Microbes and flow cytometry: from enumeration to community structure, diversity and ecosystem function

Josep M Gasol

Departament de Biologia Marina, Institut de Ciències del Mar. CSIC. Barcelona
Our ultimate goal

Dalibacter banyuleus
preferentially grazed by HNF
very sensitive to viral attack

Roundicoccus plymouthii
In summer, 75% of BCD dominates DMSP uptake

Spirovibrio kalmariensis
dominates DMSP uptake

Tinymonas bremenensis
(all names are fiction... yet)

Friday, June 1, 2012
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
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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, FACSVersa  

• **Portable cytometers**  
  Guava, Apogee, Partec, Miltenyi  
  Accuri  

• **In situ - continuously monitoring FC**  
  FlowCytobuoy, Flowcytobot  
  SeaFlow
• High-speed cell sorters
  Coulter EPICS, FACSVantage
  FACSAria - MoFlo - Influx

Thanks google for the images...

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Medium-size cytometers
- FacsCalibur, Coulter XL
- FACSCanto, FACSVerse

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Friday, June 1, 2012
• **Portable cytometers**
  Guava, Apogee, Partec, Miltenyi, Accuri

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• In situ - continuously monitoring FC
  FlowCytobuoy, Flowcytobot
  SeaFlow

LIMNOLOGY
and
OCEANOGRAPHY
METHODS

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

Jarred E Swalwell, Francois Ribolet, 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

- 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 emission peaks, extinction coefficient, and photobleaching.
BD Fluorescence Spectrum Viewer A Multicolor Tool

Options
Curves: 5
Cytometer: BD FACSArria

Excitation (nm): 355
Show Em when Ex % > 5

Wavelength (nm)

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

http://wwwbdbiosciences.com/research/multicolor/spectrum_viewer/index.jsp

Friday, June 1, 2012
http://wwwbdbiosciencescom/research/multicolor/spectrum_viewer/index.jsp

Friday, June 1, 2012
BD Fluorescence Spectrum Viewer A Multicolor Tool

Options: Curves: 5
Cytometer: BD FACS Aria
Excitation (nm): 488
Show Em when Ex % > 5

Wavelength (nm)

Fluorochrome | % | Ex | Em | Filters
---|---|---|---|---
DAPI | 0.0 | | | DAPI
FITC | 88.0 | | | FITC
PerCP | 87.4 | | | PerCP
PE | 61.6 | | | PE
Phycocyanin | 3.2 | | | Phycocyanin

http://wwwbdbiosciencescom/research/multicolor/spectrum_viewer/index.jsp

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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
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
**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

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

(from BD)
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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.

Photo: Dominique Marie, Station Biologique de Roscoff, CNRS

Image by Lucas Stal
Size and (auto)fluorescence

90° light scatter (SSC, RALS)

Orange fluorescence

Red fluorescence

Proc

PE

Syn

Peuk

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Size and (auto)fluorescence

Calvo-Diaz & Morán 2006, AME

Grob et al. 2007, BGS
Size and (auto)fluorescence

- Sulfur
- PHB
- Magnetosomes
- Vacuoles
- Differentiate bacteria
- Size bacteria

Casamayor et al. 2007-ENM
Wallner et al. 1997-AEM
Dubelaar et al. 1987-Cytometry
Allman et al. 1993-in Lloyds’ book
Troussellier et al. 1999-FEMS ME

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DNA (and RNA) fluorescence

The standard...
At least until 1995

Variability in DAPI counting

<table>
<thead>
<tr>
<th>Site</th>
<th>BA (ml⁻¹)</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eutrophic reservoir</td>
<td>$1.68 \times 10^7$</td>
<td>20 %</td>
</tr>
<tr>
<td>Med. coast-1</td>
<td>$3.63 \times 10^5$</td>
<td>15 %</td>
</tr>
<tr>
<td>Med. coast-2</td>
<td>$2.56 \times 10^5$</td>
<td>5.3 %</td>
</tr>
<tr>
<td>Mesocosm Exp.</td>
<td>$1.03 \times 10^6$</td>
<td>8.2 %</td>
</tr>
<tr>
<td>Aged seawater</td>
<td>$1.02 \times 10^5$</td>
<td>17 %</td>
</tr>
<tr>
<td>Stain</td>
<td>Binds to</td>
<td>Exc. / Em. (nm)</td>
</tr>
<tr>
<td>--------------------------------------------</td>
<td>-------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Propidium iodide (PI)</td>
<td>DNA and RNA</td>
<td>535 / 617</td>
</tr>
<tr>
<td>HOECHST 33342</td>
<td>DNA (AT)</td>
<td>350 / 461</td>
</tr>
<tr>
<td>Benzoxazinone-kanamycin (BVC kanamycin)</td>
<td>cell surfaces</td>
<td>495 / 616</td>
</tr>
</tbody>
</table>

* 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)
E. coli

Seawater

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

Seymour et al’04-AME

Seymour et al’00-AME

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Viruses and all protists get also stained
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Friday, June 1, 2012
Renewed interest into the live/dead/inactive/active bacteria

Zweifel & Hagström, 1995-AEM
Heissenberger et al. 1996-AEM
<table>
<thead>
<tr>
<th>Stain</th>
<th>Mode of action</th>
<th>Exc/Em</th>
<th>applied in</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO (acridine orange)</td>
<td>Different color when linked to DNA or other things</td>
<td>460 / 650</td>
<td>cultures</td>
<td>Nishimura et al. 1995, Darzynkiewicz and Kapuscinski 1990, McFeters et al. 1991</td>
</tr>
<tr>
<td>PI (propidium iodide)</td>
<td>excluded by living cells</td>
<td>536 / 623</td>
<td>cultures</td>
<td>Jepras et al. 1995, López-Amorós et al. 1995b</td>
</tr>
<tr>
<td>EthBr (ethidium bromide)</td>
<td>excluded by living cells</td>
<td>510 / 595</td>
<td>cultures</td>
<td>Paau et al. 1977, Pinder et al. 1990</td>
</tr>
<tr>
<td>FDG (fluorescein-galactopyranose)</td>
<td>Activity of the enzyme β-galactosidase</td>
<td>494 / 518</td>
<td>cultures</td>
<td>Nir et al. 1990, Miao et al. 1993</td>
</tr>
<tr>
<td>Fluorescein diacetate (FDA)</td>
<td>cleaved by intracellular enzims</td>
<td>492 / 517</td>
<td>cultures</td>
<td>Diaper et al. 1992</td>
</tr>
<tr>
<td>CTC</td>
<td>indicator of respiratory-chain activity</td>
<td>480 / &gt;585</td>
<td>cultures</td>
<td>Porter et al. 1995a</td>
</tr>
<tr>
<td>c-SNARF-1 AM</td>
<td>intracellular pH</td>
<td>~488 / ~610</td>
<td>cultures</td>
<td>Leyval et al. 1997</td>
</tr>
<tr>
<td>Calcefluor white, Tinopal CBS-X...</td>
<td>excluded by living cells</td>
<td>347 / 436</td>
<td>cultures</td>
<td>Mason et al. 1995</td>
</tr>
<tr>
<td>SYTOX Green</td>
<td>excluded by living cells</td>
<td>325 / 430</td>
<td>cultures</td>
<td>Davey and kell 1997</td>
</tr>
<tr>
<td>TOPRO-1</td>
<td>excluded by living cells</td>
<td>504 / 523</td>
<td>cultures</td>
<td>Roth et al. 1997, Veldhuis et al. 1997</td>
</tr>
<tr>
<td>TOPRO-3</td>
<td>excluded by living cells</td>
<td>515 / 531</td>
<td>freshwater</td>
<td>del Giorgio et al., in press</td>
</tr>
<tr>
<td>16S rRNA probes</td>
<td>attach to ribosomes</td>
<td>642 / 661</td>
<td>cultures</td>
<td>Davey et al. 1999</td>
</tr>
<tr>
<td>DVC*</td>
<td>live cells elongate when in presence of ABs</td>
<td>-</td>
<td>cultures</td>
<td>Amann et al. 1990, Wallner et al. 1993</td>
</tr>
<tr>
<td>BacLight Live / Dead</td>
<td>(Syto9 / PI)</td>
<td>-</td>
<td>seawater</td>
<td>Thorsen et al. 1992, Joux et al. 1997, Nishimura et al. 1995</td>
</tr>
</tbody>
</table>

* 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.
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)
Heat treatment

Unfiltered sample

% of initial

Log time

UVC treatment

< 0.8 μm

% of initial

Log time

CTC

NADS-live

Leu
Falcionei et al. 2008-AEM.

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< 0.8 µm

Live
Dead

Falcioni et al. 2008-AEM.
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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

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The physiological-state continuum

- High activity
- Medium activity
- Low activity (Dormancy)
- Death
- Lysis

- CTC
- Microautoradiography
- DNA content
- Dibac (depolarization)
- PI (damage)
- TEM

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)

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• 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

Friday, June 1, 2012
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- 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?
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. © 2007 Elsevier B.V. All rights reserved.

Sahara dust particles

image: M. Weinbauer & R. Cattaneo
Sahara dust particles

Black Carbon (soot) particles

Fossil fuel BC emissions (Tonnes/1° x 1°)
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
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- 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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- Not yet: Build bacterioplankton size spectra
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Can we define and use the cytometric diversity?


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
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
Cytometric diversity
Pyrenees lakes, summer 2008

20 lakes (3 depths per lake)
Abundance picoalgae: $3 \times 10^{-2}$ - $2.1 \times 10^{-5}$ (average, $1.3 \times 10^{-4}$)
Number of different populations: 1-11 (average, 4.7)
Simpson diversity index: 1 - 6.9 (average 2.4)

Antarctic lakes, summers 2003-2004
11 lakes (some, in 2 yr)
Abundance picoalgae: $1 \times 10^{-3}$ - $3.3 \times 10^{-5}$ (average, $3 \times 10^{-4}$)
Number of different populations: 1-4 (average, 2.2)
Simpson diversity index: 1 - 2.8 (average 1.4)

FC and OM diversity, 1/D

Diversity by molec techniques (18S rRNA), 1/D

$r^2$ between 0.73 and 0.93
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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)
- Measuring 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

Experiment Impresión-1 (Vigo, Jan’05)

Bacterial respiration (mmol O₂ h⁻¹ d⁻¹)

Time (days)

BR = 0.163 + 9.41 x 10⁻⁶ x CTC, r² = 0.88

Gasol & Serret, unpubl.

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Diel pigment variability and growth

150°W 5°S

Equatorial Pacific

Prochlorococcus

Synechococcus

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

\[ \text{H}_2\text{S} - S^0 \]

\[ S^0 - \text{SO}_4 \]

intracellular or attached \( S^0 \) changes SSC

\[ y = 1.569 + 0.0675x \quad R^2 = 0.992 \]

\[ y = 1.842 - 0.025621x \quad R^2 = 0.9289 \]
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.)
An example of C(S)-flow Cell sorting

Sunlight modulation of the relative activity of bacteria and picophytoplankton

Flow cytometry cell sorting

NW Mediterranean

Prokaryotic phytoplankton, major primary producers in oligotrophic systems

3H-leucine or 35S-DMSP

DARK  PAR  PAR+UVA  PAR+UVR

Het. Bac.  Syn

Vila-Costa et al. 2006, Science
Ruiz-González et al. ISME J. 2012
Sunlight modulation of the relative activity of bacteria and picophytoplankton

Flow cytometry cell sorting

3H-leucine

No changes in their relative contribution to leucine uptake

Ruiz-González et al. ISME J. 2012
Sunlight modulation of the relative activity of bacteria and picophytoplankton

Flow cytometry cell sorting

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

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

Identification

PCR & Sequencing of 16S rRNA genes
Phylogenetic affiliation, Designing specific probes
FISH confirmation of taxon domination

Function

Determining functional role of the groups
Pulse loading with radiolabelled compounds (e.g. $^{35}$S-Methionine)
Measuring rate of precursor uptake

Graph by M. Zubkov & B. Fuchs
DMSP producing phytoplankton bloom in the North Sea
Emiliania huxleyi y Prorocentrum minimum

Abundance highly correlated with DMSP consumption

Zubkov et al. 2001-EMI
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