personal view of the last 20 yr. highlights

- Routi
- Not y
- Routi
- Routi
- Enum



Rose, JM, Caron, DA, Sieracki, ME, and Poulton, N. 2004. Aquatic Microbial Ecology **34**: 263-277.

. V., P.H. Burkill, Topping. 2007. Journal of Plankton Research **29**: 79.

A personal view of the last 20 yr. highlights

- Routine enumeration of pico- and nanoalgae (>80's)
- Not yet: enumeration of large and "rare" cells
- Routine enumeration of heterotrophic bacteria (90's)
- Routine enumeration of planktonic viruses (00's)
- Enumeration of heterotrophic protists (05's)
- Not yet: enumeration of BChlorophyll containing microorganisms
  oxygenic BChla containing organisms: AAPs
  BUT anoxygenic BChla, b, c, d, e- containing organisms

A personal view of the last 20 yr. highlights

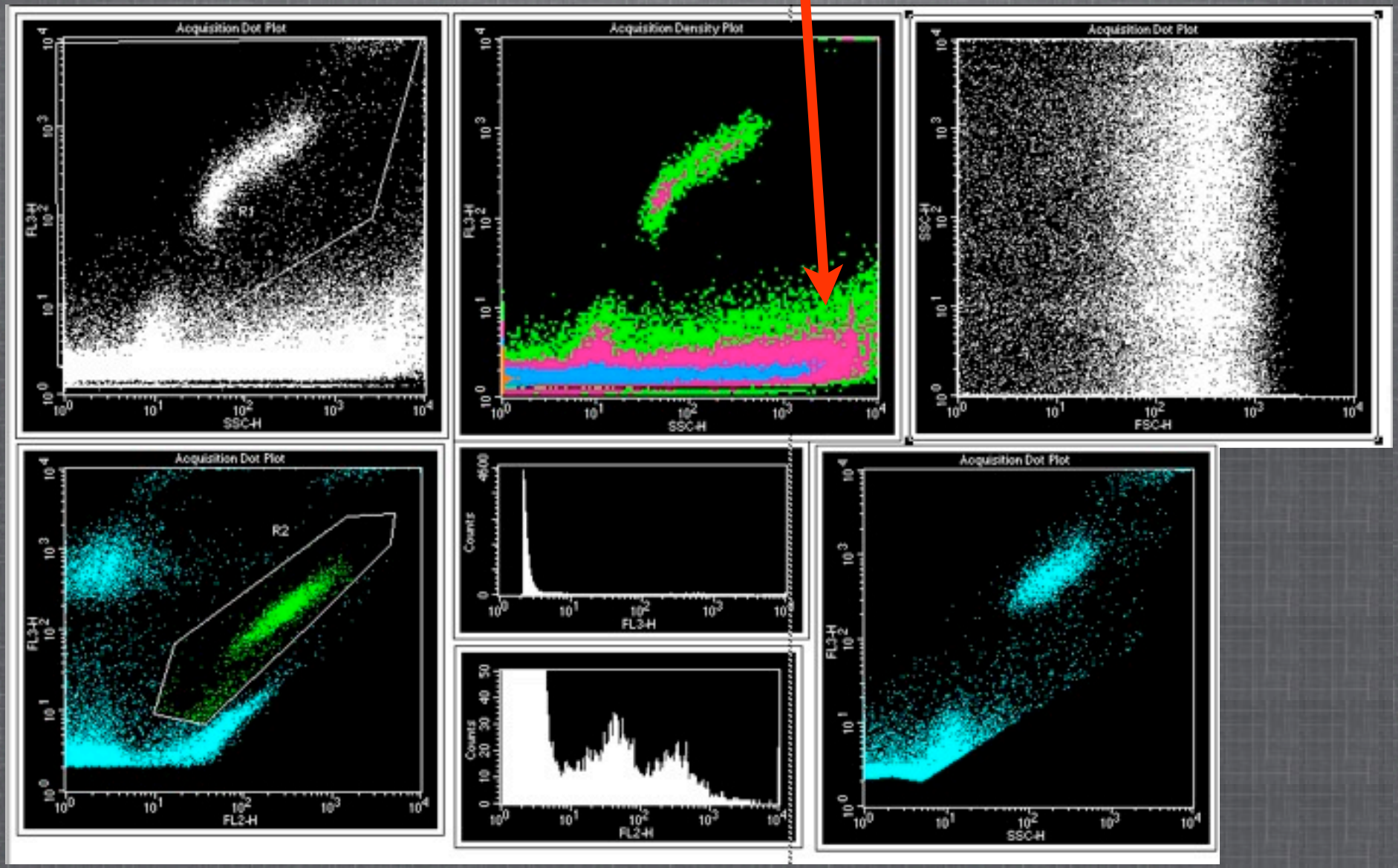
- Routine enumeration of pico- and nanoalgae (>80's)
- Not yet: enumeration of large and "rare" cells
- Routine enumeration of heterotrophic bacteria (90's)
- Routine enumeration of planktonic viruses (00's)
- Enumeration of heterotrophic protists (05's)
- Not yet: enumeration of BChlorophyll containing microorganisms
- Not yet: non-living particles, and particle-attached microbes

  organic particles (gels...)

  inorganic particles (Saharan dust, black carbon)

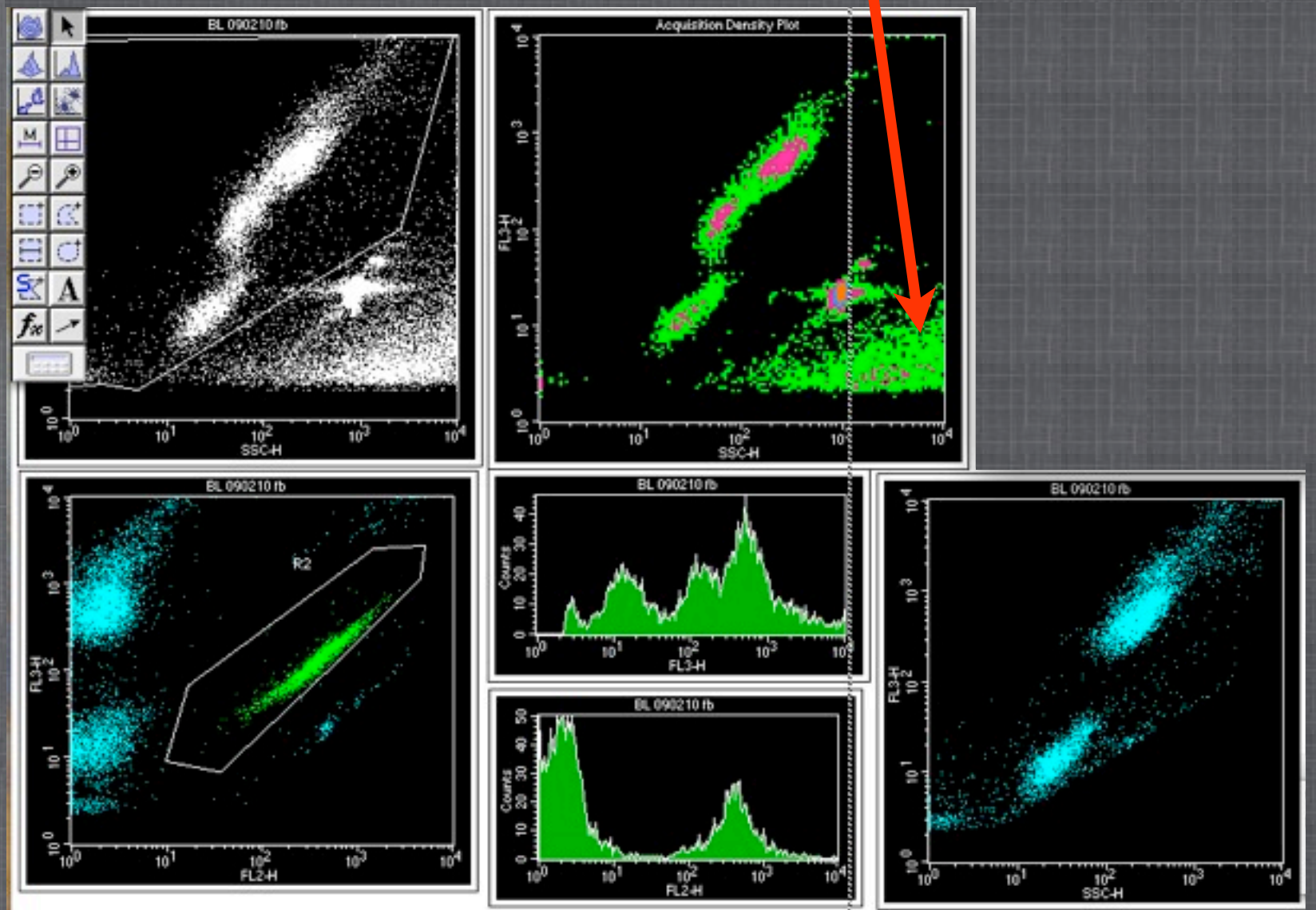
  particle-attached microbes

Anything worth looking at, in here?



Barcelona Olympic Harbor, March 13, 2010

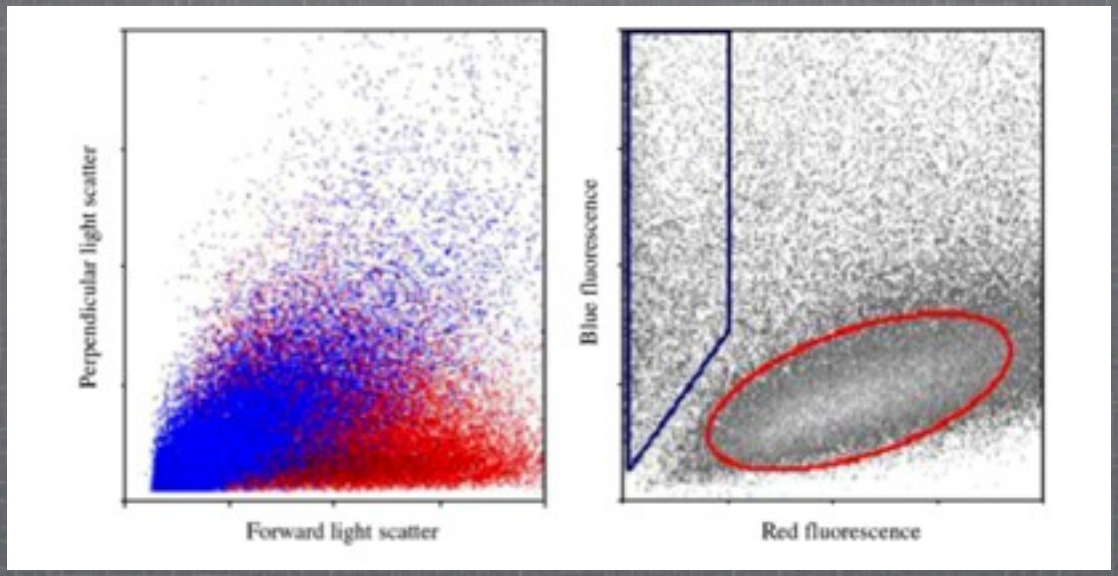
Anything worth looking at, in here?



Blanes Bay, February 10, 2009

Marine microgels: Optical and proteomic fingerprints

Mónica V. Orellana [a,*], Timothy W. Petersen [a,1], Alan H. Diercks [a], Samuel Donohoe [a,2], Pedro Verdugo [b], Ger van den Engh [a,1,3]



Dissolved organic matter (DOM) is a major carbon reservoir for the global carbon cycle, and its molecules play a key role in the biogeochemistry of the ocean. Colloidal DOM macromolecules assemble to form polymer hydrogels known as marine microgels. Marine microgels represent one of the most dynamic pools of organic carbon in the ocean. However, their optical characteristics and their contribution to ocean optical properties are largely unknown. In this work, we explore the optical and proteomic properties of spontaneously assembled DOM polymer microgels. Microgels from cultures and from Puget Sound seawater were sorted and counted using a dual-laser (365 nm/365 nm) high-speed cell sorter. This sorter has been adapted to interface with a scanning monochromator to measure the fluorescence emission spectrum of the microgels over the range from 300 to 850 nm. Surprisingly, the microgels show a broad fluorescence emission from 420 to 520 nm when excited with UV light. The microgels were classified according to their blue autofluorescence, and by three criteria that are used to define microgels: 1) staining with chlortetracycline 2) the ability to undergo phase transitions at low pH, and 3) dispersion following calcium chelation by EDTA.

Orellana, MV, Petersen, TW, Diercks, AH, Donohoe, S, Verdugo, P, and van der Engh, G. 2007. Mar Chem **105**: 229-239.

Sahara dust particles

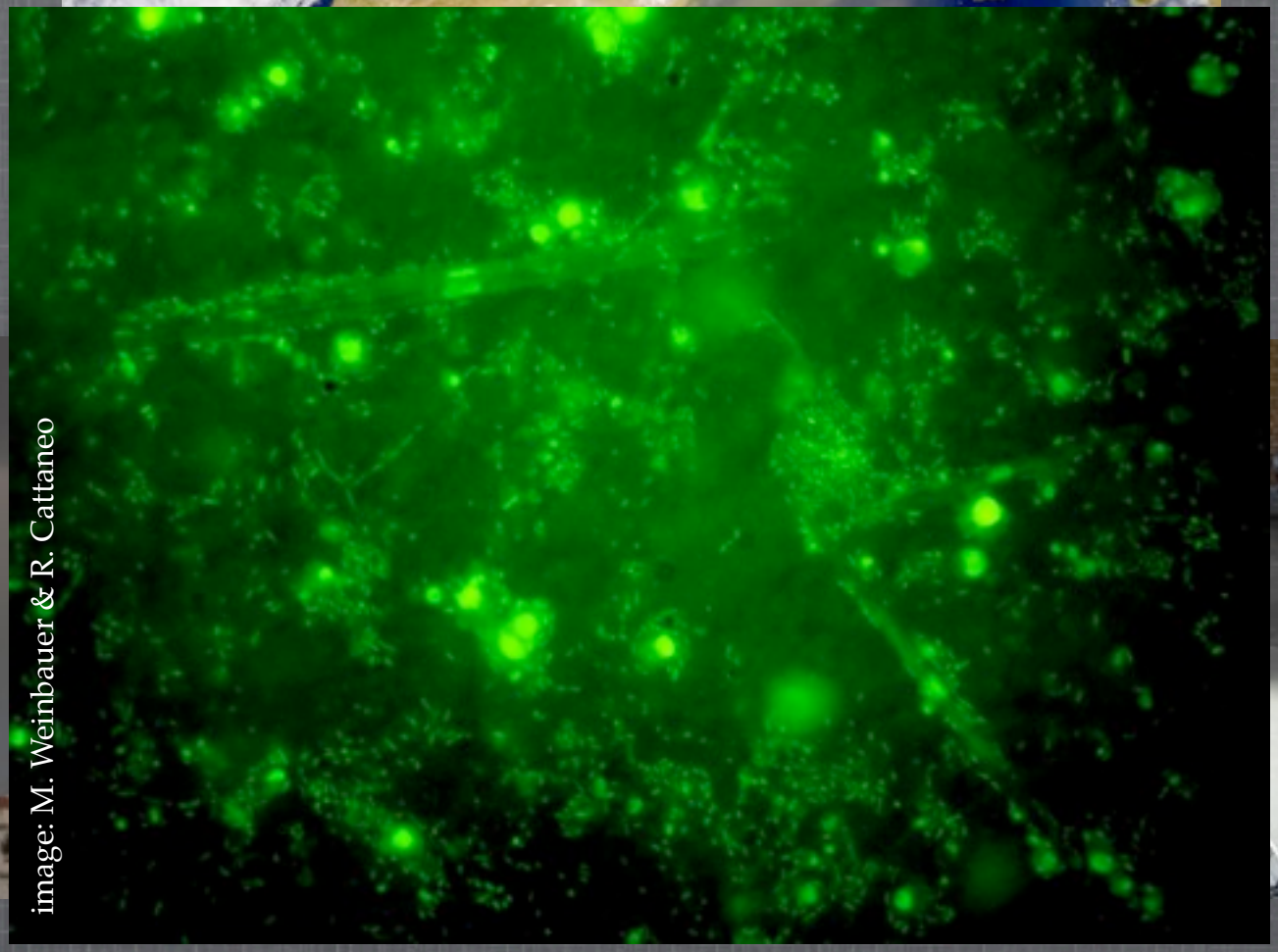
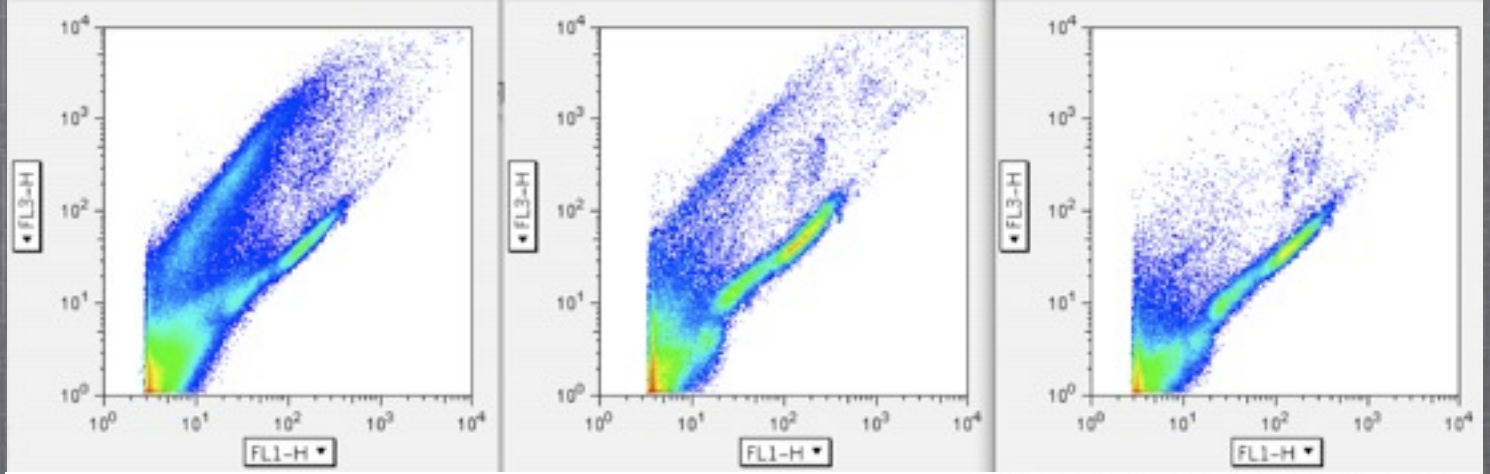
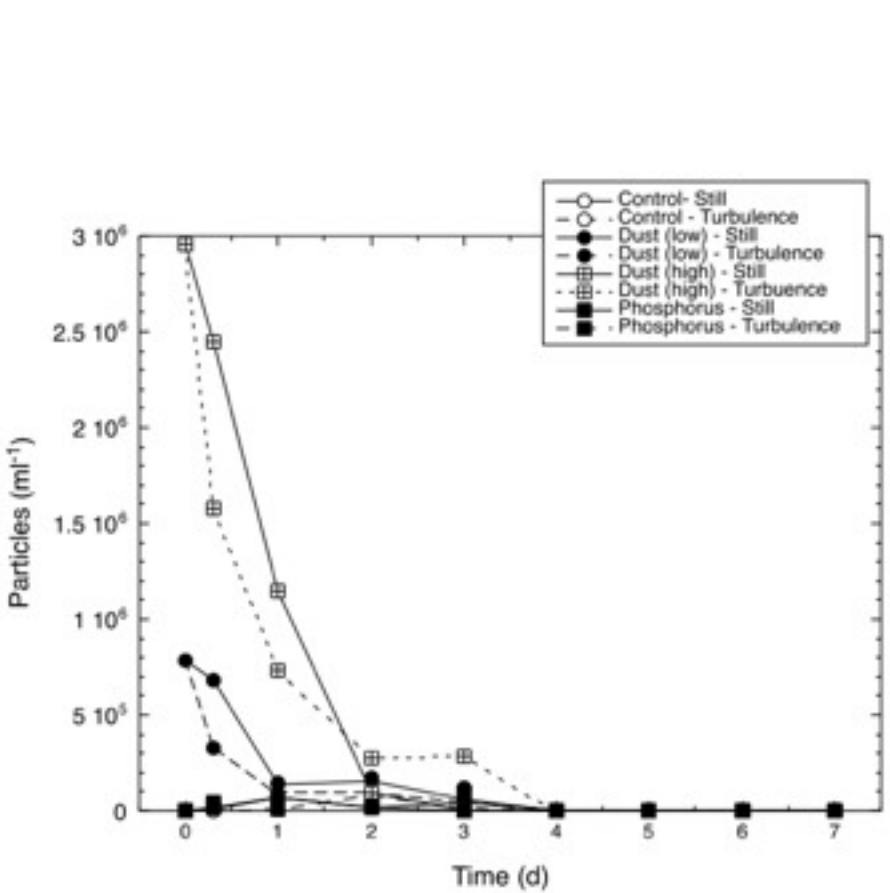
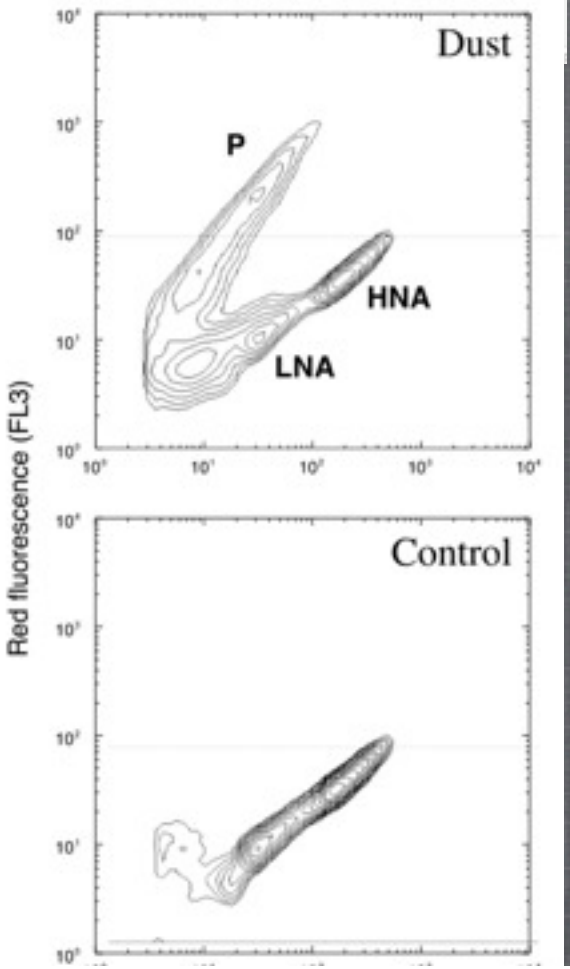


image: M. Weinbauer & R. Cattaneo

Sahara dust particles

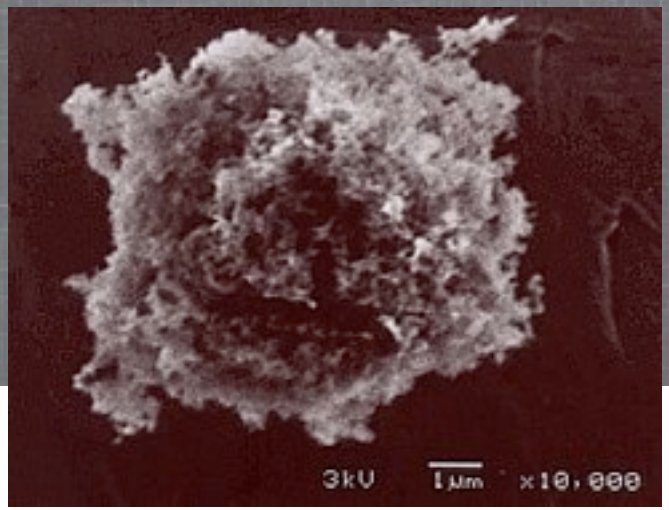


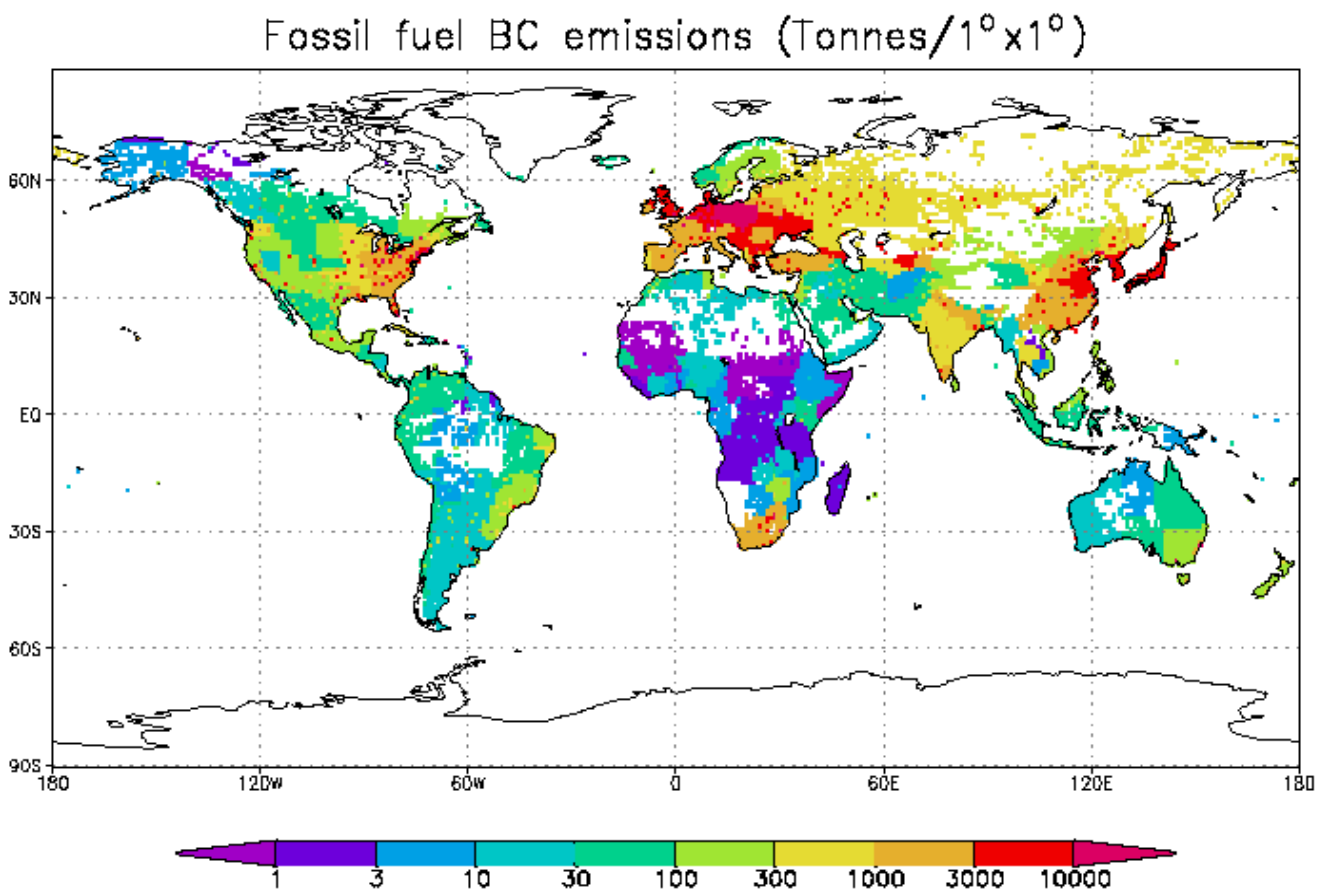




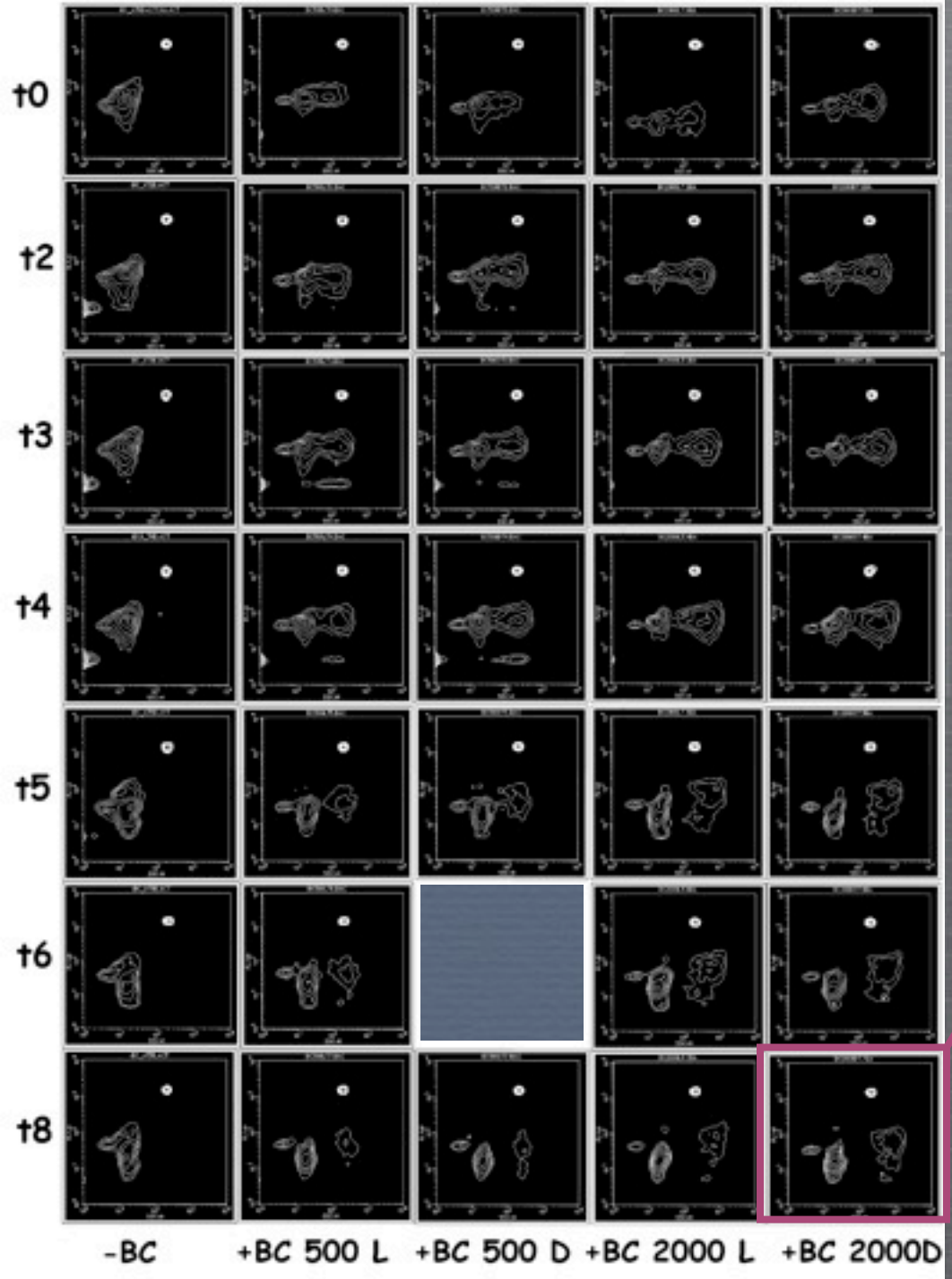
Lekunberri, I., T. Lefort, E. Romero, E. Vazquez-Dominguez, C. Romera-Castillo, C. Marrase, F. Peters, M. Weinbauer, and J.M. Gasol. 2010. Journal of Plankton Research **32**: 381-396.

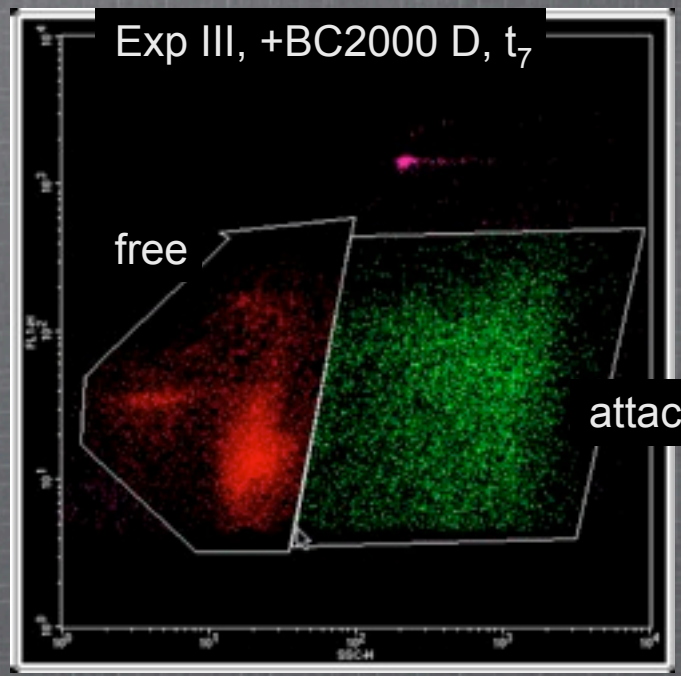
Black Carbon (soot) particles





Experiment III:
Range of BC concentrations:



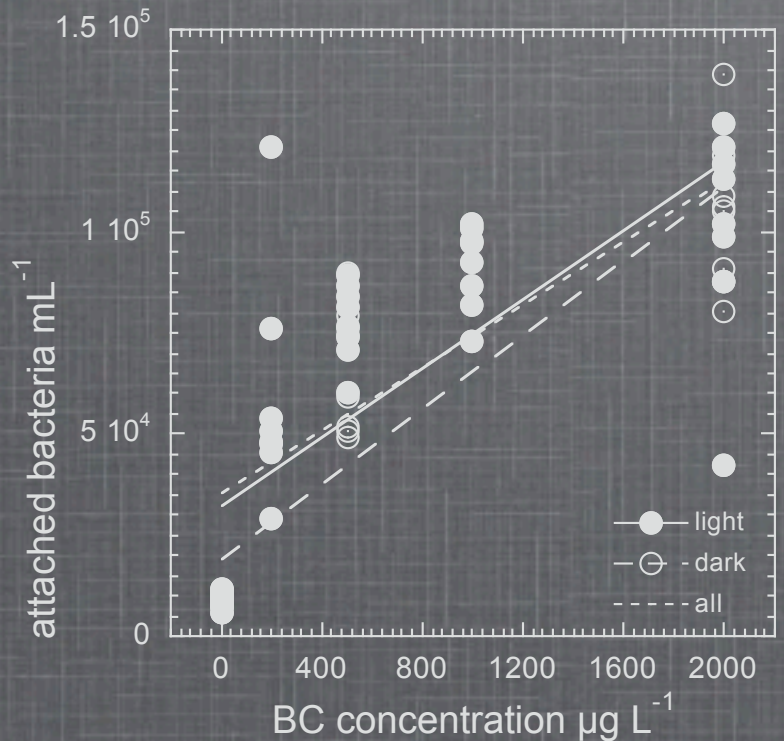


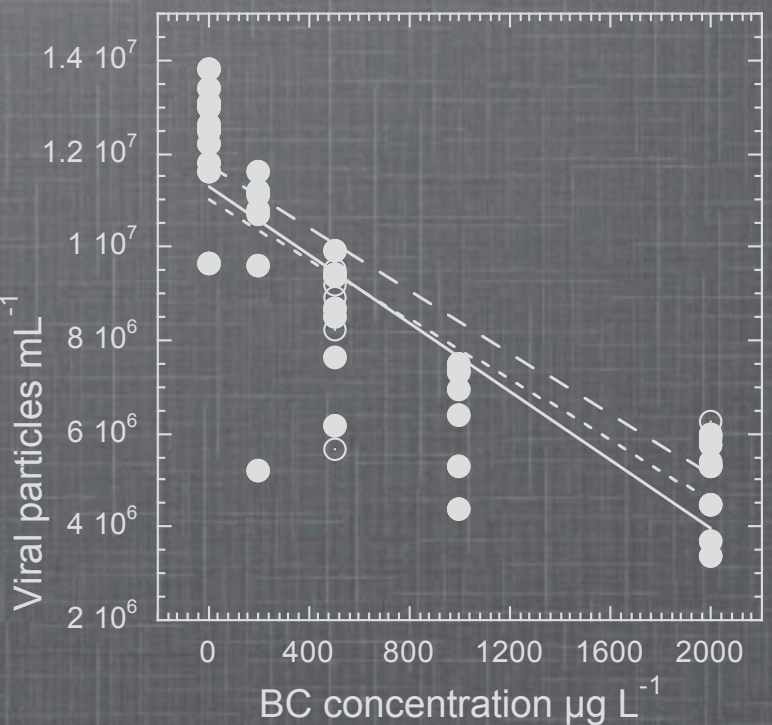
Malits et al., in prep.

Experiment III:

abundance of attached bacteria increases with BC concentration

abundance of viral particles decreases with BC concentration

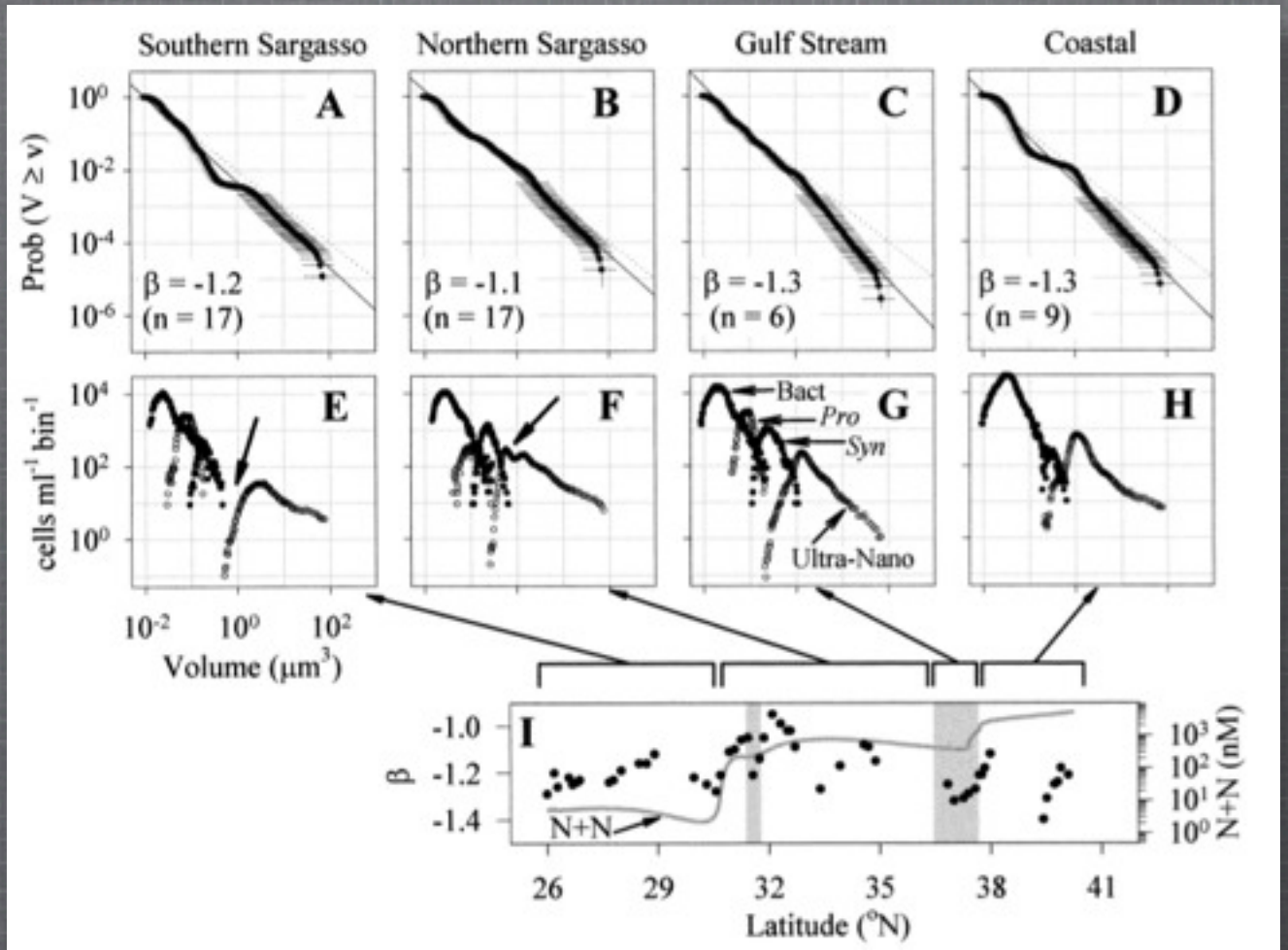




Malits et al submitted

A personal view of the last 20 yr. highlights

- Routine enumeration of pico- and nanoalgae (>80's)
- Not yet: enumeration of large and "rare" cells
- Routine enumeration of heterotrophic bacteria (90's)
- Routine enumeration of planktonic viruses (00's)
- Enumeration of heterotrophic protists (05's)
- Not yet: enumeration of BChlorophyll containing microorganisms
- Not yet: non-living particles, and particle-attached microbes
  - organic particles (gels...)
  - inorganic particles (Saharan dust, black carbon)
  - particle-attached microbes
- Many physiological probes been tried (for live/dead - active/inactive)
- Not yet: good understanding of "microbial death" and physiological community structure
- Relate scatter and cell size and build phytoplankton Size spectra



Cavender-Bares et al. 2001-L&O

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Microbes and flow cytometry (bias to heterotrophs)

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6) Going further: cytometric diversity
7) Going further: Probing ecosystem function
   Relating community structure to ecosystem functioning

Can we define and use the cytometric diversity ?

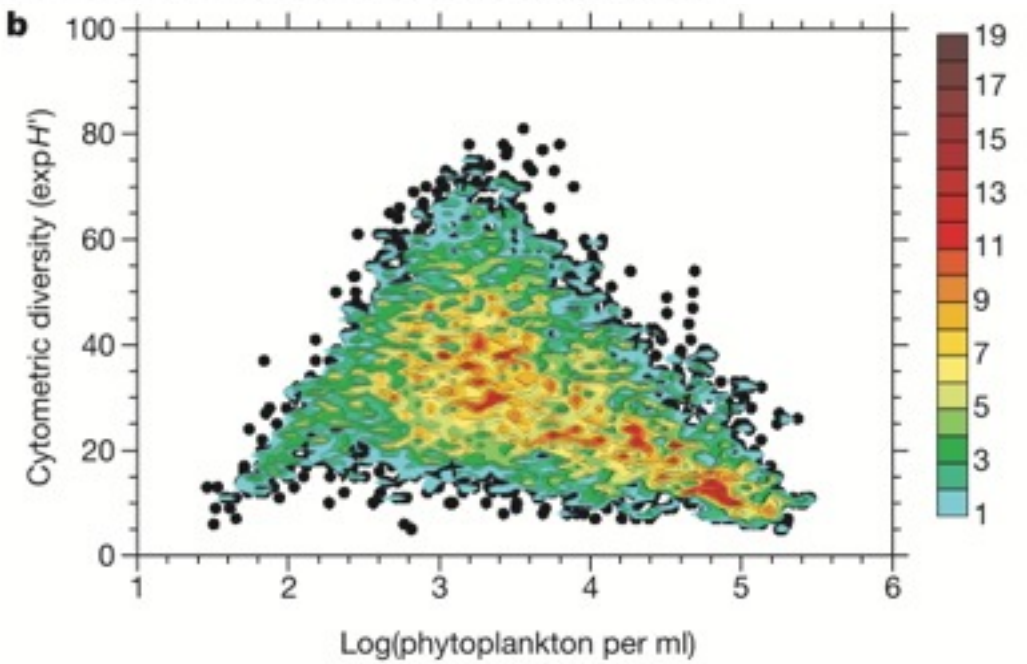
Limnol. Oceanogr., 42(5), 1997, 874–880

Cytometric diversity in marine ultraphytoplankton

W. K. W. Li
Biological Oceanography Section, Bedford Institute of Oceanography, P.O. Box 1006, Dartmouth, Nova Scotia B2Y 4A2

Abstract

The concept and methods of ecological diversity in communities were applied to phytoplankton categorized by flow cytometric measurements related to size and chlorophyll content. Each cytometric signature was condensed to single numerical values indicative of diversity and evenness. Measurements pooled from studies disparate in temporal and spatial scales indicated greater chlorophyll biomass and primary production with greater cytometric diversity and evenness. Future development of these ideas may help link biological oceanographic processes with patterns established through ecological processes at the community level.



Li, W. K. W. 1997. Cytometric diversity in marine ultraphytoplankton. Limnology and Oceanography **42**: 874-880.

Li, W. K. W. 2002. Macroecological patterns of phytoplankton in the northwestern North Atlantic Ocean. Nature **419**: 154-157.

Santa Pola Salterns'99

Picophytoplankton

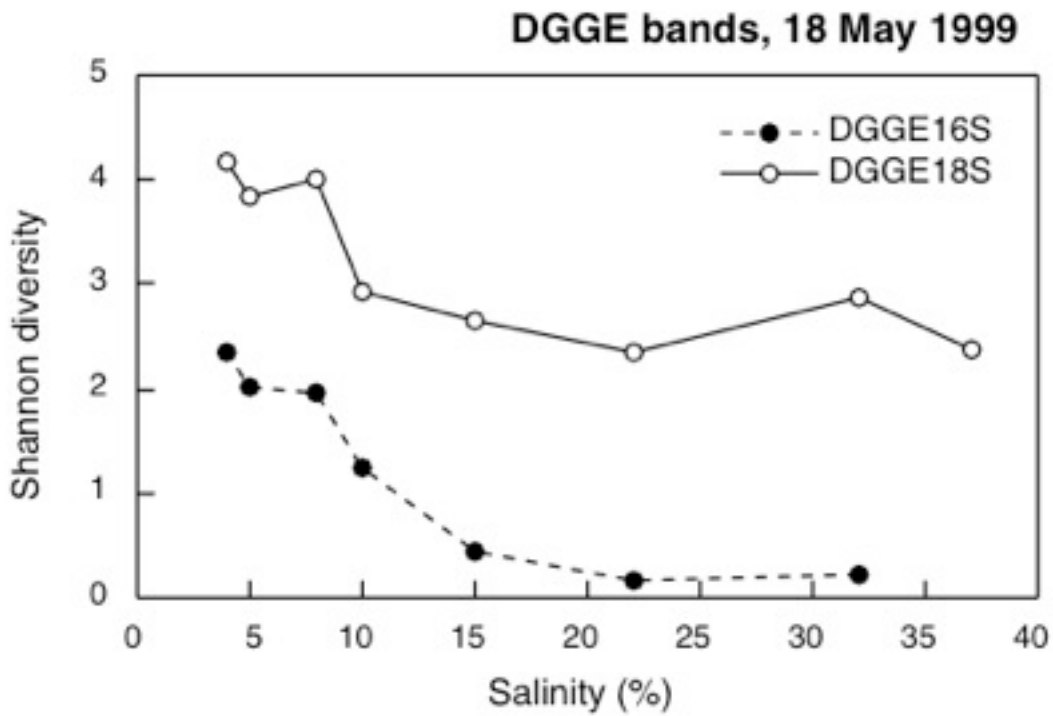
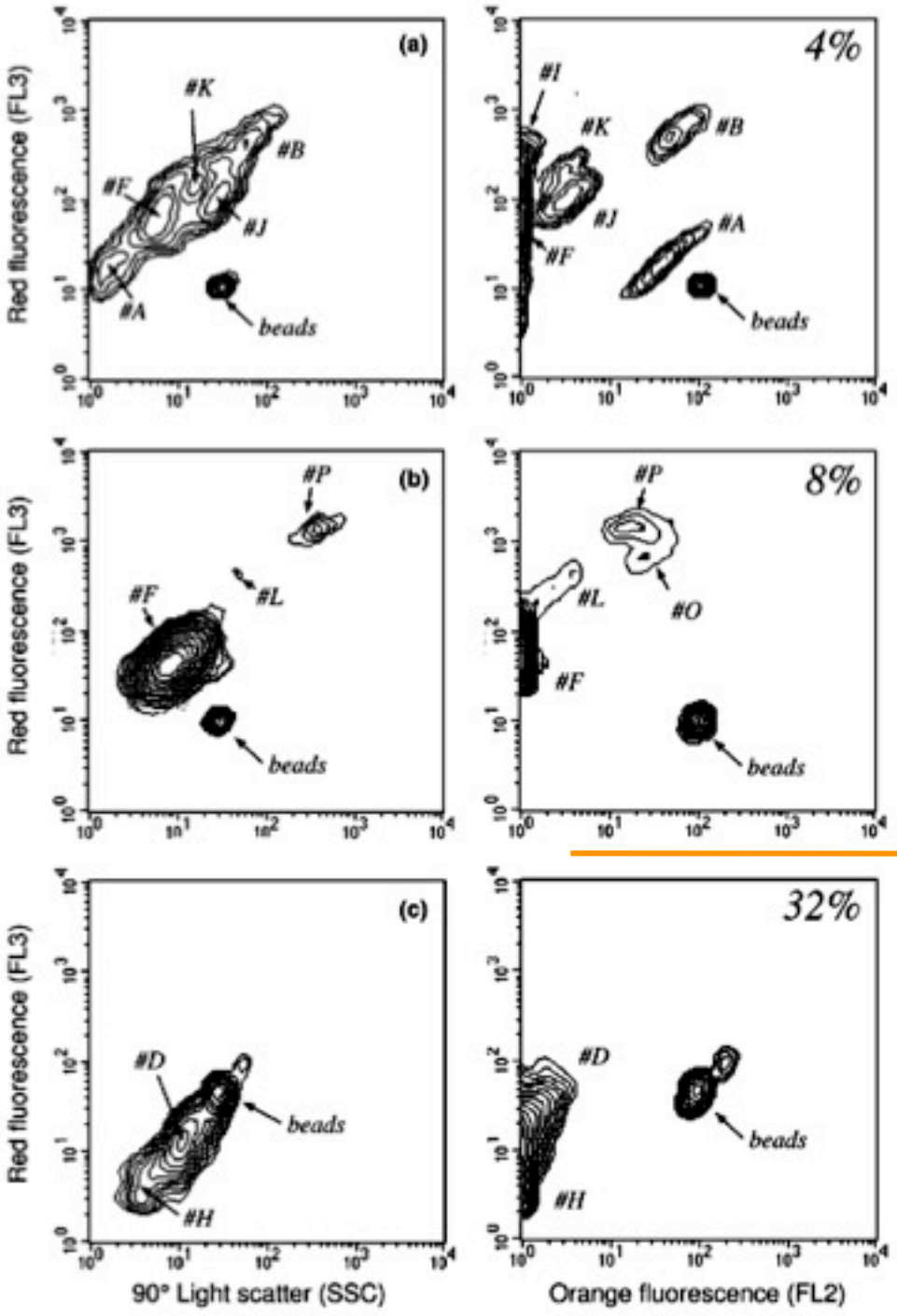


Fig. 8. Distribution of the Shannon diversity index based on the number and intensity of bands in DGGE gels from samples along the salinity gradient on 18 May, after a PCR with primers for 16S rRNA (D_{16S}, filled symbols) or for 18S rRNA (D_{18S}, empty symbols).

Estrada et al. 2004. FEMS-ME



Red

Orange

Estrada et al. 2004. FEMS-ME

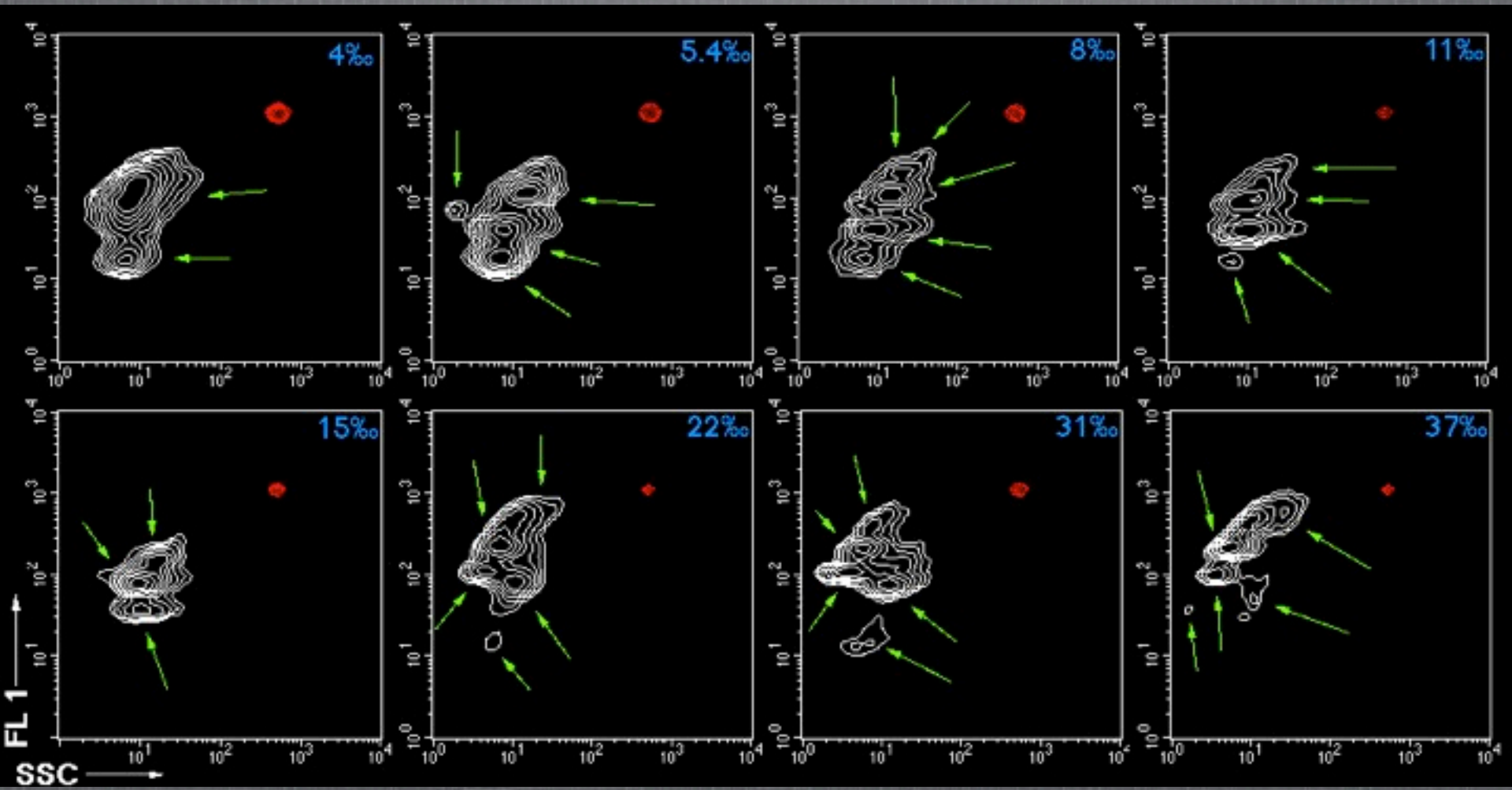
4.4. Diversity patterns

As can be seen in Table 3, the number of classes of the different variables considered (S_x) tended to decrease with salinity and reached the minimal values in the crystallizers. All S_x indices were significantly correlated (Table 4). Due to the variety of methods used, different numbers of classes must be expected, even when dealing with the same organisms. For example, morphological differences among filamentous cyanobacteria, will not

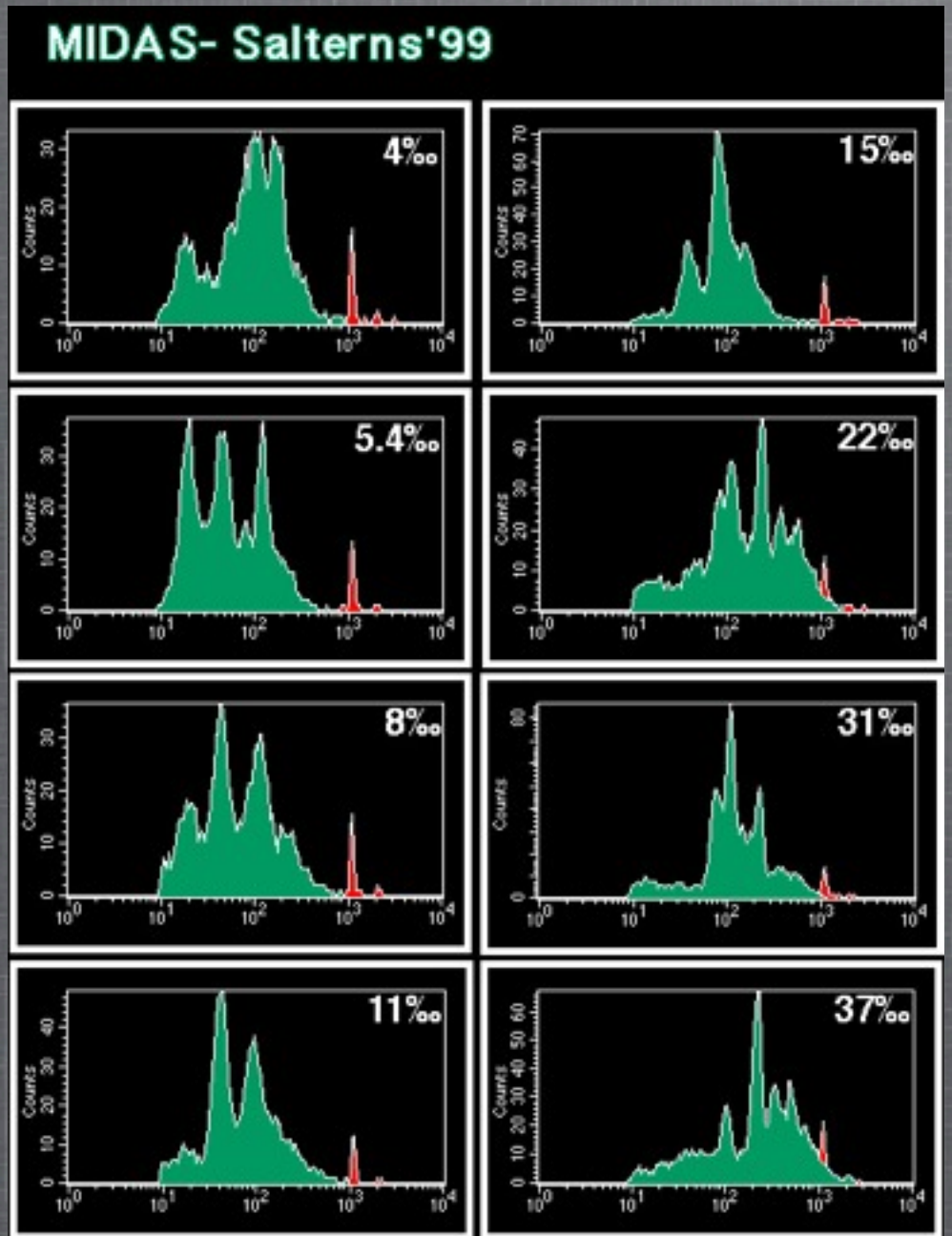
Fig. 7. Distribution of the Shannon diversity indices on 18 (filled symbols) and 26 May 1999 (empty symbols) for (a) pigment concentrations determined by HPLC (D_P), (b) phytoplankton counts by the inverted microscope technique (D_M) and (d) fluorescent picoplankton counts (DF). (c) Distribution of the K_M diversity index for phytoplankton (see text).

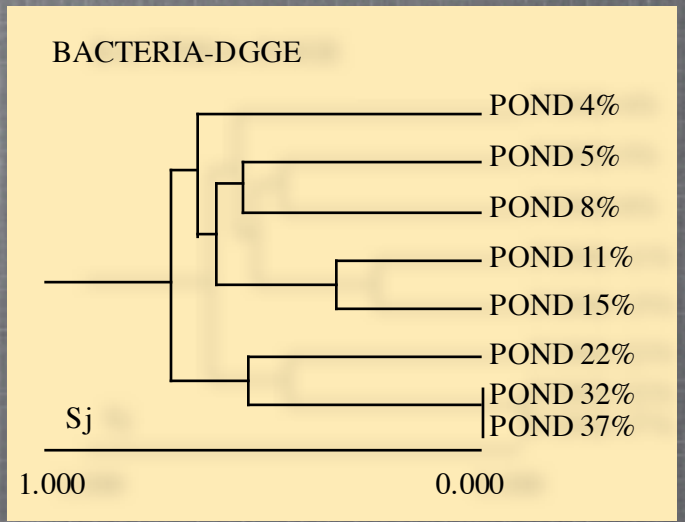
Estrada et al. 2004. FEMS-ME

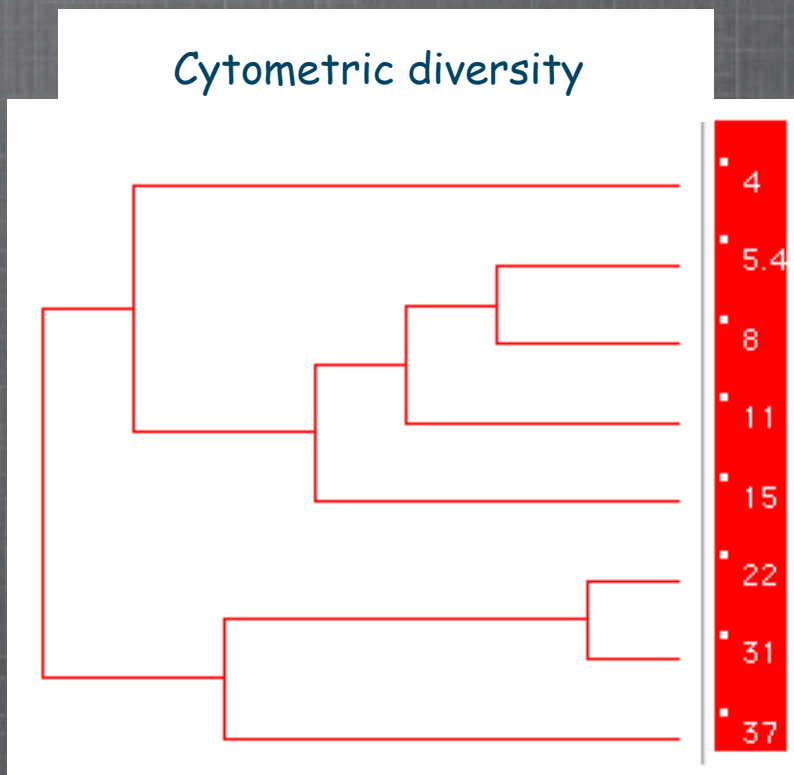
Santa Pola Salterns'99

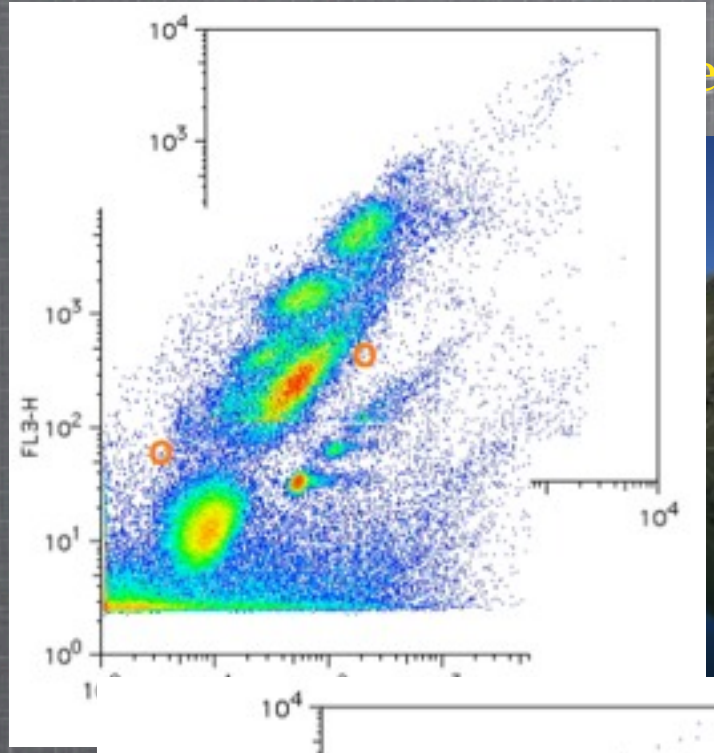


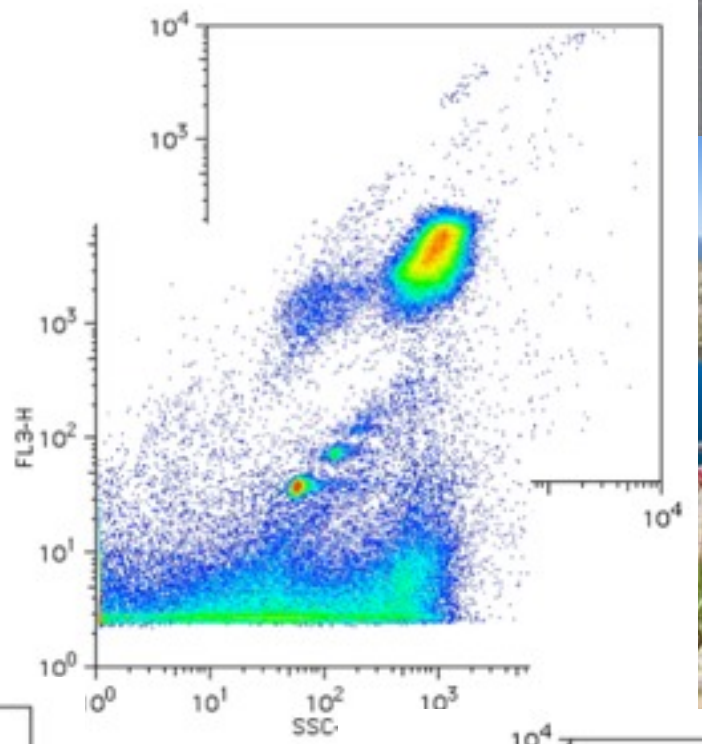
Gasol, J. M., E.O. Casamayor, I. Joint, K. Garde, K. Gustavson, S. Benlloch, B. Díez, M. Schauer, R. Massana, and C. Pedrós-Alió. 2004. Aquatic Microbial Ecology **34**: 193-206.

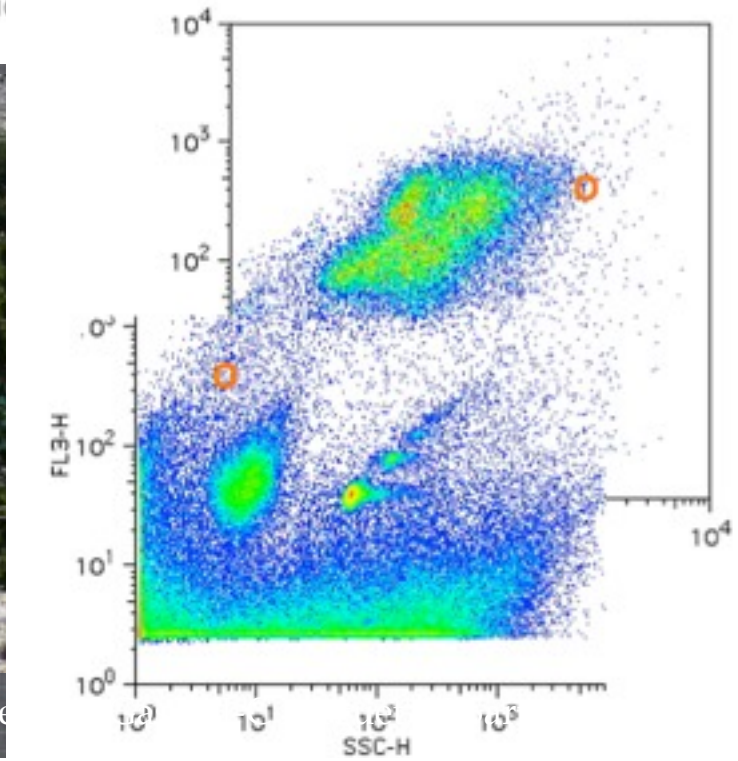

MIDAS- Salterns'99
4‰
15‰
5.4‰
22‰
8‰
31‰
11‰
37‰
Counts

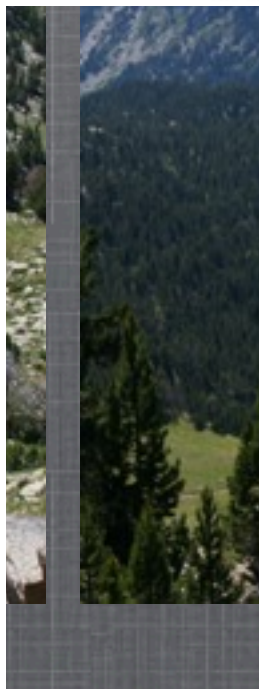

BACTERIA-DGGE
POND 4%
POND 5%
POND 8%
POND 11%
POND 15%
POND 22%
POND 32%
POND 37%
Sj
1.000
0.000

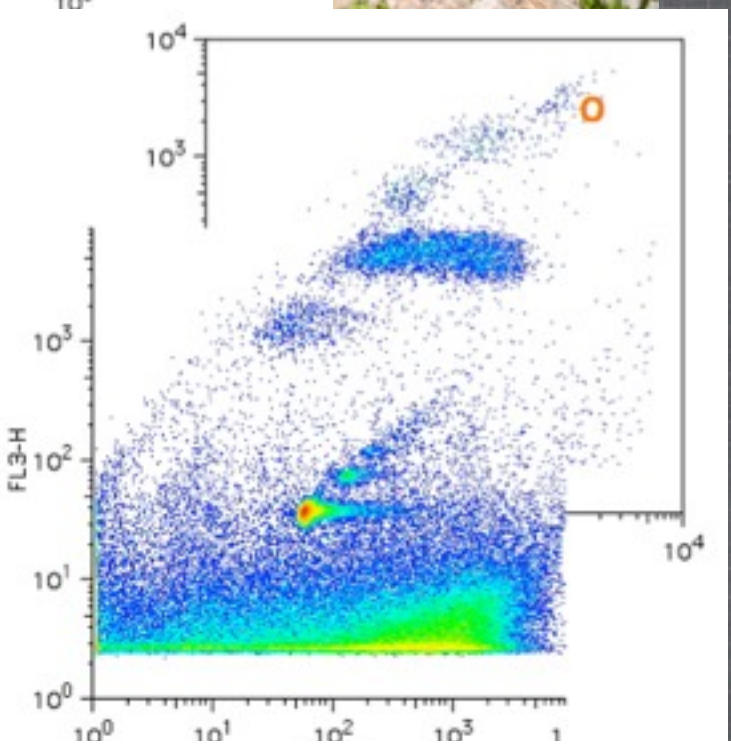

Cytometric diversity
4
5.4
8
11
15
22
31
37





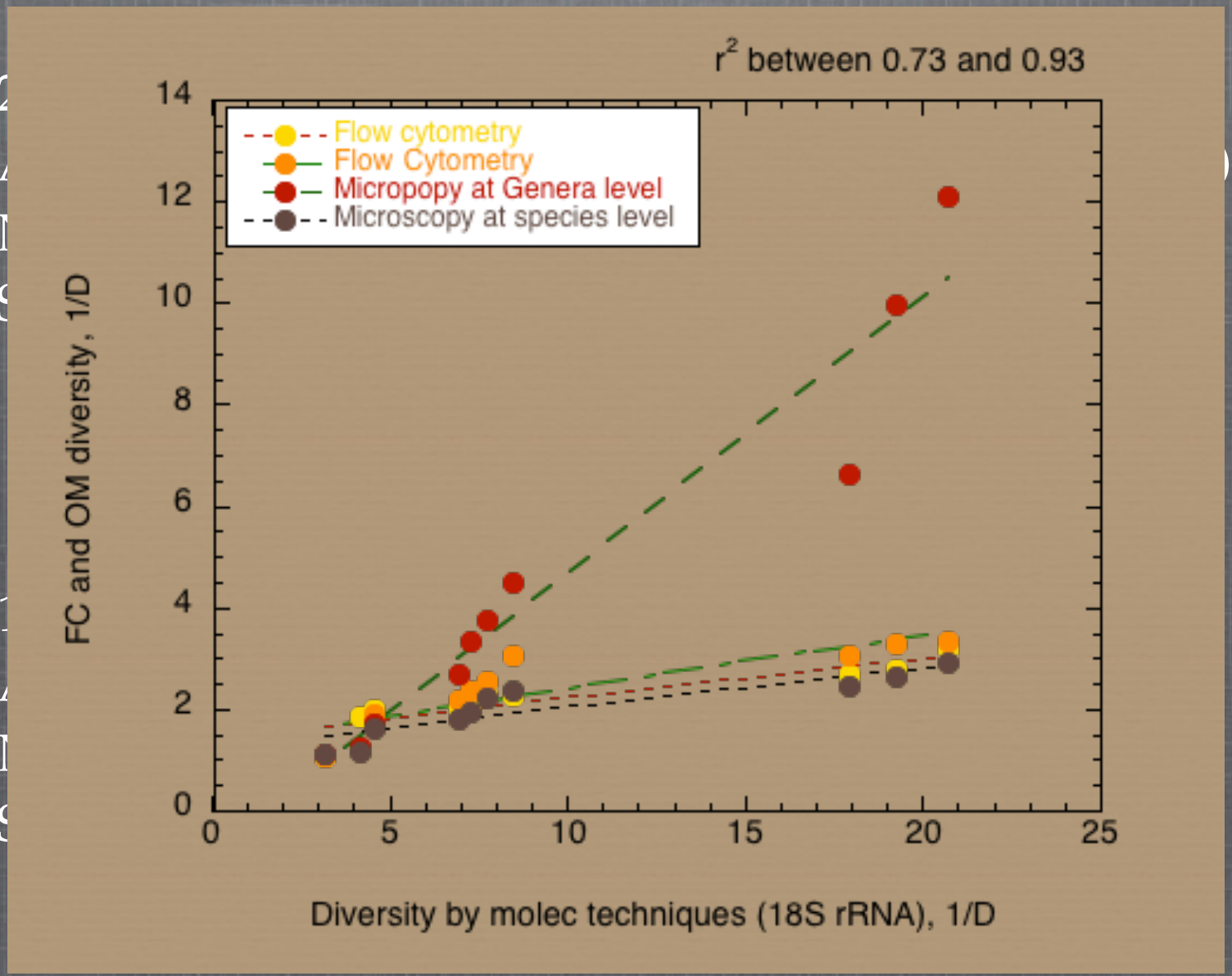






Image

Pyreenes lakes, summer 2008



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   Relating community structure to ecosystem functioning

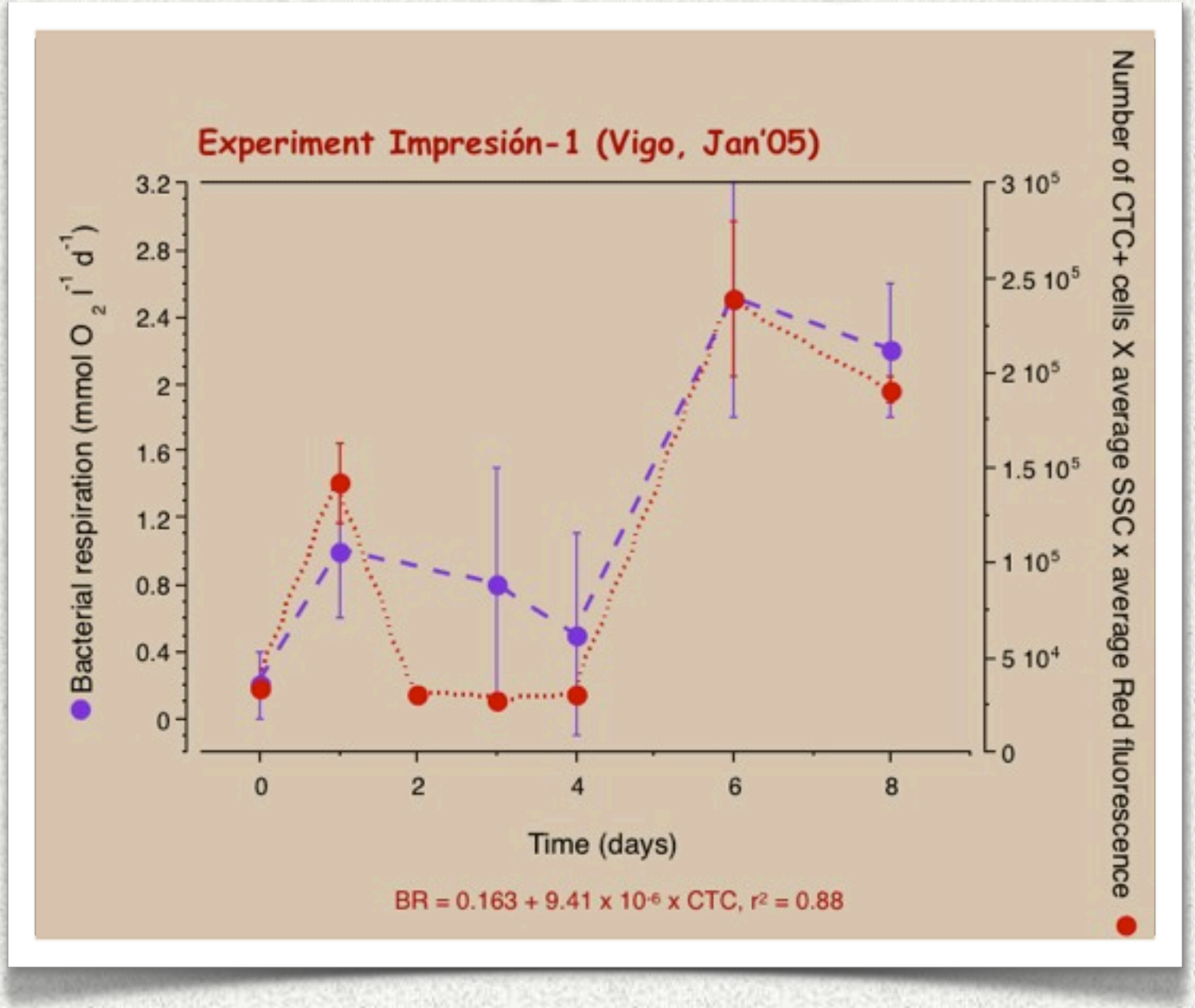
Probing ecosystem function

Relating diversity to ecosystem function

- Metabolic and indicator probes
  - Respiratory probes (e.g. CTC)
  - Phosphatase act (Dignum et al 2004-FEMSME; Duhamel et al. 2008-JMM)
- Mesuring processes
  - diel cycles and growth rates
  - viral infection and mortality (Brussaard et al. 2001-AME)
  - sulfur accumulation
  - bacterial losses rates to grazers (Vazquez-Dominguez et al. 2005-AME)
  - single-cell HNF activity (Sintes & del Giorgio 2010-EMI)
- C and Nut. flows through different populations
- Population identification
- Linking diversity with function

  radioactive incorporation and cell sorting and molecular analyses

CTC as a respiration probe

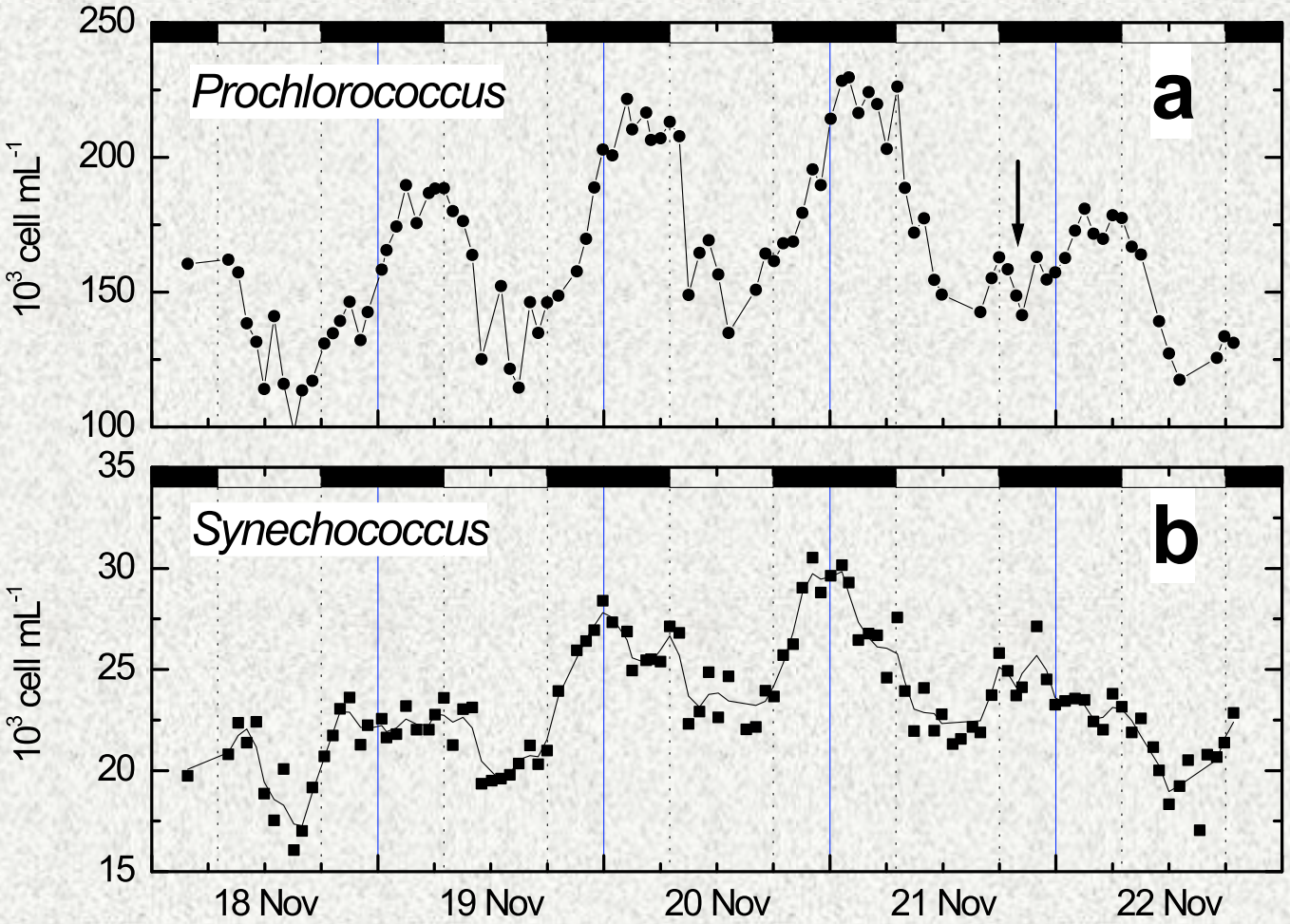


Gasol & Serret, unpubl.

Diel pigment variability and growth

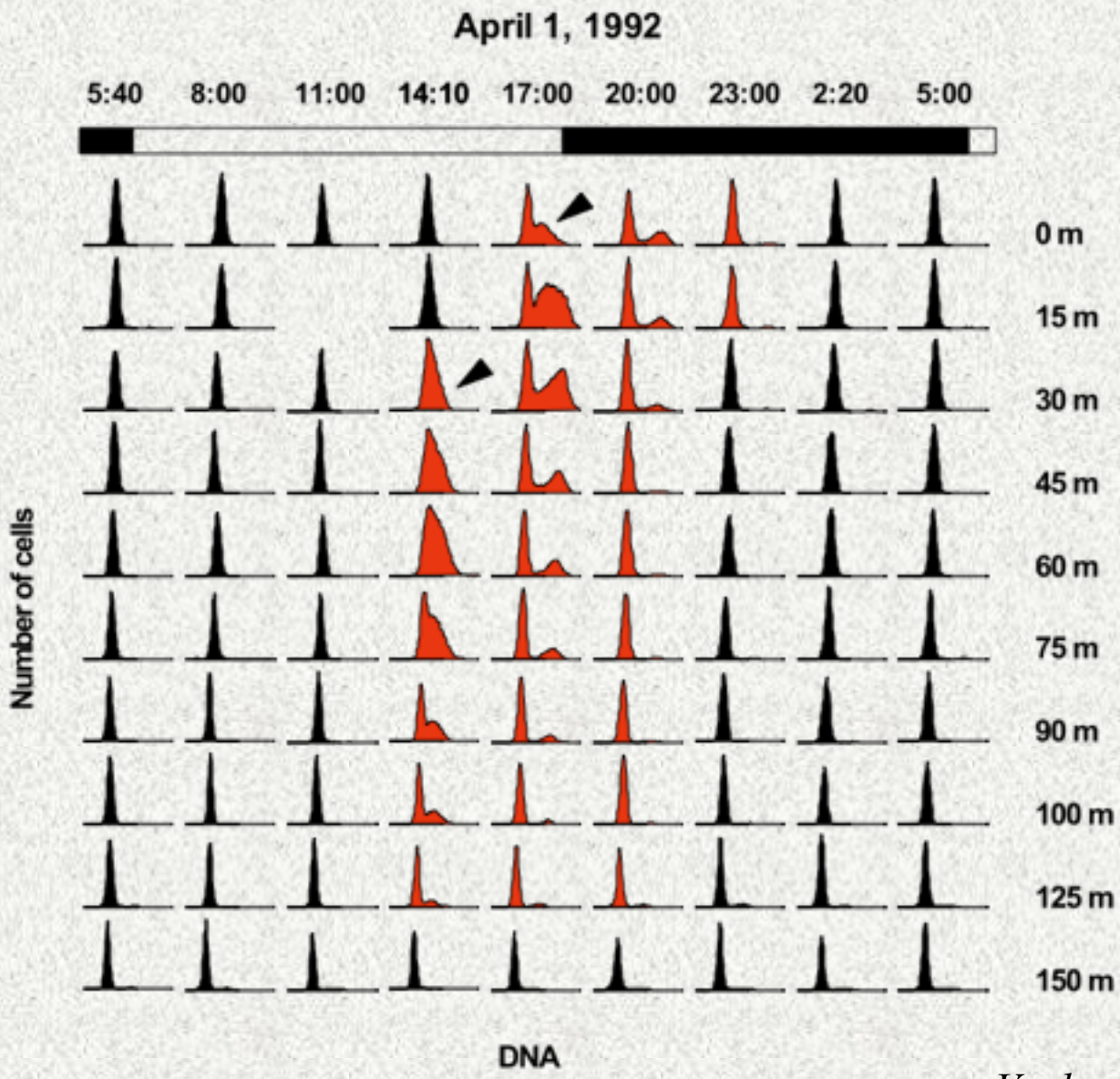
150°W 5°S

Equatorial Pacific



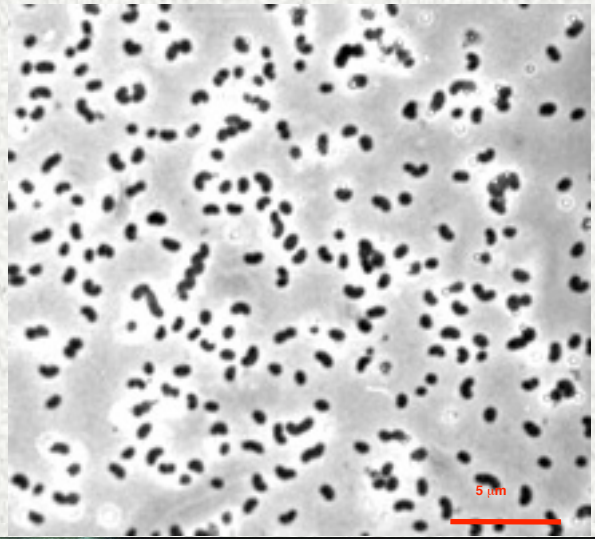
Vaulot and Marie, 1999 JGR

Diel pigment variability and growth

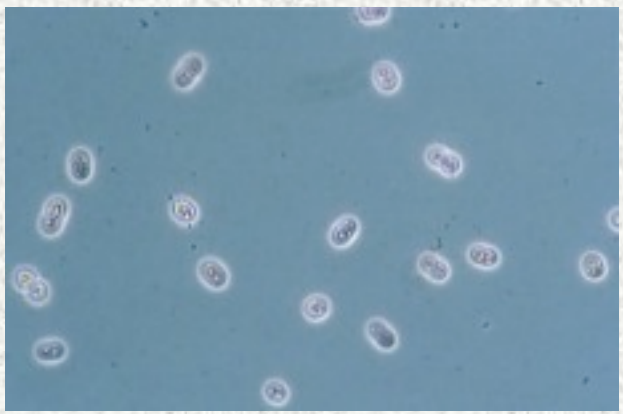


Vaulot et al., 1995 Science

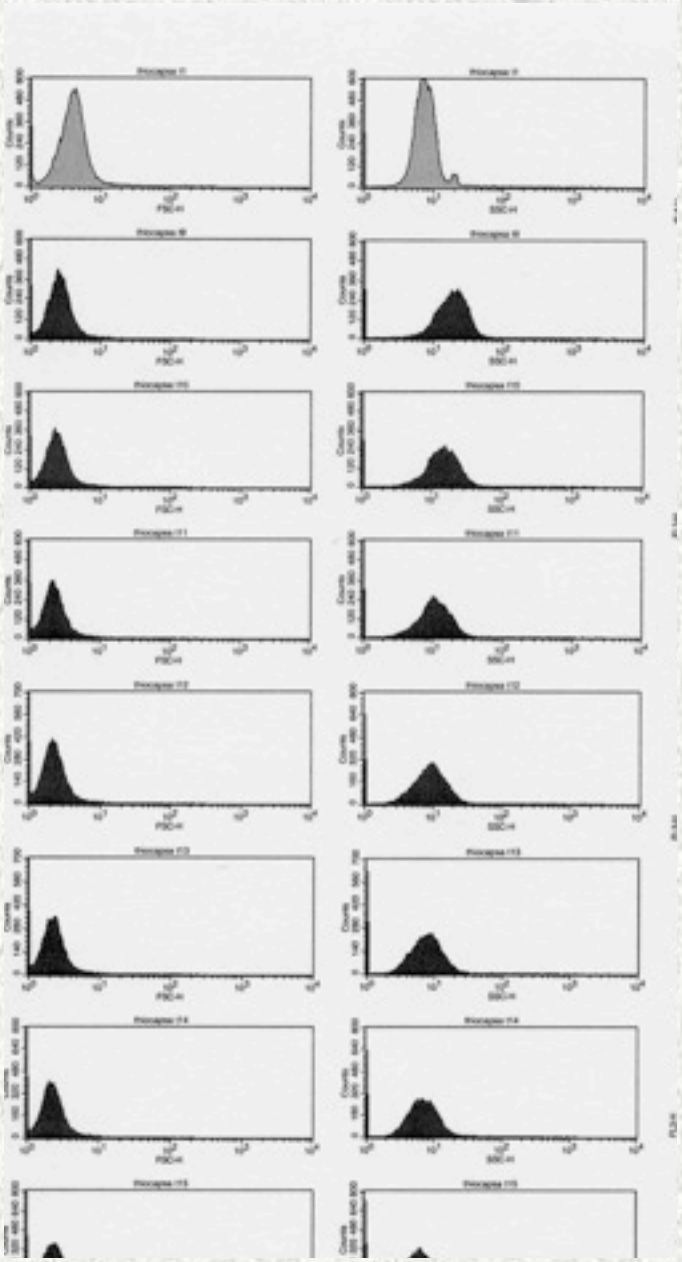
Photosynthetic activity of AnAnB



Anoxygenic
anaerobic
photosynthetic
prokaryotes



Photosynthetic activity of AnAnB

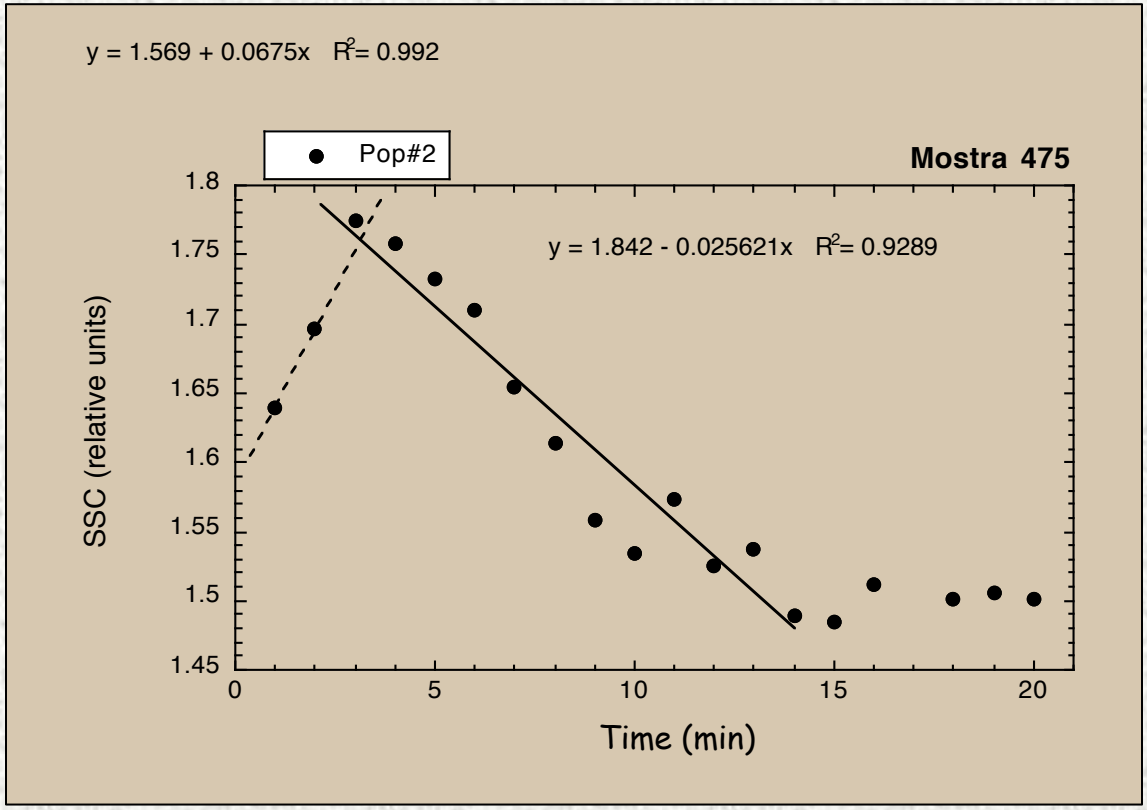


Sulfur as a source of reducing power for photosynthesis

$H_2S - S^0$

$S^0 - SO_4$

intracellular or attached S^0 changes SSC

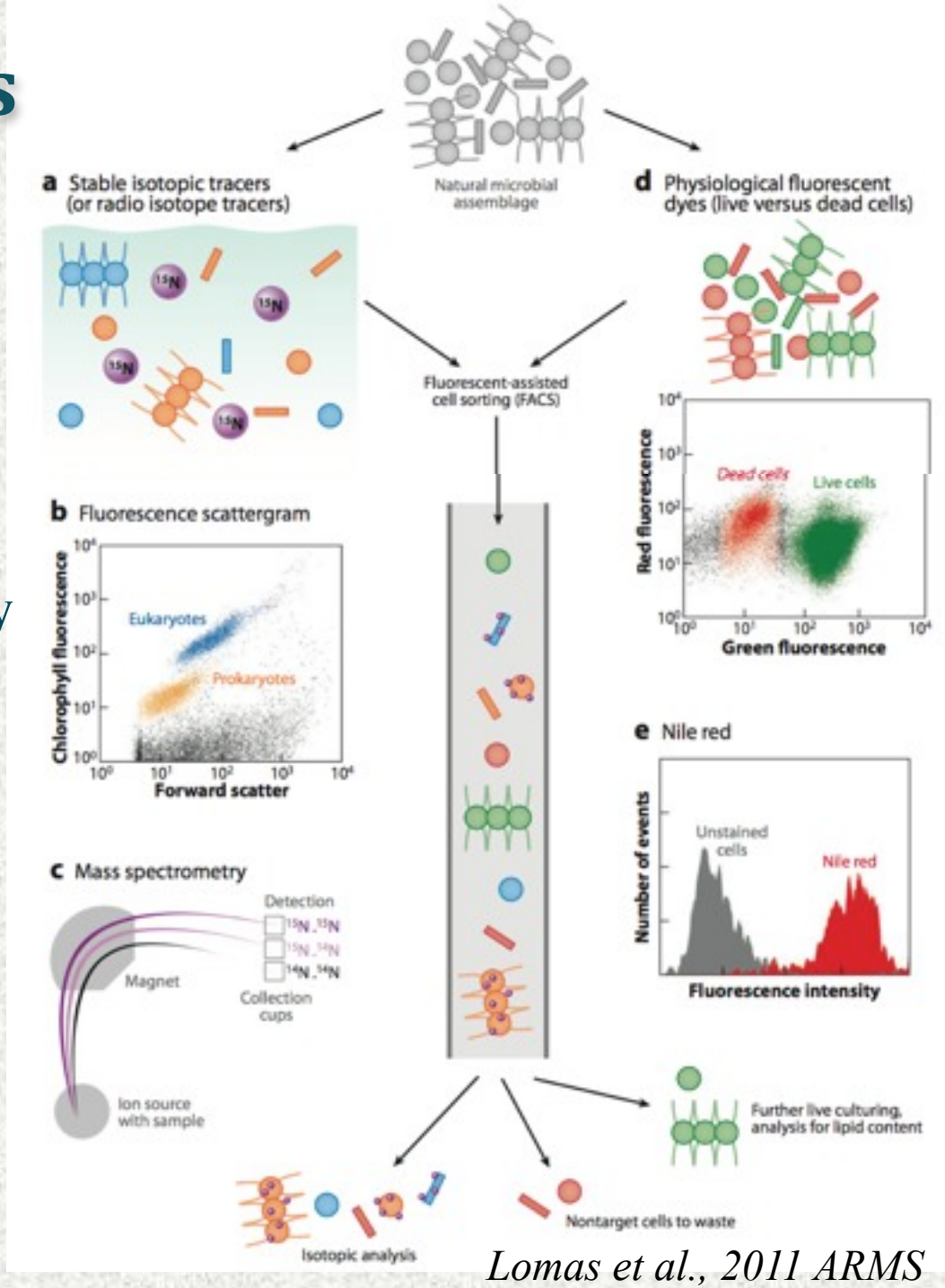


Casamayor et al.2007. EMI

C and nutrient flows through different populations

- by size, natural fluorescence, induced fluorescence, or activity

- downstream
  - rad. labeling
  - stable isotopes
  - chemical analyses
  - microscopy
  - DNA & RNA analyses
  - cultivation



Lomas et al., 2011 ARMS

Radioactivity/stable isotope Cell sorting

- ^{14}C-uptake (Rivkin et al. 1986, Li 1994)
- ^{15}N-uptake (Lipschultz 1995)
- ^{3}H-leucine (Servais et al.'99, '00, '03, Zubkov et al'04)
- ^{35}S-methionine (Zubkov et al.'03)
- ^{35}S-DMSP (Zubkov et al'01, Vila et al., Maelstrom et al.)

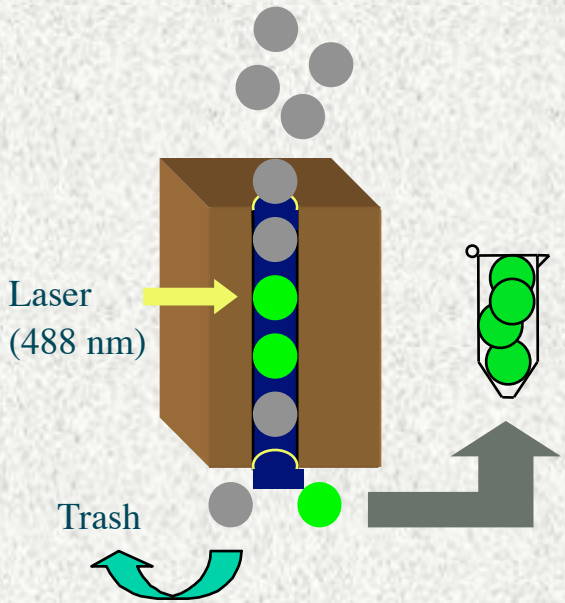


Table 1. Primary production of three North Atlantic ultraphytoplankton samples.

Sta.	Location	z (m)	%I_0*	Chl (μg liter^{-1})	Group†	n (cells liter^{-1})	P_c (fg C cell^{-1} h^{-1})	P‡	% agg
14	32°39.5'N, 26°39.2'W	60	4	0.21	PRO	1.62×10^{8}	0.27	44.2	57
					CYA	2.35×10^{6}	0.82	1.9	2
					LEUC	1.02×10^{8}	24.09	24.5	32
					sum			70.6	91
					agg			77.4	
20	29°00.1'N, 19°16.3'W	60	2	0.15	PRO	1.57×10^{8}	0.03	4.6	16
					CYA	3.34×10^{6}	0.19	0.6	2
					LEUC	1.24×10^{8}	17.93	22.3	79
					sum			27.5	97
					agg			28.3	
30	31°13.9'N, 10°48.0'W	1	91	0.37	PRO	6.63×10^{7}	0.81	53.6	11
					CYA	1.27×10^{7}	7.68	97.8	20
					SEUC	8.49×10^{6}	7.32	62.1	13
					LEUC	1.25×10^{8}	193.16	241.1	49
					sum			454.5	92
					agg			493.4	

* Irradiance as percent of intensity at sea surface.
† PRO—prochlorophytes; CYA—cyanobacteria; SEUC—small eucaryotic ultraphytoplankton; LEUC—large eucaryotic ultraphytoplankton; sum—PRO+CYA+SEUC+LEUC; agg—aggregate from filtration of bulk sample ≤3 mi.
‡ $P = (n \cdot p)10^{-3}$, ng C liter^{-1} h^{-1}.

Li 1994, L&O

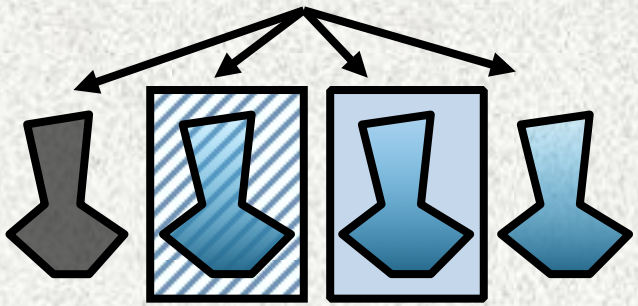
An example of C(S)-flow Cell sorting

Sunlight modulation of the relative activity of bacteria and picophytoplankton

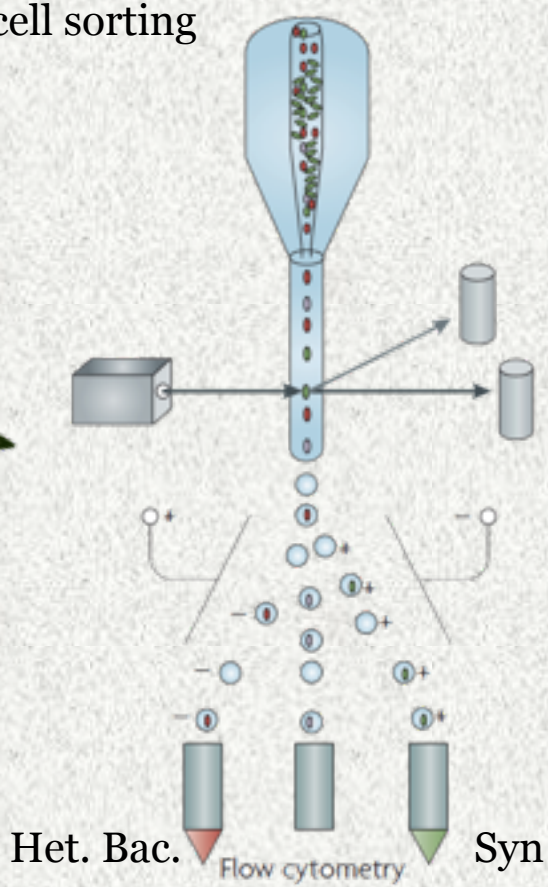
NW Mediterranean

Prokaryotic phytoplankton, major primary producers in oligotrophic systems

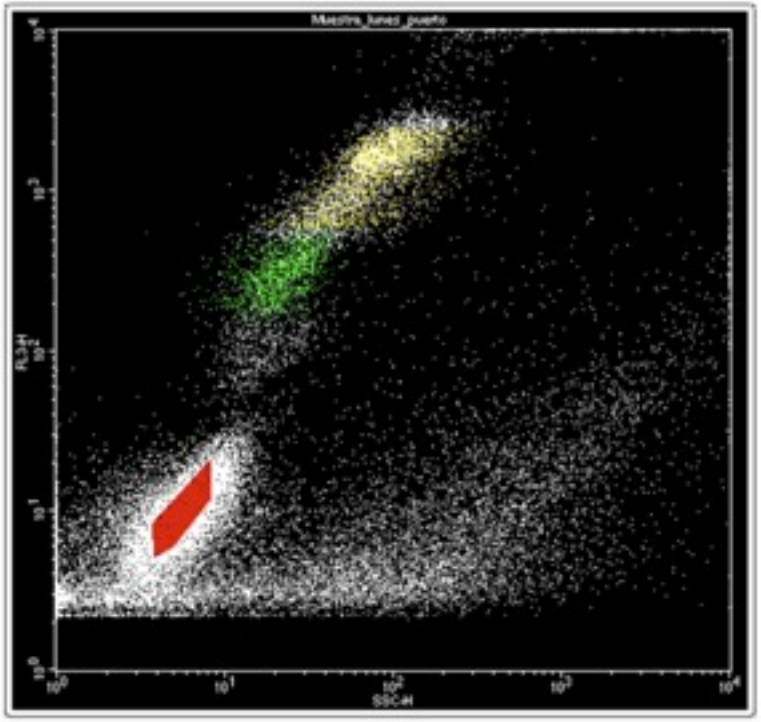
^{3}H-leucine or ^{35}S-DMSP

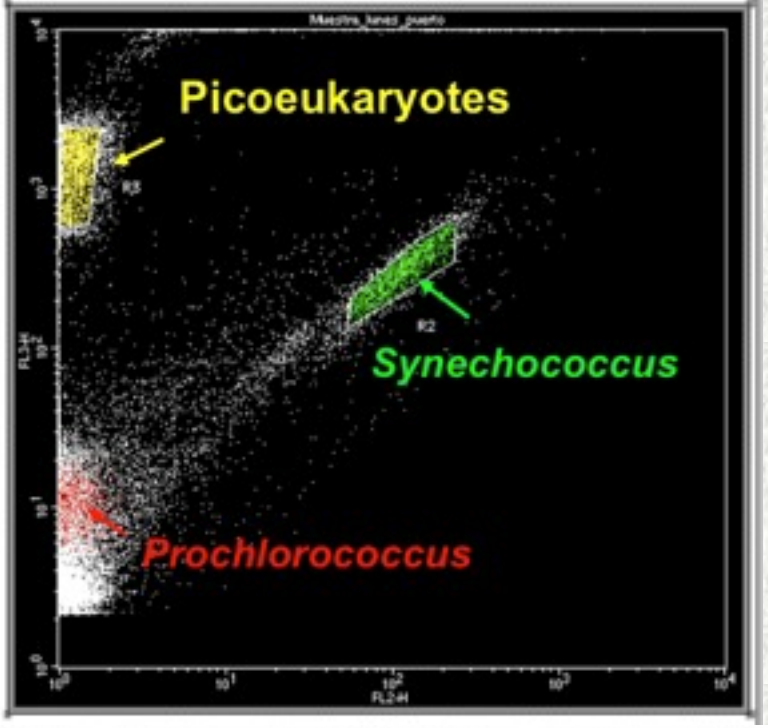


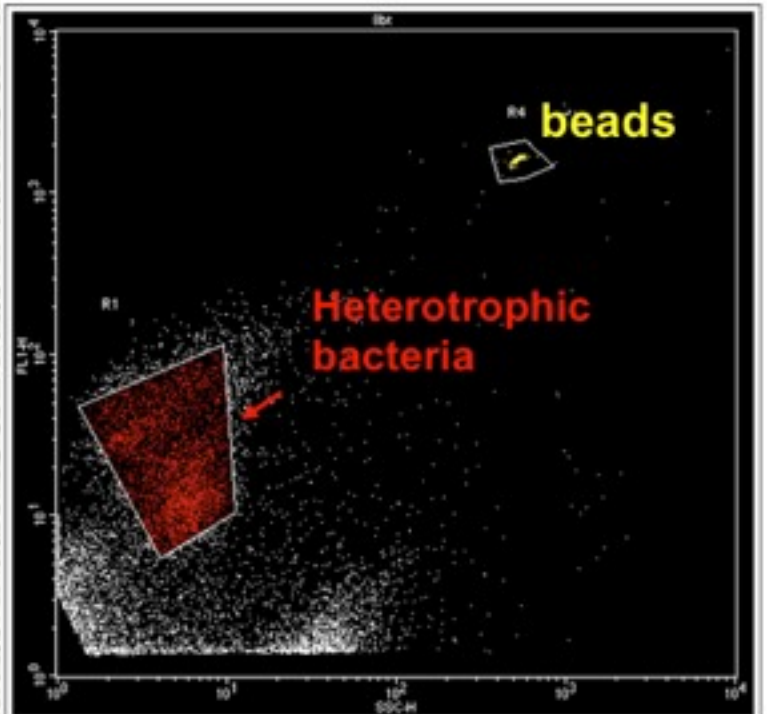
Flow cytometry cell sorting

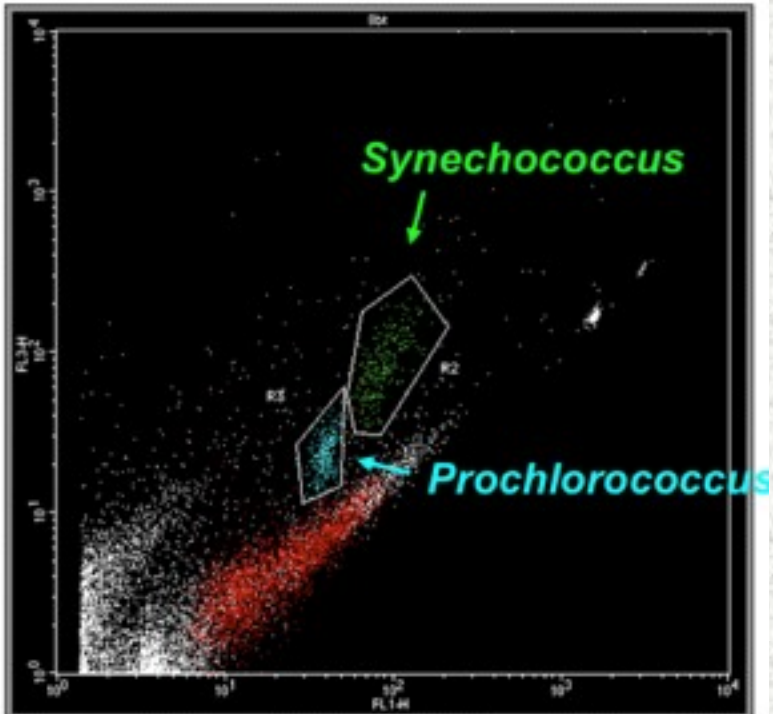


Vila-Costa et al. 2006, Science
Ruiz-González et al. ISME J. 2012




Picoeukaryotes
Synechococcus
Prochlorococcus

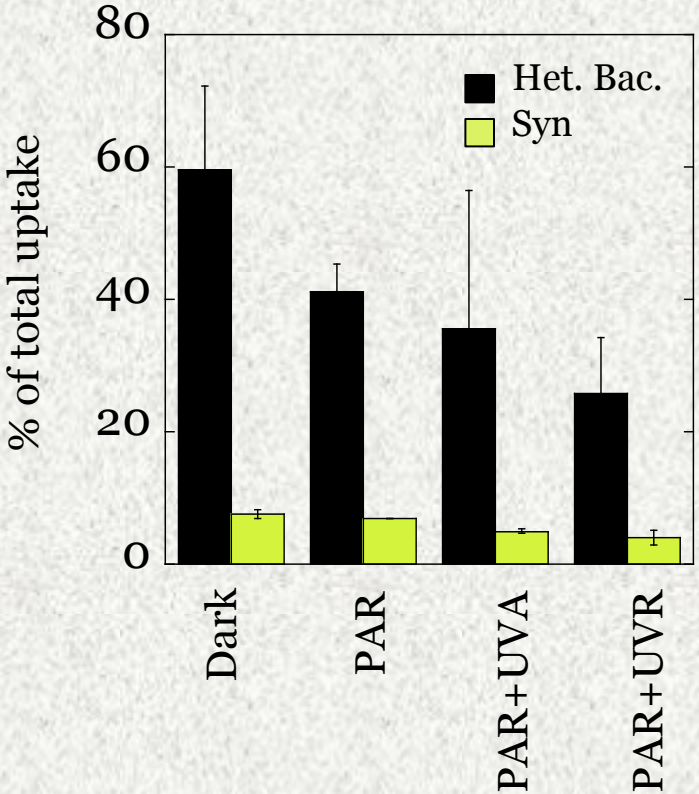

beads
Heterotrophic
bacteria

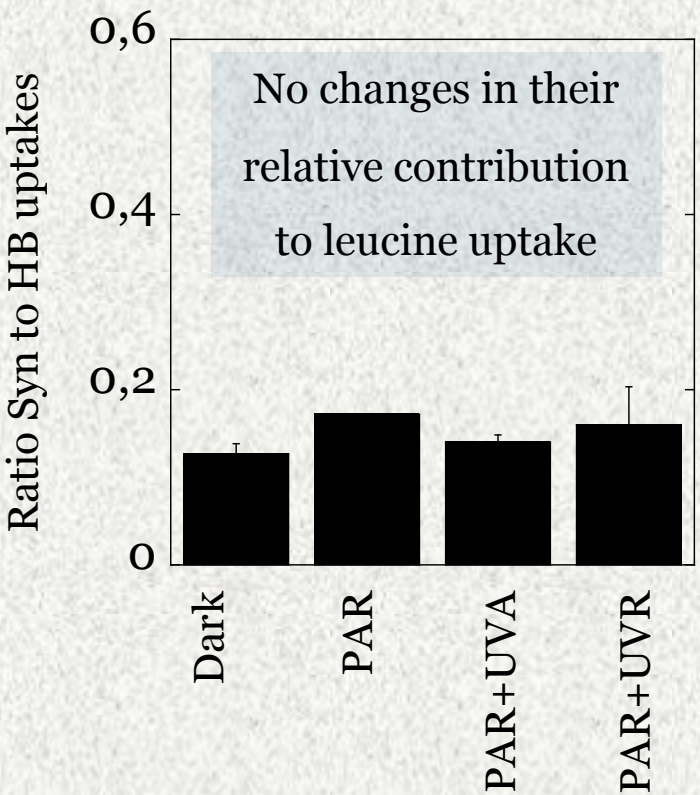

Synechococcus
Prochlorococcus

Sunlight modulation of the relative activity of bacteria and picophytoplankton

Flow cytometry cell sorting

^{3}H-leucine



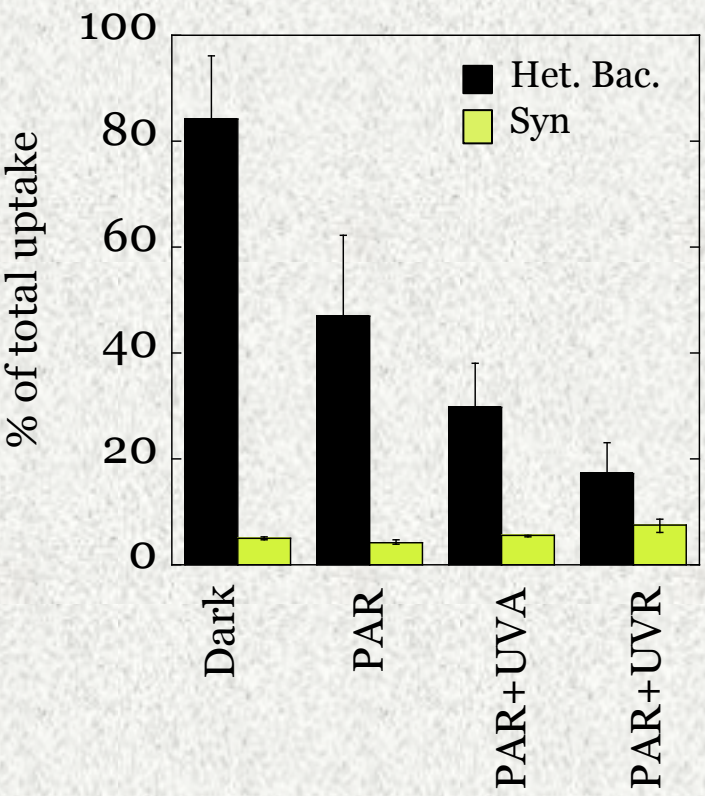


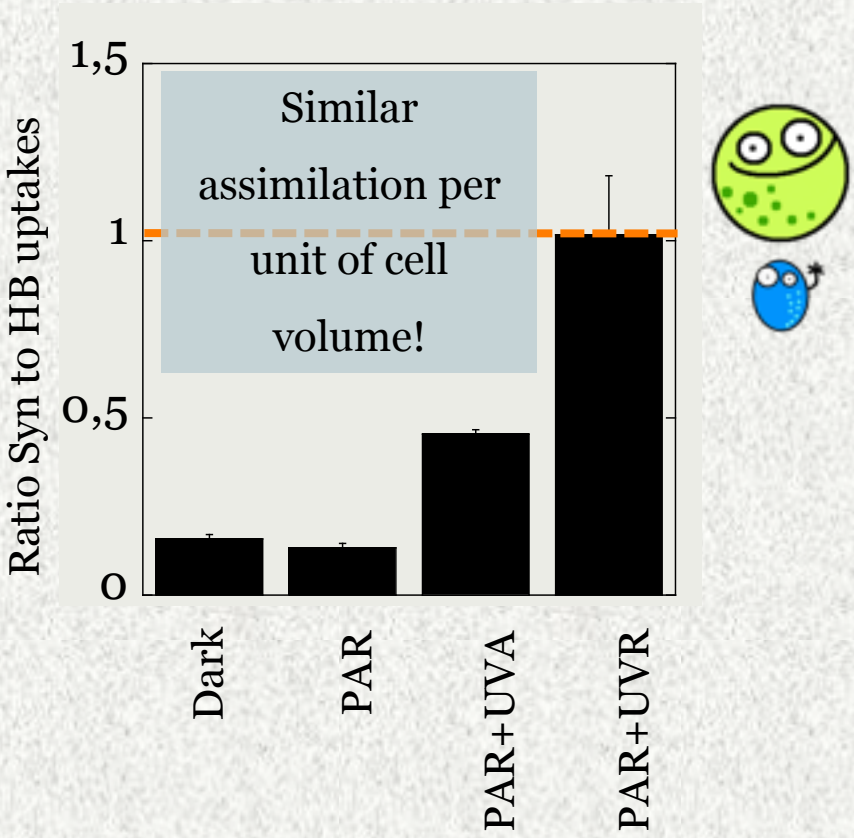
Ruiz-González et al. ISME J. 2012

Sunlight modulation of the relative activity of bacteria and picophytoplankton

Flow cytometry cell sorting

^{35}S-DMSP





Ruiz-González et al. ISME J. 2012

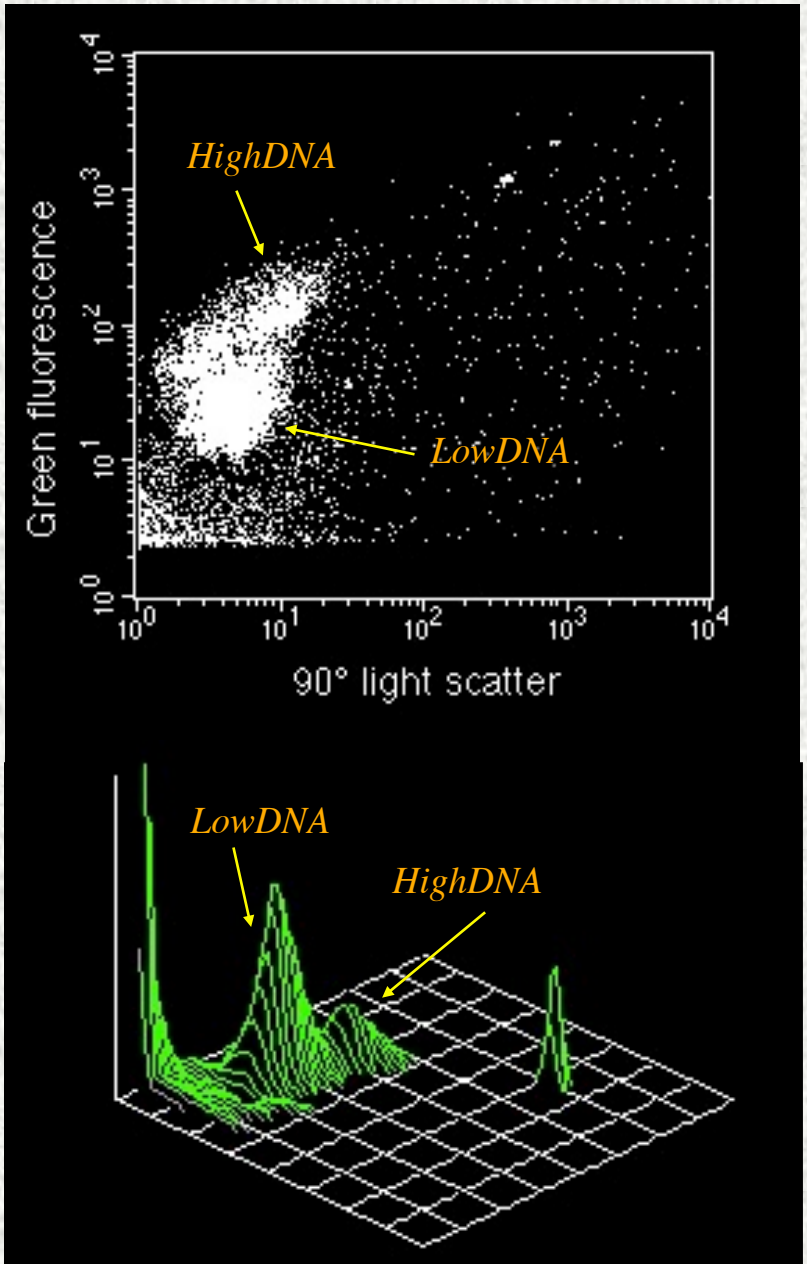
An example of cell sorting and diversity

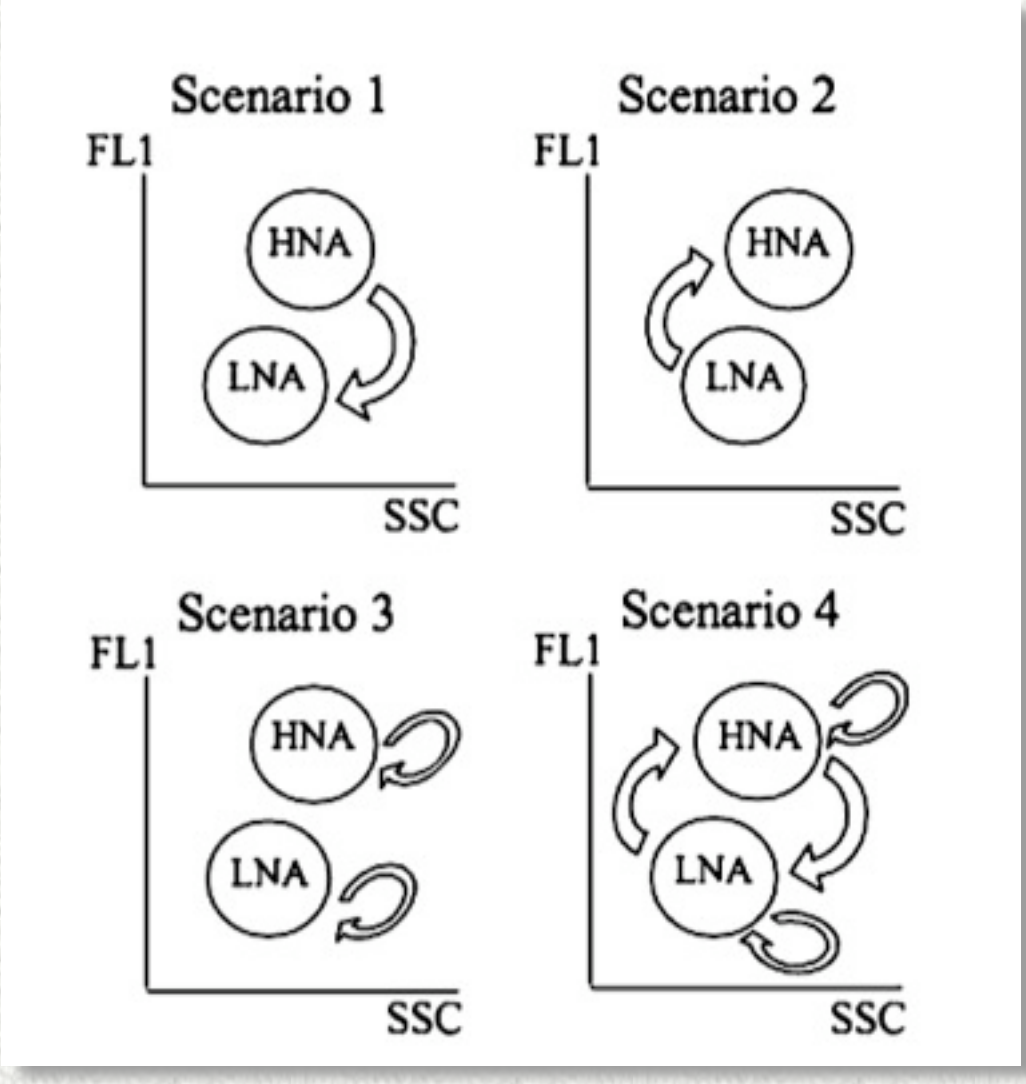
- Bacteria can be detected by flow cytometry after DNA staining

- It works similarly well with most DNA stains (Syto9, SybrGreen...)

- A plot of "size" (light scatter) vs. DNA fluorescence allows enumeration

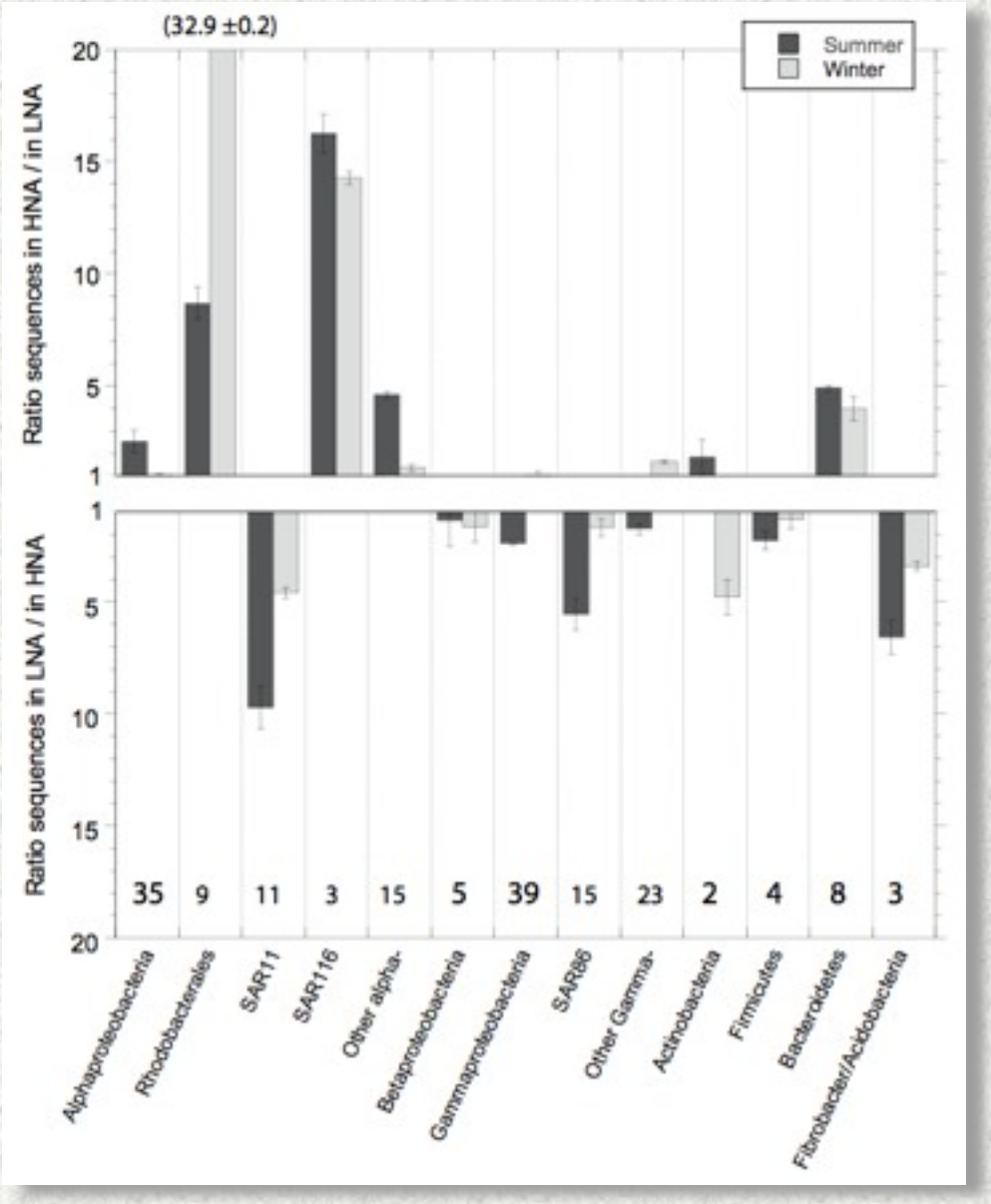
- Surprinsingly, at least two clear populations can be seen: HNA and LNA in almost all types of samples

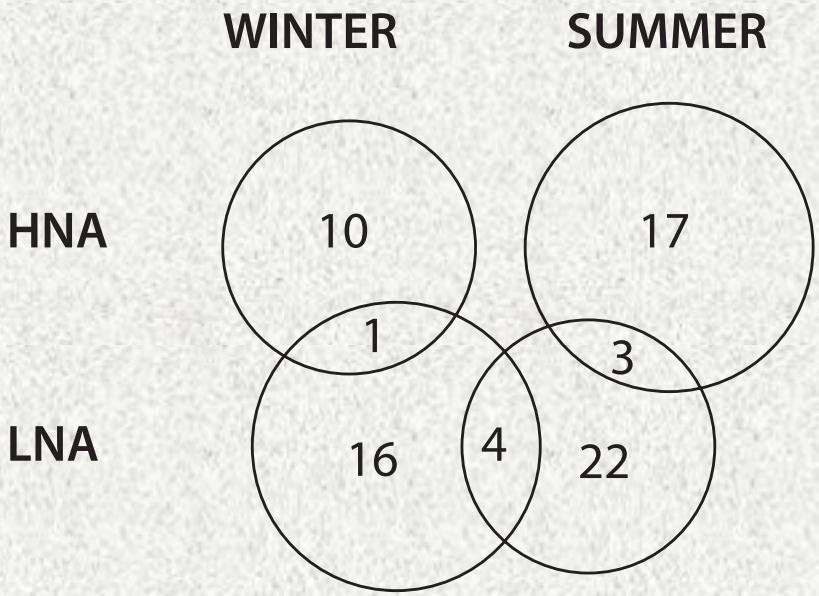




Bouvier et al'07-ENM

OK. Let's sort them out and deep sequence the populations
How many (OTUs) are common to the HNA and LNA?

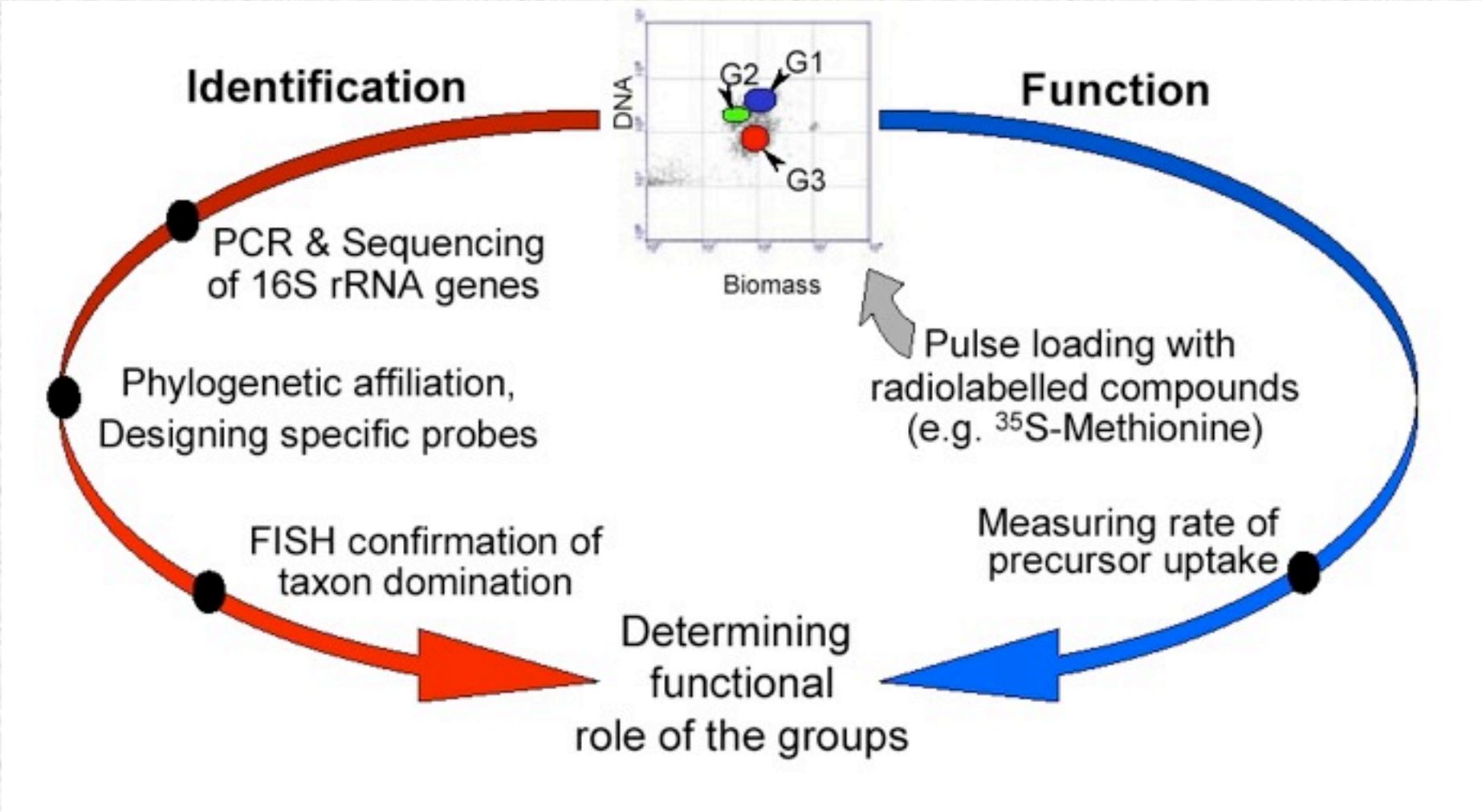




Abundant (>1% sequences) OTUs

Vila-Costa et al., 2012-EMI

Linking function and diversity



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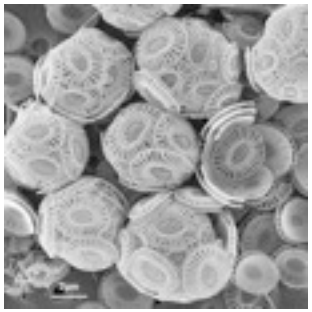
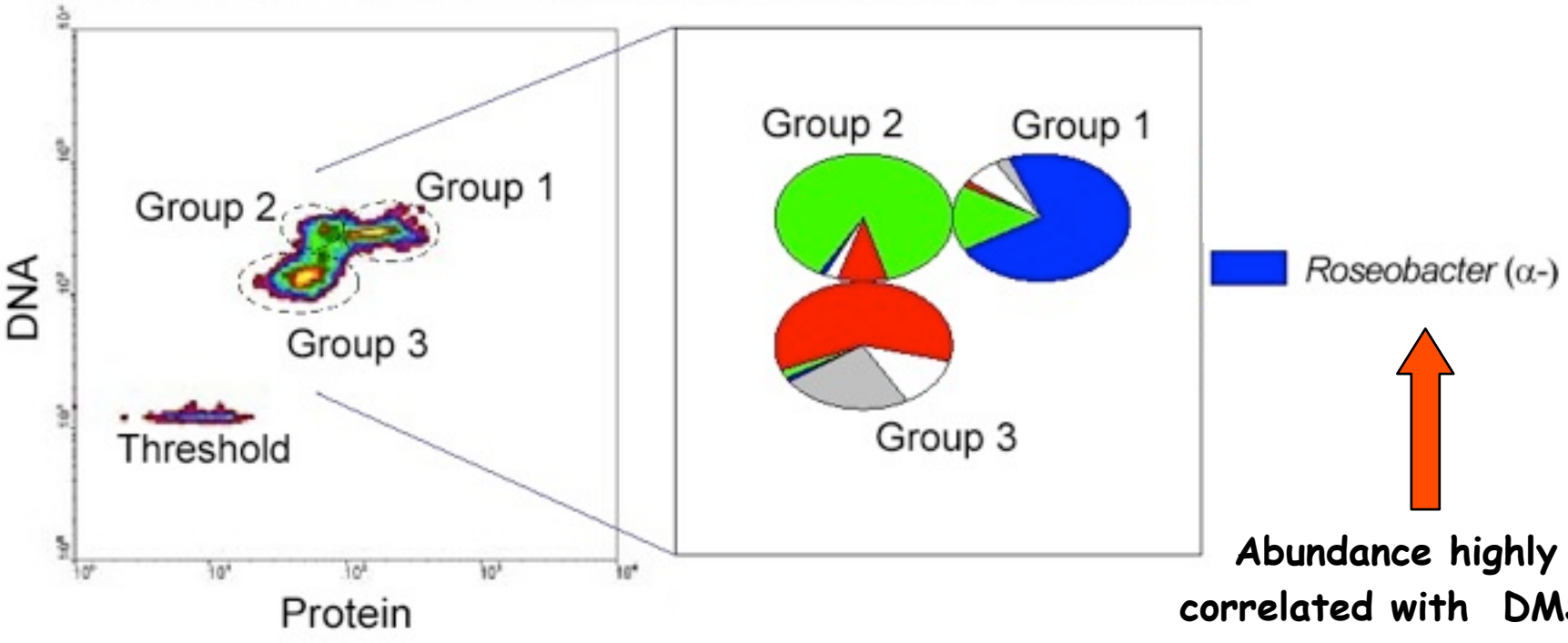
Graph by M. Zubkov & B. Fuchs



DMSP producing phytoplankton bloom in the North Sea
Emiliania huxleyi y Prorocentrum minimum

Flow cytometric signature & pylogenetic composition of the groups



Abundance highly correlated with DMSP consumption

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Zubkov et al. 2001-EMI

Microbes and flow cytometry (bias to heterotrophs)

1) Introduction: what is CF?
2) Cellular size and structure, and pigment detection
3) Detecting bacterial, viral and protistal DNA (and RNA)
4) Measuring Bacterial activity and physiological status
5) Where are we? A personal view of our achievements (and lack of)
6) Going further: cytometric diversity
7) Going further: Probing ecosystem function
   Relating community structure to ecosystem functioning

Summary