How to count picoalgae and bacteria with the FACScalibur flow cytometer

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• What is this ?

This manual contains instructions for the routine use of the FACScalibur for counting bacterial and picoalgal concentrations in natural samples. Part of the instructions (like turning on and off or cleaning the machine) will be useful to users of the machine for other applications. However, any user that intends to develop new methods or approximations or that will work with unusual samples will have to manage her/himself on his/her own. I recommend that in such a case, the cytometer manual, and a good cytometry book (like Shapiro's "Practical Flow Cytometry") are read and understood before starting to play around. I also recommend that enough time is allotted to development of procedures before actual samples are run.

Needless to say, I offer this information with the assumption that you will report back to me any errors or missunderstandings and you will also report any improvements you make.

It took me some months to setup the procedures that are explained below, and some details are still not completely understood. I will appreciate if new information or procedures are reported to me so that I can add them to the document.

• Contents

A. Principle.

A fluidics system uses pression to force the sample to go through a fixed point in a way in which one particle, in a single drop, at a time circulates in front of a laser beam. Our system has a blue (488 nm) laser. Light dispersed by the particle, and fluorescence emitted by the particle after the laser excitation, are collected in photomultipliers and sent to the computer where data are processed. The procedure is fast, and thousands of particles can circulate per minute. Information about light dispersion as well as fluorescence emitted is collected, combined, and saved to disk.

Figure 1, created by G. Dubelaar, and obtained from http://home.wxs.nl/~dubelaar/icesrep.html, shows an overview of the process:

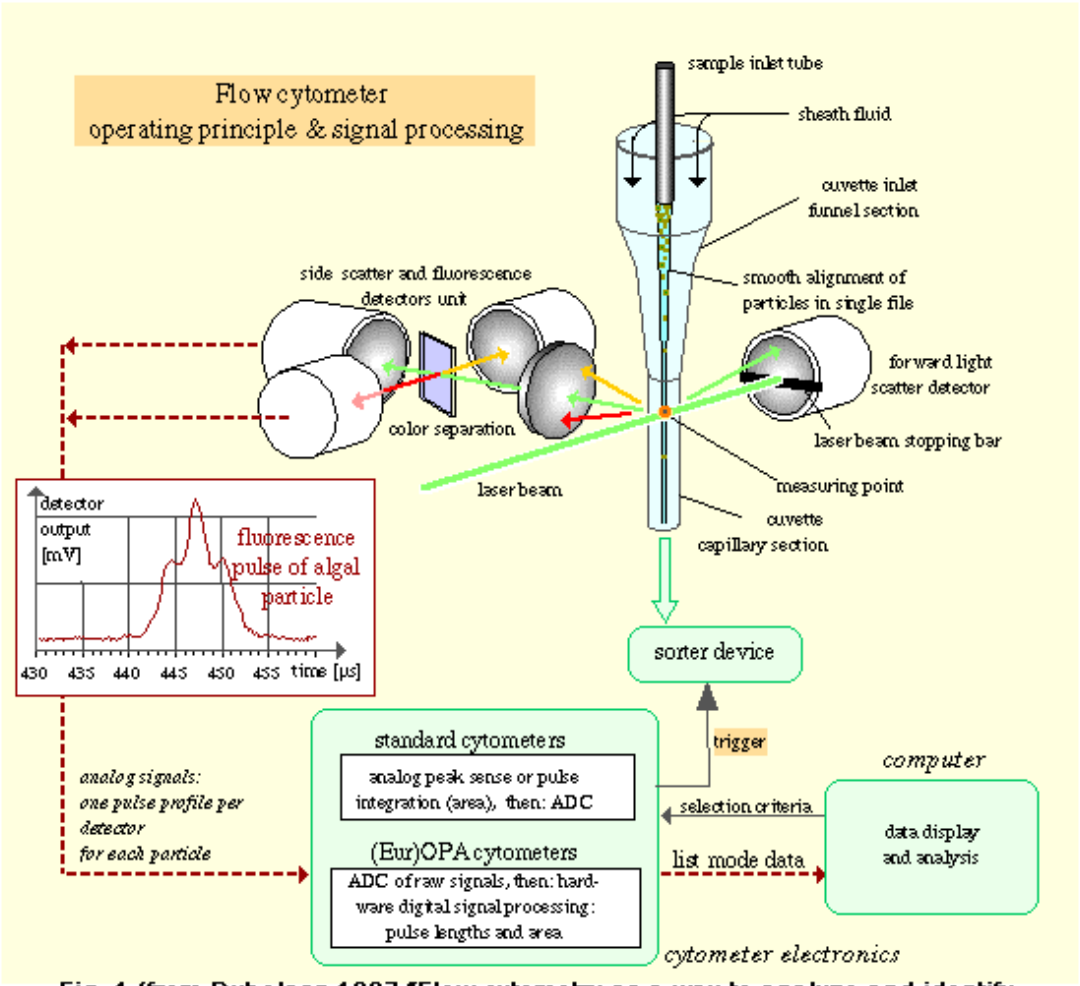


Fig. 1 (from Dubelaar-1997 "Flow cytometry as a way to analyze and identify phytoplankton" - (http://home.wxs.nl/~dubelaar/icesrep.html)

B. The FACSCalibur

All this is implemented in the following machine, common in many oceanography labs:

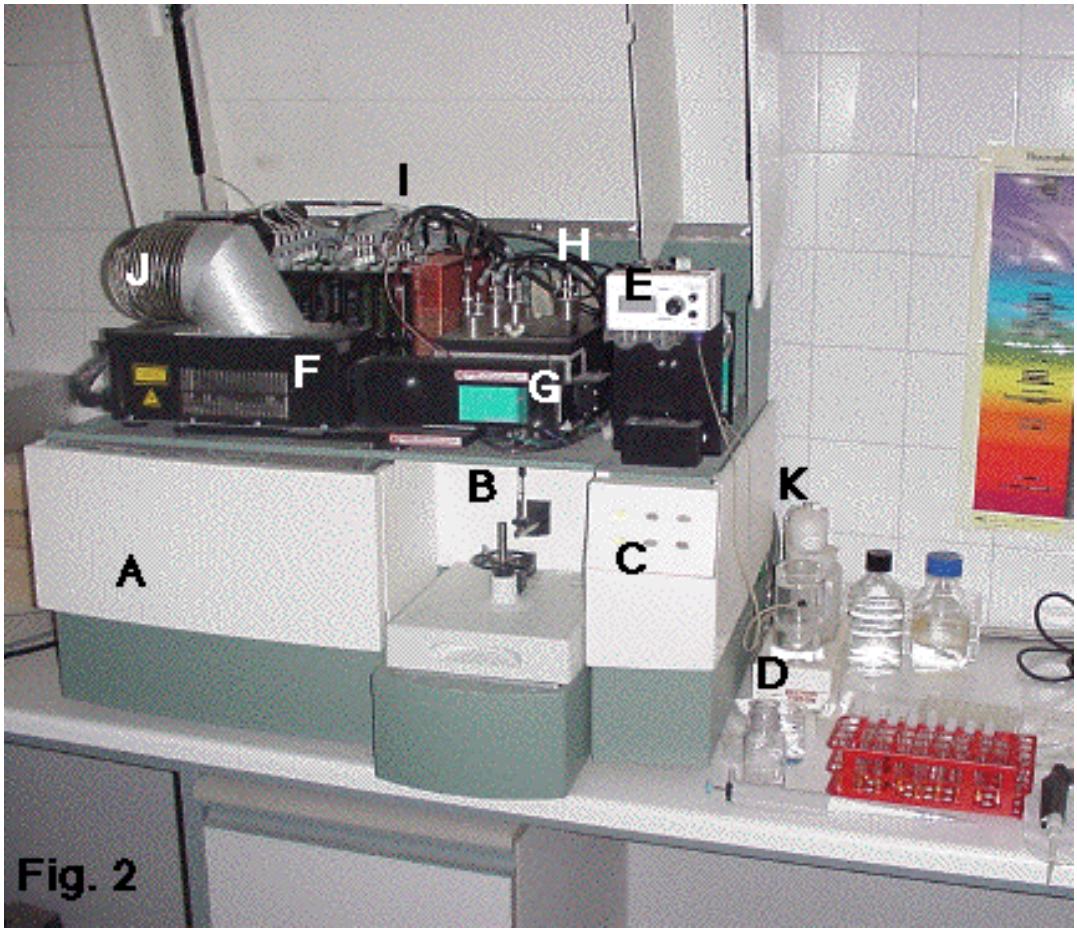
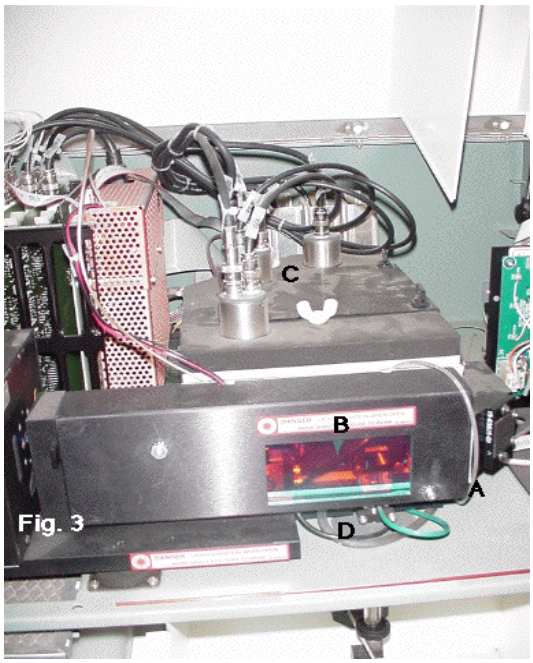


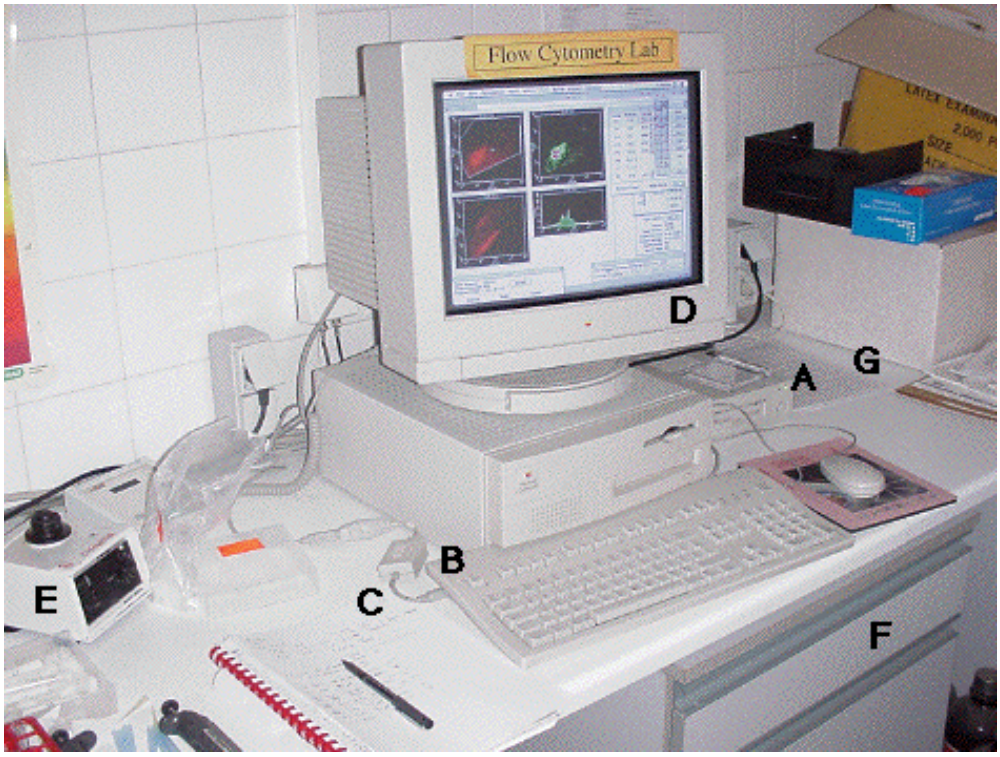
Fig. 2

A.- Liquids (sheath and waste) tanks
B.- Sample (in a tube)
C.- Main controls
D.- Filtration stuff for sorting
E.- Controls of sorting
F.- Laser
G.- Analysis chamber
H.- Photomultipliers and optical filters
I.- Electronics
J.- Laser refrigeration
k.- Switch (on/off)

In a close-up, we can appreciate the details (Fig. 3), in particular, the entrance of the sample (D), the chamber where paricles interact with the laser (B), the photomultiplers (C), and the forward scatter detector, which is the only one that is not a photomultiplier (A).



The machine is controlled by a computer (Fig. 4), which can be turned on by pressing the key in B. The magnetooptical drive A has to be turned on before the computer. The software is protected by a keylock (C) that, once in a while gets disconnected.



C. Sampling and fixation.

Fixation of samples is a necessary procedure both because we don't want to loose cells with time and also because fixation aparently facilitates probe entrance into the cell (Bullock 1984). We are currently fixing samples with P+G (1% paraformaldehyde + 0.05% glutaraldehyde final) followed by deep-freezing in liquid nitrogen. Vaulot et

al.1989 suggested fixation with 1% glutaraldehyde and Premazzi et al.1992 developed a protocol with 0.1% to 0.5% paraformaldehyde (methanol-free formaldehyde). A good comparison performed by Troussellier et al. (1995) showed that PFA or formalin are better than glutaraldehyde both for cell permeabilization (helping the dye getting in) and for fluorescence maintenance. PFA also did not modify the size characteristics of the cells. Other fixatives used are formaldehyde, paraformaldehyde alone and glutaraldehyde. Furthermore, glutaraldehyde smells really bad. The protocol we use is the same recommended for fluorescent in situ hybridization, and can be cited as Marie et al. (1997).

Some details have to be taken into account for the P+G fixation, :

Preparation of P+G (1 liter):

- Weight 100 g. of PFA (Sigma P6148).
- Place in 880 ml dH_2O in a beaker, covered with Parafilm (to reduce water loss through evaporation), and with a magnetic fly. The water has to be very hot. Near boiling.
- Leave for > 24 h in a fume hood on a heater (90°) with magnetic stirring
- After dissolution (it make take days), add 100 ml PBS (phosphate-buffered saline solution). The PBS is prepared with a Sigma P4417 pill (1 tablet in 200 ml)
- Add 20 ml Glutaraldehyde 25%
- Filter through polycarbonate 0.2 µm in the fume hood and with a “dead” filtration system
- Distribute in 5, 20 ml and 50 ml tubes as desired.
- Freeze them in the -70°C freezer.
- Store the tubes at -20°.
- Once unfrozen, a tube should be used within a week or discarded.

Fixation:

- Add freshly unfrozen P+G to sample (10% of sample volume).
- Leave on bench in the dark for 10 min to allow complete cell fixation.
- Deep freeze in liquid N_2
- After some hours, the sample can be placed at -20 °C or, much better, at -70°C

D. Before switching on

Before switching the machine on, the following rules should be complied with:

- Write your name in the log file (usually placed in Fig. 4, G) and fill in the details.
- Get a removable magnetooptical disk (nothing but settings can be stored in the computer’s hard disk).

- Get yourself a bead solution (see below), sonicate the beads (5 min), and count them under the epifluorescence microscope (see below).
- Take the samples and the stain out of the freezer and let them unfreeze.
- Get a small (50 ml) amount of MilliQ water
- Make sure there are enough tubes for the number of samples you've got.
- If there aren't enough tubes or dye, talk to the facility supervisor.
- The needed automatic pipettes should be in the drawer (F) just below the computer.

E. Switching on

Our machine has to be switched on 15 min before the computer is turned on. It has to warm up or else the computer will not recognize the machine. In some models you can hear a "click" when the machine is ready and the computer can be turned on.

Once the machine is on, the first step is to check for how much sheath fluid is there. Do so by opening the left container in the machine. You will see two plastic containers with liquid:

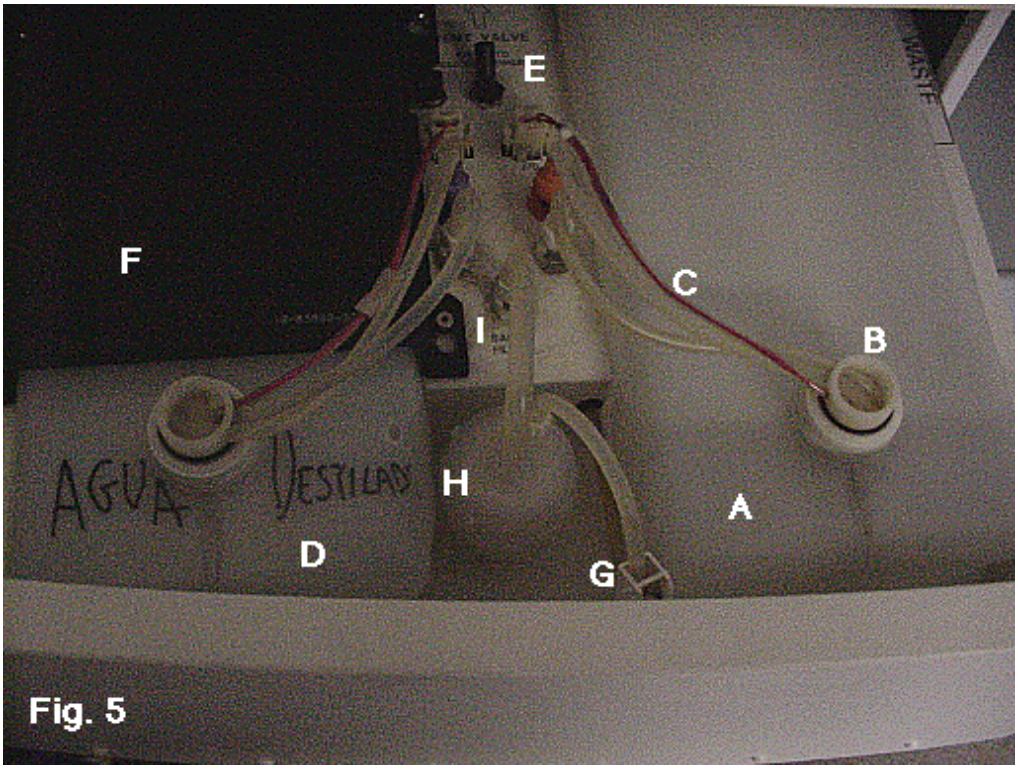


Fig. 5

- Make sure there is a water-filled tube in position in the machine (A in Fig. 6).

- The one at the right side (A, Fig. 5) is the Waste tank. It should be emptied. Unscrew the connections (B) at the top (CAREFULLY, it's not necessary to break the tubing, C). Throw the liquid through the sink.

- The left one, covered by a black metal jacket, is the Sheath liquid tank (D). To remove it, you have to: First, get the pressure out by moving the black valve (E) towards you. Second, remove the black jacket (F) by pushing it backwards. Third,

unscrew the connections at the top (CAREFULLY, it's not necessary to break the tubes).

- Go upstairs and fill the tank with MilliQ water. (this requires opening the MilliQ machine 10 min in advance, writing down your name and amount of water used, removing the filter at the MilliQ water outing -it might contaminate water instead of filtering it-, and getting the water).

- Put both tanks in their place. Close the connections (CAREFULLY, it's not necessary to break the tubs). Put the black jacket back in place. Put pressure on (valve towards the wall).

- Let water circulate by opening the white valve (G) at the front. This action should remove all air bubbles from the tubing. Close that valve afterwards. No air bubbles (or very small ones) should be visible. If a big bubble is in the filter (H) or around it, remove the connection of the filter from the central panel (I), and push with your nails the side of the tip until water comes out.

- Close the Liquid receptacle, and push the “Prime” pilot light (Fig. 6, F, red color) on the machine. Once it turns orange again, throw away the tube with the liquid, and put MilliQ water. Push “Run” (D) and “Hi” speed (C). This action will further clean the tubing. To place a tub in, you have to move sideways the B switch, and be careful at placing the tube so as not to break the metal rod.

Fig. 6

By now 15 min have passed so you should be able to open the computer. Open FIRST the magnetooptical. Then the computer. Select your username and type your password.

If the computer has been connected before the machine warmed up, the last icon to appear at the bottom of the screen while the computer initializes will appear with a black cross. If

that happens, you have to shut down the computer, wait 10 min and turn it on again. This happens often !

Once in a while, even after rebooting the computer, the cytometer is not seen by the computer. In that case, you have to turn off both computer and cytometer, and on. It has happen often that it's not necessary to wait the 15 min, but you may need to.

Use the time before starting the computer to sonicate your beads for 5 min.

F. Checking that it all works.

At this point the program CellQuest should be opened. (Go to the **Apple** menu, there under **Aplicacions Citometria**, you will find an access to CellQuest). An untitled window will appear. Close it. Open the "General" folder which is placed inside the "Users" folder of the Hard disk. There should be a folder called "Fulls". There, two files can be found: one called "Bacteria" and another one called "Picoalgae". Open one or the other depending on what intend to count.

Next, under the menu **Acquire**, open "Connect to cytometer". If this takes a long time (> 30") before appearing, turn down the computer and start again.

After the Acquisition Control window appears, select, under the menu **Cytometer** the windows "Threshold", "Detectors/Amp" and "Compensation". Place the windows wherever you like on the desktop. You should also call for the window "Status" and the window "Counters" (under menu **Acquire**).

Now, under the menu **Cytometer** call "Instrument settings". Open, under the "General" folder which is placed inside the "Users" folder of the Hard disk the folder called "Settings". Two files can be found there: one called "Bacteria settings" and another one called "Fitoplàncton settings". Select whichever you need for your analysis but don't forget that the Fitoplancton one is optimized to count prochlorophytes, cyanobacteria and picoeukaryotes in oceanic samples. Once this is done, the button called "Set" has to be pushed. And then "Done".

Two processes should be checked befor starting to work. One is slightly more technical ("alignement") and should only be done routinely by the facility superviser. The other process ("cleanness") must be done always.

• Alignement. Check that the B&D beads have the right CV in all the detectors. This is done, once a month, by the facility supervisor.

> All coefficients of variation of fluorescent 2 µm beads should be < 2%
> With linear adcquisition at gains ~ 400.

• Cleanness. Fill half a tube with MilliQ water. Place it in the cytometer. To do so, you have to:

a) make sure the cytometer is in "Standby" (Fig. 6, E)
b) carefully move to right or left the black round thing onto which tubes are placed (Fig. 6, B).
c) bring down the tube. Do so smoothly, with care not to break the metal tubing
d) place your tube.
e) push up your tube.
f) close the black thing.
g) Push "Run" (Fig. 6, D).

The “Run” button should turn green. If it stays in orange, first push up the tube. Secondly change the tube (see troubleshooting). Thirdly, call the facility superviser.

In the “Acquisition Control” window, make sure that “Setup” is selected (it has a cross in the square). Push Acquire. Now the machine starts seeing things.

It is possible that many particles appear initially. Wait for some time (~10 min). The rate of particles appearing should be below 20 per $second^{-1}$. If it stays high, the “cleaning protocol” (see below) should be followed. You can see the rate of particle passage in the Counters window.

G. Running samples

Your bead solutions should be sonicated for 5 min. before the start of the session.

a. Pico and nanophytoplankton.

Standard settings that work for me are: FSC: E01, SSC: 380, FL1: 400, FL2: 505, FL3: 590 (Threshold at FL3: 60). All acquisition in Log.

Standard run between 0.6 and 1.0 ml of sample, at HI speed (~46 µl min^{-1}) and with 10 µl of a 10^5 beads ml^{-1} solution. I usually terminate the run after 10 - 12 minutes or when 10.000 particles have been recorded.

By default a dot plot of SSC vs. FL3 and another one of FL2 vs. FL3 will be displayed together.

DON'T FORGET to write down in your notebook the sample volume and the volume of beads added.

Procedure:

a) Put the sample in the tube.
b) add the beads, on the wall and making sure the drop doesn't remain in the tip of the pipette.
c) vortex the tube.
d) put the machine in “Standby”.
e) change the tube.
f) put the machine in “Run” (if the light stays in orange, see below).
g) In the Acquisition Control window, push “Pause”, then “Abort”, then make sure “Setup” is on, and then push “Acquire”.
h) Let the machine run for a few seconds. Watch that the populations appear. When happy with what you see, prepare the machine for saving the data. You should see the beads appear in channels numbers SSC: 3E2 and FL3: 1E2.
i) Go to the menu **Acquire**, and select “Parameter description”. Give a name to the sample, and select the folder where it will be stored. Remember that it MUST be in a magnetooptical disk. By default 10.000 particles will be acquired.
j) In the Acquisition Control window, push “Pause”, then “Abort”. Click on “Setup” to remove the cross, and now push “Acquire”. The data will start to be acquired.

Notes:

- If the sample runs out before the end of the acquisition, air will enter the sample and it will be tough to analyze, so keep an eye on the sample.
- Once you are fed up with waiting, you can always store what you got by pushing “Pause” and then “Save” in the Acquisition Control window.
- Don't ever even think of changing any of the public settings. If you consider yourself a power user, save your modifications with a different name and in your folder. And READ THE MANUAL !
- If you only see noise (but you are sure you had your sample), a gate can be placed (see below for instructions).

b. Bacteria.

Standard settings that work for me are: FSC: E02, SSC: 400; FL1: 511, FL2: 400, FL3: 590 (threshold a FL1: 72). All acquisition in Log.

Standard run 0.4 ml of sample, at LO speed (~19 µl min^{-1}) and with 10 µl of a 10^6 beads ml^{-1} solution. I usually terminate the run after 10.000 particles have been recorded which should happen in around 1-2 minutes.

By default a dot plot of SSC vs. FL1 and another one of FL1 vs. FL3 will be displayed together.

DON'T FORGET to write down in your notebook the sample volume and the volume of beads added.

Procedure:

a) Put the sample in the tube.
b) add 4 µl of stain solution (see below). Vortex. Wait 10 minutes with the tube in the dark. This is a high concentration (5 µM final of stain). It will stain equally well with 2 µl (the recommended 2.5 µM), but this is difficult to pipette. And increasing the sample volume to 1 ml implies that you will spend all your sample in a single run. However, I have checked that there is no change in the response of the sample with varying concentrations from 1 µM until 25 µM. We can always dilute more the stain, but we won't do it in routine counting mode.
c) add the beads, on the wall and making sure the drop doesn't remain in the tip of the pipette.
d) vortex the tube.
e) put the machine in “Standby”.
f) change the tube.
g) put the machine in “Run” (if the light stays in orange, see below).
h) In the Acquisition Control window, push “Pause”, then “Abort”, then make sure “Setup” is on, and then push “Acquire”.
i) Let the machine run for a few seconds. Watch that the populations appear. When happy with what you see, prepare the machine for saving the data. You should see beads fall in channels ~ 10^3 for SSC and FL1, and bacteria fall in the channels around 10^1 for SSC and 10^2 for FL1.
i) Go to the menu **Acquire**, and select “Parameter description”. Give a name to the sample, and select the folder where it will be stored. Remember that it MUST be in a magnetooptical disk. By default 10.000 particles will be acquired.
j) In the Acquisition Control window, push “Pause”, then “Abort”. Click on “Setup” to remove the selection, and now push “Acquire”. The data will start to be acquired.

Notes:

- Don't ever even think of changing any of the public settings. If you consider yourself a power user, save your modifications with a different name and in your folder. And READ THE MANUAL !
- If you only see noise (but you are sure you had your sample), a gate can be placed (see below for instructions).
- Keep an eye at the speed of sample passage. If it is above 800 particles s^{-1}, then you will be underestimating true concentration. You will have to dilute your sample. See below for instructions.

Speeds of sample passage (Calibrated March'98)
LO: 18.4 events s^{-1}
ME: 24.6 events s^{-1}
HI: 45.9 events s^{-1}

C. Gating the sample.

Sometimes there is a lot of noise coming at low fluorescent levels. If you store all the data, maybe only 500 out of the 10000 events acquired are of interest. To avoid that happening, we can always gate the sample.

Noise usually occurs at high SSC and low FL3 when running for picoalgae (but it might also occur at low SSC and low FL3), and at low SSC and low FL1 when running for bacteria.

To gate the sample, you have to:
a) Stop acquiring data. Push “Abort” in the Acquisition Control window.
b) Call the Tool palette and select the “Polygonal-region tool” (sixth in the right side).
c) create a CLOSED window, that includes beads and cells and lets out noise.
d) Go to the menu **Acquire** and select “Acquisition and storage”.
e) Select “R1 = G1” in all the menus where “All” is selected (that is, in “Accept”, in “Event count” and in “Data file will contain”).
f) Now you can proceed as usual.

Note that if you want to see the rate of cell passage, in the Counters window you have to extend it down (by clicking in the upper-right little square). Now the “true” number of events stored and the true rate is the one that you see in the lower left part of the square.

H. Preparing the stains

A description of how to prepare a new batch of Syto13 follows. Preparing other stains (PicoGreen, SybrGreen I and II...) follows pretty much the same protocol.

• Buy the stuff from Molecular Probes (fax: 31 71 5233419). Catalog number S-7575, size 250 µl. If you buy them 5 at a time it is way cheaper. Keep searching their web (www.probes.com) for updates. ICM costumer number is F7364. Give the signed “vale” to Justo at the same time.

• The stuff will come next day. Label the box (owner and date). Place it in the freezer.

• When ready, get yourself DMSO (bought from SIGMA), 20 Eppendorf vials, a plastic bag, a >25 ml plastic tube, and pipettes and tips. Wear gloves all the time.

• Let the MP vial with the stuff unfreeze. Pipete the 250 µl and place them in the plastic tube. The stuff is heavy and some of it will remain on the tip and on the walls of the vial. With a new tip, dispense 200 µl of DMSO in the MP vial, vortex, pipette it out to the plastic tube. Repeat three times. Fill up to 2.5 ml of DMSO (that is add: 2500 - 250 – (6*(200)) = 1050 µl).

• Clean the first tip with the DMSO-Syto13 solution and add it back to the plastic vial.

• Distribute the solution in the Eppendorf tubes (≤ 200 µl per eppendorf). It is now at 500 µM concentration. Label them with S13. Put them in a plastic bag that you label with your name and date. Put them in the freezer.

I. The bead solutions

Prepare the bead solutions always in glass Pyrex bottles. Keep them always refrigerated. Count them every day. Stain with Syto13 (or with DAPI) once in a while and after a long time without being used.

Preparation of the “Backup solution”:
Place 1 drop of beads in 18 ml MilliQ water + 2 ml of Glutaraldehide 10%

Preparation of a 10^6 solution:
20 ml MilliQ + 3 ml of backup solution (previously sonicated for 5 min)

Preparation of a 10^5 solution:
20 ml MilliQ + 0.7 ml of Backup solution (previously sonicated for 5 min)

Label the bottles with your name, the date and the approx. concentration.

Write down in the book date and characteristics of the prepared bead batches.

Counting the bead solutions

Get 1 ml of bead solution and place in a tub together with 2 ml of MilliQ or PBS.

Prepare a 0.2 µm black Nuclepore -GTBP- (or Poretics) and a backing 0.8 µm AA Millipore filter. Place the backing filter in the tower, place a drop of water. Connect vacuum until water is gone. Disconnect vacuum. Place the 0.2 µm filter. Place the tower. Vortex the tube. Place the sample in the tower. Connect the vacuum until all water is gone. Remove tower, remove filter and let dry. Disconnect the vacuum.

Place a drop of low fluorescence oil on the slide. Place the filter, place another drop of oil and the coverslip. Flat down the coverslip with the help of the forceps .

Count the beads under epifluorescence (blue or UV light, it doesn't matter). COUNT ONLY SINGLE BEADS. At least 30 fields, and at least 200 beads. Try to count a whole transect, because beads tend to concentrate together at the sides of the filter.

Conversion factor for the “upright” Nikon microscope with the “new” towers:

Concentration = Average per whole field divided by volume filtered and times 54119.

Be warned that if you count only lines or small squares you've got to convert that value to the whole field (big square)

Write down in the book the concentration you have measured and the date.

NOTE: We are now using 1 µm (in fact I think they are 0.95 µm) yellow-green beads from Polysciences. I am extremely unhappy with these beads. Sometimes they aggregate a lot, some other times they don't aggregate at all. Sometimes they get easily contaminated, some other times you can leave a bottle on the counter for weeks without any contamination occurring... Some beads are clearly smaller than the assumed average size, dilute concentrations lose beads (e.g. Olson et al. 1993), and all sorts of nightmares. *We are in the process of substituing these beads for other brands and qualities.*

J. Diluting the samples

As told before, when the rate of particle passage is above 800 s^{-1}, chances are that you are underestimating true concentration because some cells may pass together ("coincidence"), and also because particles may pass in the "dead process time" zone: the time that the machine (& computer) electronics uses to process the signals obtained before. This "dead time" is difficult to calculate, and everybody says that we shouldn't have problems for rates below 2000 s^{-1}. However, and empirically, we have found evidence that suggests that we should avoid rates of particle passage above 800 s^{-1}. I haven't noticed any systematic patterns when using speeds below 800 s^{-1}. We may as well stay on the safe side and dilute our sample always when we suspect cells are very concentrated (cultures, eutrophic sites) or when we see that the rate is too high.

By using the protocol that follows, we have been able to count samples from Salt lagoons with concentrations above 10^7 cells ml^{-1}, and salinities above 250‰. Salinity interfers with DNA staining. However, a 20-fold dilution of these samples generated both concentrations and salinities within limits of procedure. Note that the samples should have been fixed if we intend to dilute the,

For dilution, use always the same liquid that you are using as Sheath fluid (usually MilliQ water). If you don't, SSC signals are off and fluorescent signals may also be off.

Test ALWAYS that this liquid is bacteria-free.

DON'T dilute more than 20 times, without checking what happens if you do.

Process:

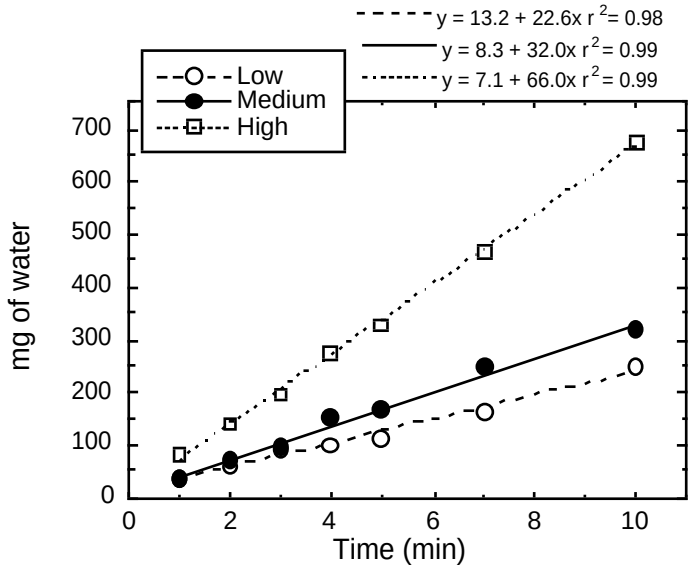
a) Put the sample (0.4 ml)
b) Put the stain, vortex and wait for 15 min.
c) dilute (with e.g. 0.4 ml of MilliQ).
d) Immediately add the beads, vortex the sample, and run it.

K. Calibrating the flow rate

An alternative way of performing direct concentration measurements relies on a good calibration of the sample flow rate. To determine it the following procedure can be done:

Fill at least 5 tubes with 1-2 ml of water.
Weight the tubes to the third decimal mg.
Run the samples for different times (1 to 10 min)
Reweight the samples
Plot weight difference (in mg) againsts time (in min)
Regress it and determine the slope. This is the flow rate.

This is an example for the three sample flow rates ("Lo", "Me", "Hi")



Note that there is no guarantee that the flow rate is constant. In particular, it is thought that when the sheath fluid reservoir is completely full, or almost empty, the flow rate probably varies. However, using the flow rate is a simple alternative to measuring concentrations using beads and can be used as a complementary method to be more certain about the calculated concentrations. Some researchers calibrate dailly the flow rate with this purpose.

L. Troubleshooting

Typical problems are:

- The cytometer does not respond	Rebooth cytometer & computer
- The concentrations have nonsense	Pipettes are disequilibrated
- The cytometer never gets clean	Use the bleach solution
- The "Run" button stays in orange	
check the tube	The tube has a crack. Change it.
check for air bubbles	Remove air (see above).
- A window saying "The cytometer is not ready" appears	
	A problem with the laser (talk to the superviser).
	Sheath fluid run out.

M. Cleaning

When you are finished, you have to clean the machine. If you did not use any stains, you can simple run MilliQ water for 10-15 min at "HI" speed. This should be enough for removing the rests of samples and beads accumulated in the tubing.

If you used stains, cleaning has to be done carefully. We currently set four levels of cleaning

Level A: 6-10 min of MilliQ water
Level B: 6-10 min of
Level C: 6-10 min of FACSRinse (detergents)
Level D: 6-10 min of diluted commercial bleach

We would use A if not very dirty, A+B+A if more dirty, A+B+C+A, or if very dirty, A+B+C+A+D+A.

Check always the rate of particle passage which should stay at below 20 s^{-1} (always in setup mode).

N. Switching off and leaving

The following order is mandatory.

1.- Go under the **Acquisition** menu, and select "Disconnect to cytometer". This separates the cytometer from the computer.

2.- Put a new tube with MilliQ water in the cytometer, and turn it off.

3.- Quit from the different programs in the cytometer. Do not save settings or CellQuest files unless you know esxactly why you do it. If you save the files, change their name and save them in your folder.

4.- Store the pipettes in the drawer. Save any unused stain in the freezer and the bead solutions in the fridge.

5.- Turn off the vortex

6.- Clean the work surface

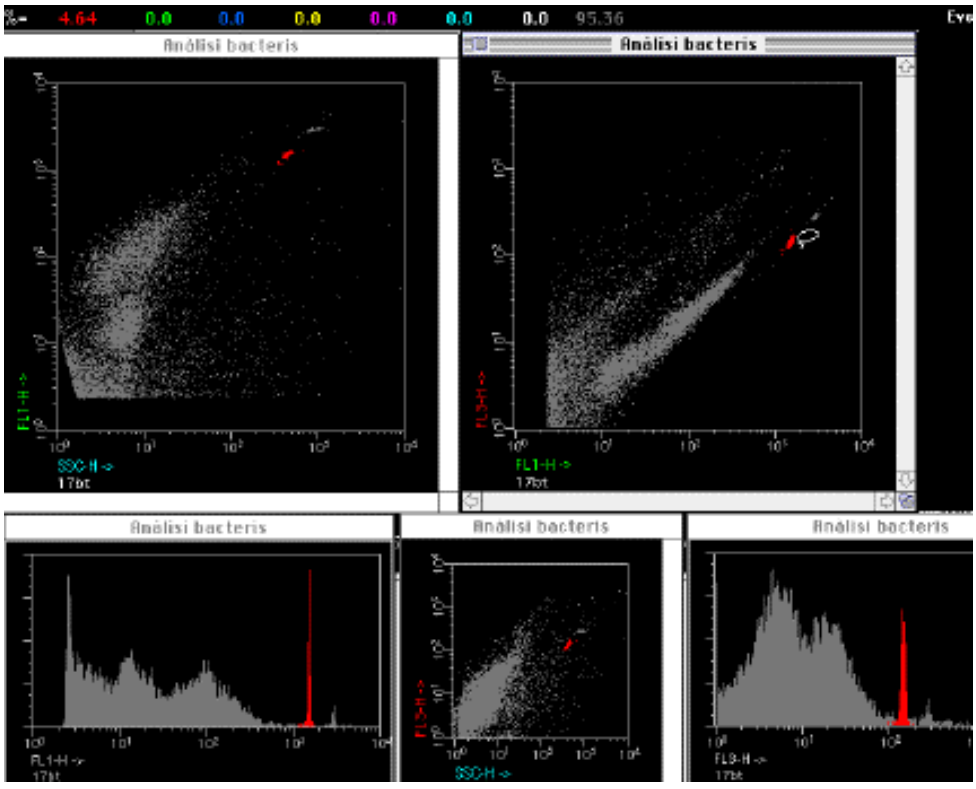
7.- **WRITE DOWN** in the Log-book the number of samples processed and time of disconnection.

O. Data processing

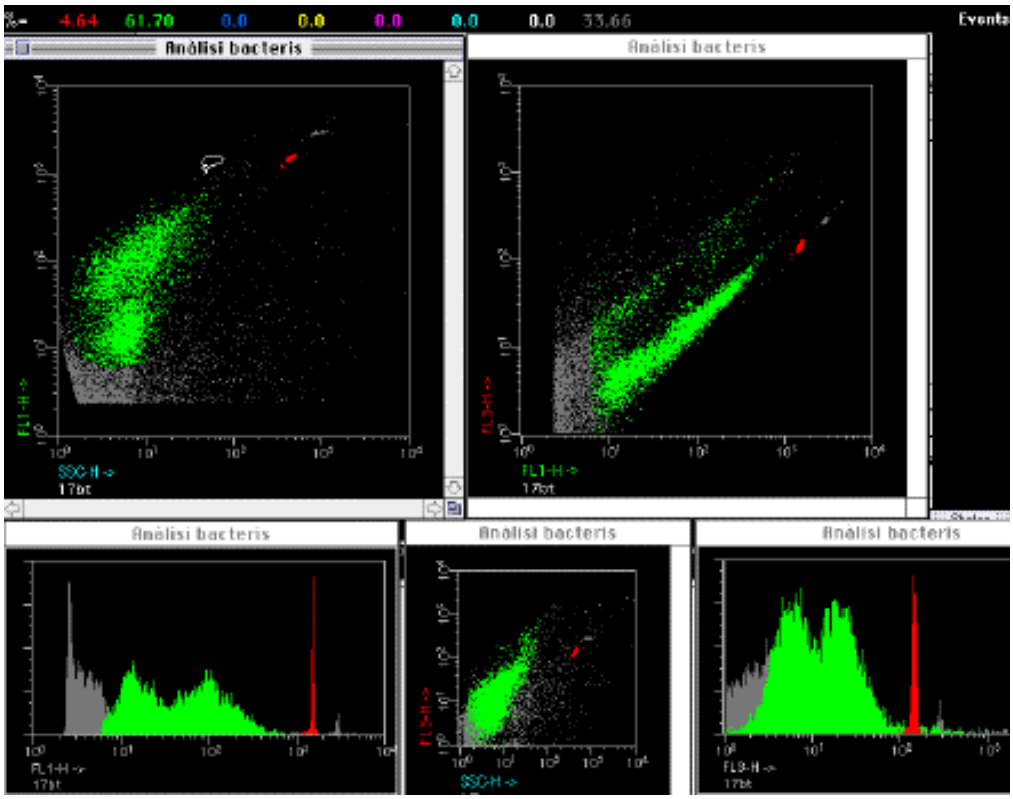
Follows a step by step description of how to process the data using the program Paint-A-Gate.

a.- <u>Bacteria</u>

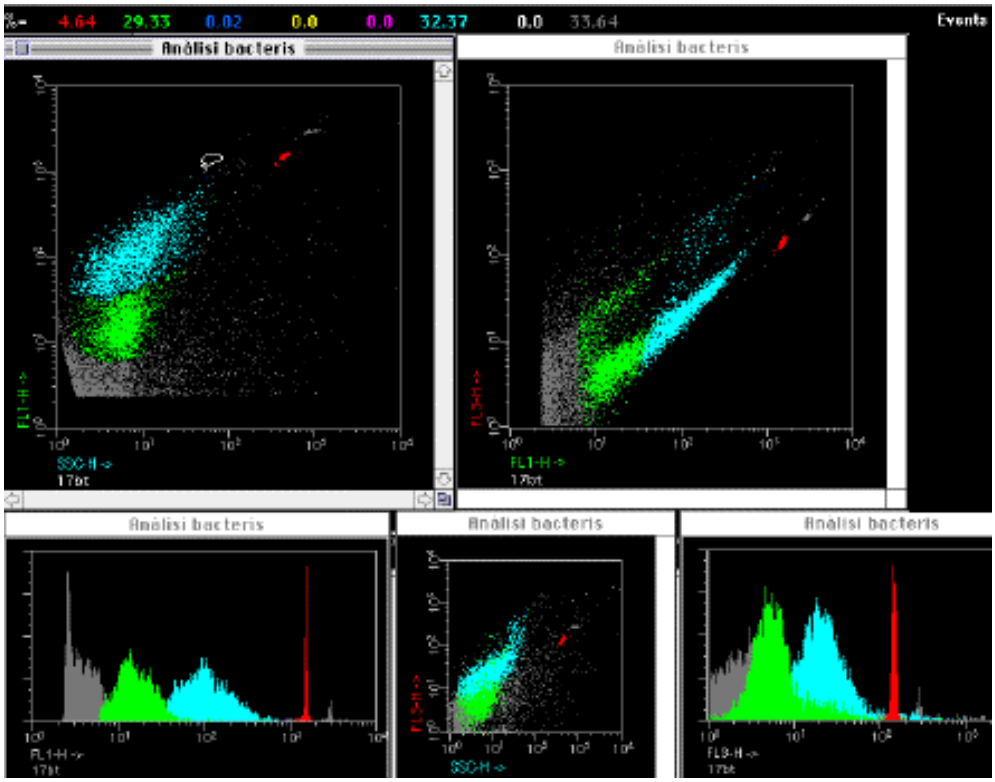
- Open Paint-A-Gate vs. 3 (Apple menu).
- Go to the menu **File**, under **Open PAG set** and select in the Users folder, the file "Análisis Bacteria". Nothing will happen. Go to the menu **Process** and select **Load FCS file** Select one data file of this kind. A window like the one below will appear.
- Select the red color (menu **Paint**), and paint the beads as in the right panel of the graph.



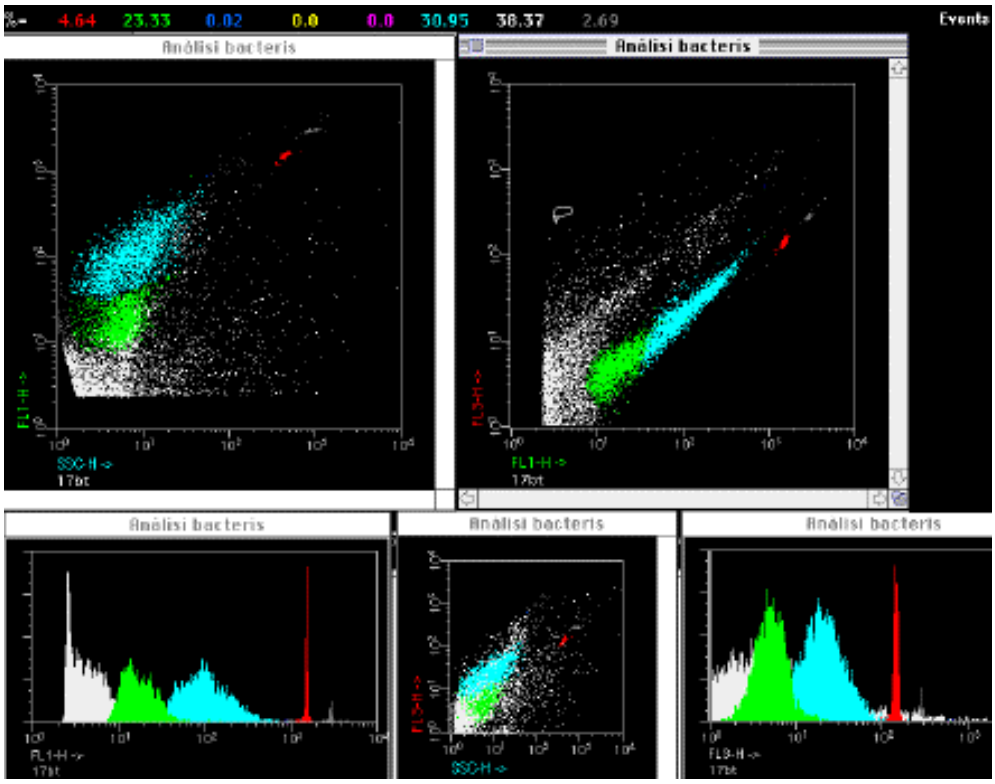
- Select the Green color (menu **Paint**), and paint all the bacteria as in the left panel of the graph.



- Select the blue color (menu **Paint**), and paint the high DNA bacteria as in the right panel of the graph.

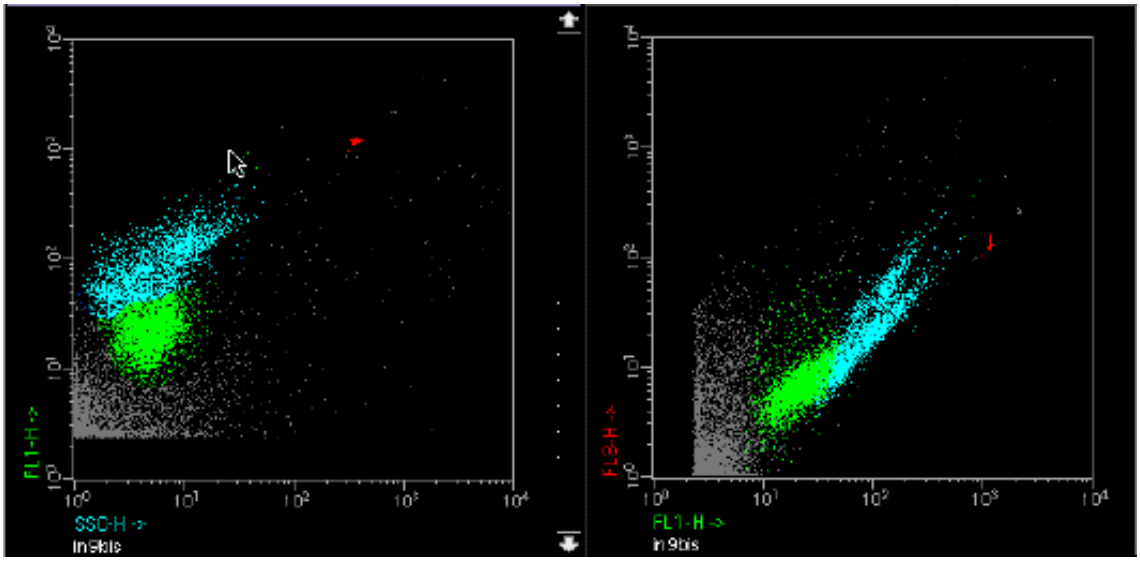


- Finally, with this process you included some noise particles that happened to be in the middle of the bacteria. To remove them, select the white color (menu **Paint**), and paint the noise as in the right panel of the graph.

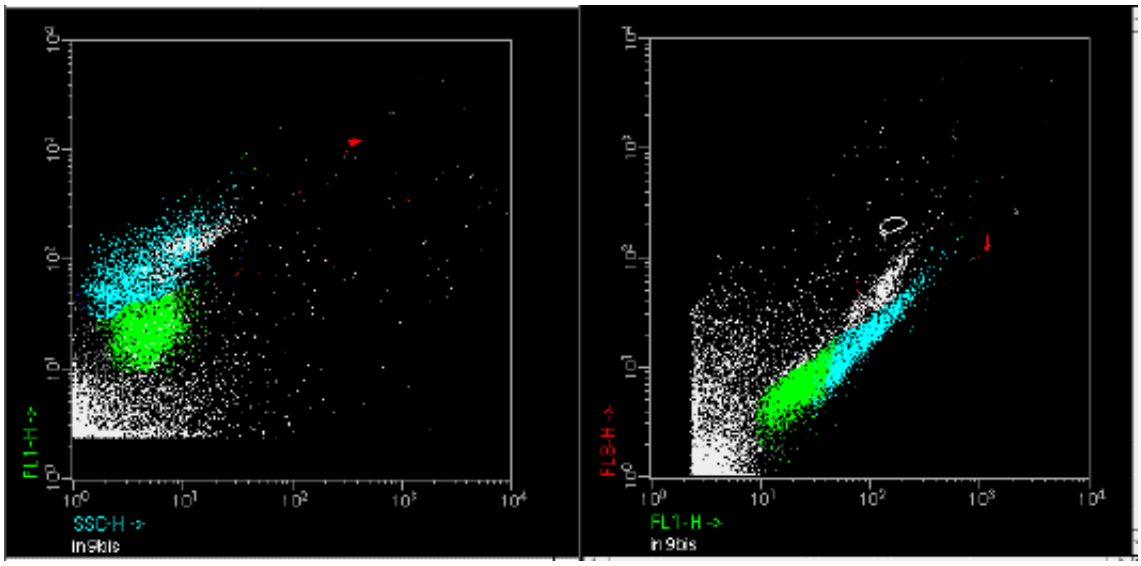


- If the sample had lots of *Prochlorococcus* or *Synechococcus*, they will be stained as chemotrophic bacteria. Then, two options can be done.

- You either consider them bacteria (they are bacteria, I know), count them in an unstained sample and substract the counts



- Or else you delete them using the white color, as in the example below. Note where in the left graph these populations appear.



- Now the data has been collected. Send the data to your file. To do so, you have first to go to menu **Report**, and "Open new spreadsheet". Give a name to the file.
- Now you can **Report**, "Send data to spreadsheet".
- Your data is now in the spreadsheet. Now you can open Excel, select the data of interest and paste it, for example, in a spreadsheet like this one:

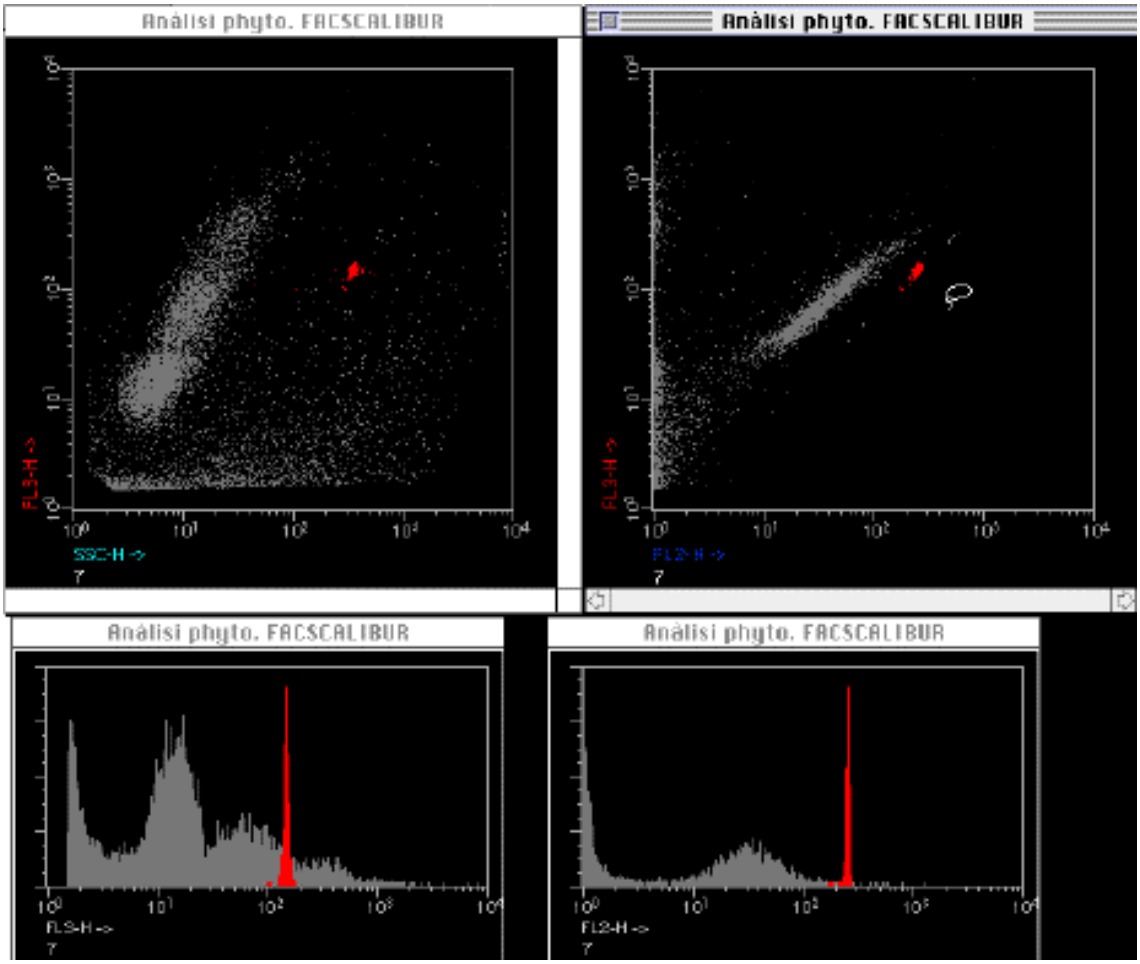
Bacex.xls

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1				Speed	TIME 0	TIME F	TIME (S)	VOL	Factor	Vol(ml)	Vol(µl)	Conc	Beads			LDNA
2				µl min-1				(µL)	dilució	passat	beads	beads	#	SSC	FL1	#
3	1A 1/4 bt			20.7	14:09:10	14:10:40	90.00	31.05	0.9091	1	10	1.50E+06	440	660.86	803.61	1730
4	1B 1/4 bt			20.7	14:09:10	14:10:40	90.00	31.05	0.9091	1	10	1.50E+06	333	660.06	802.73	1485
5	1B 1/4 bt			20.7	14:09:10	14:10:40	90.00	31.05	0.9091	1	10	1.50E+06	333	660.06	802.73	1485
6																

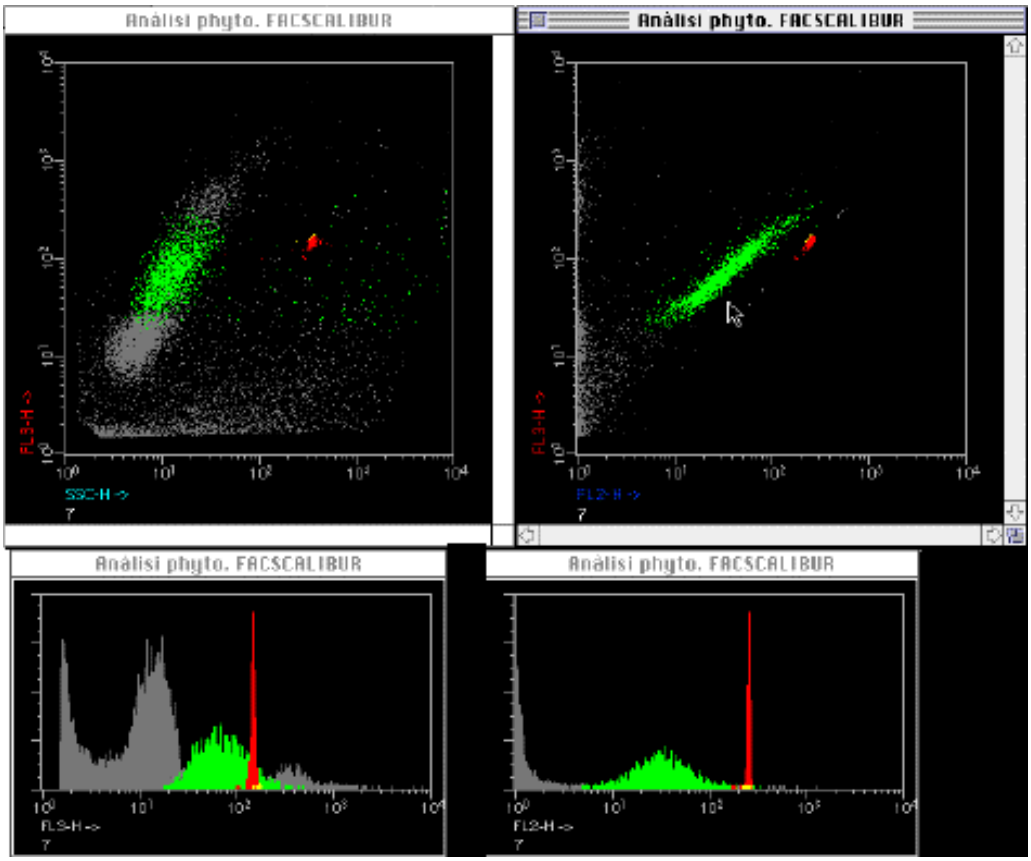
Bacex.xls

b.- <u>Picoalgae</u>

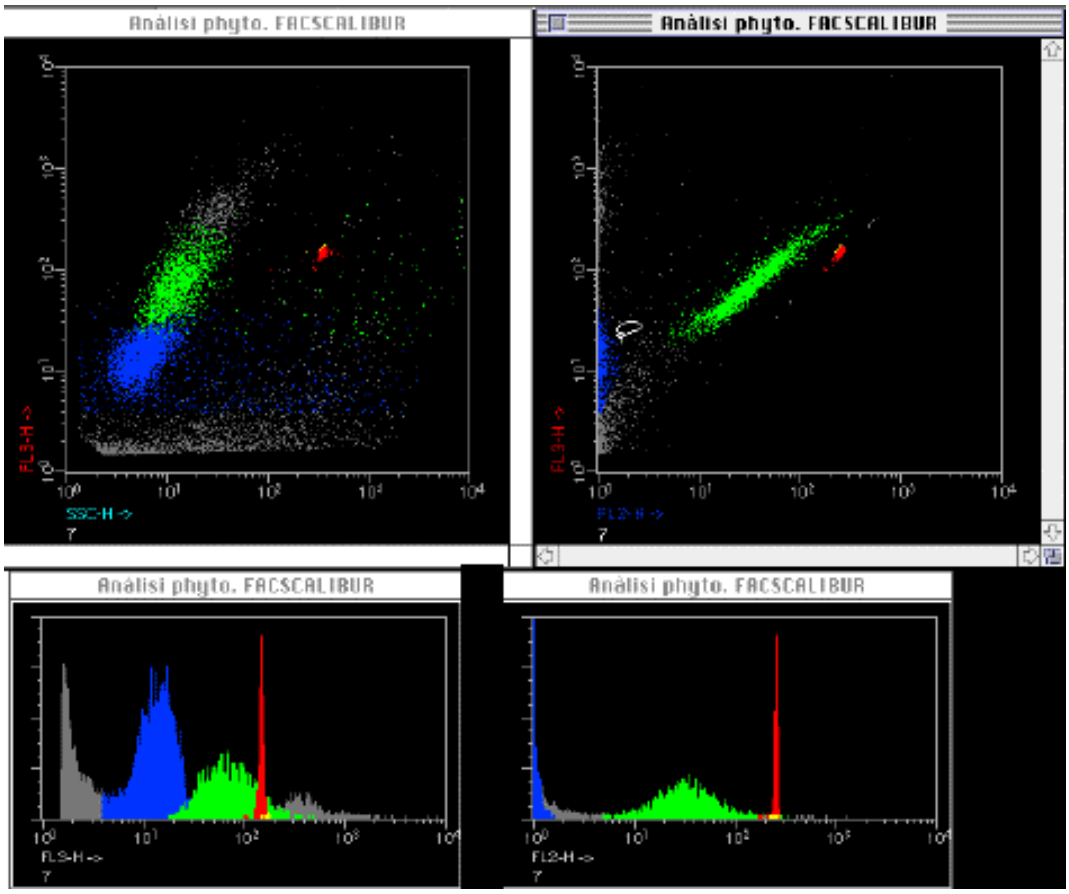
- Open Paint-A-Gate vs. 3 (Apple menu).
- Go to the menu **File**, under **Open PAG set** and select in the Users folder, the file "Análisis Fito". Nothing will happen. Go to the menu **Process** and select **Load FCS file**. Select one data file of this kind. A window like the one below will appear.
- Select the red color (menu **Paint**), and paint the beads as in the right panel of the graph.



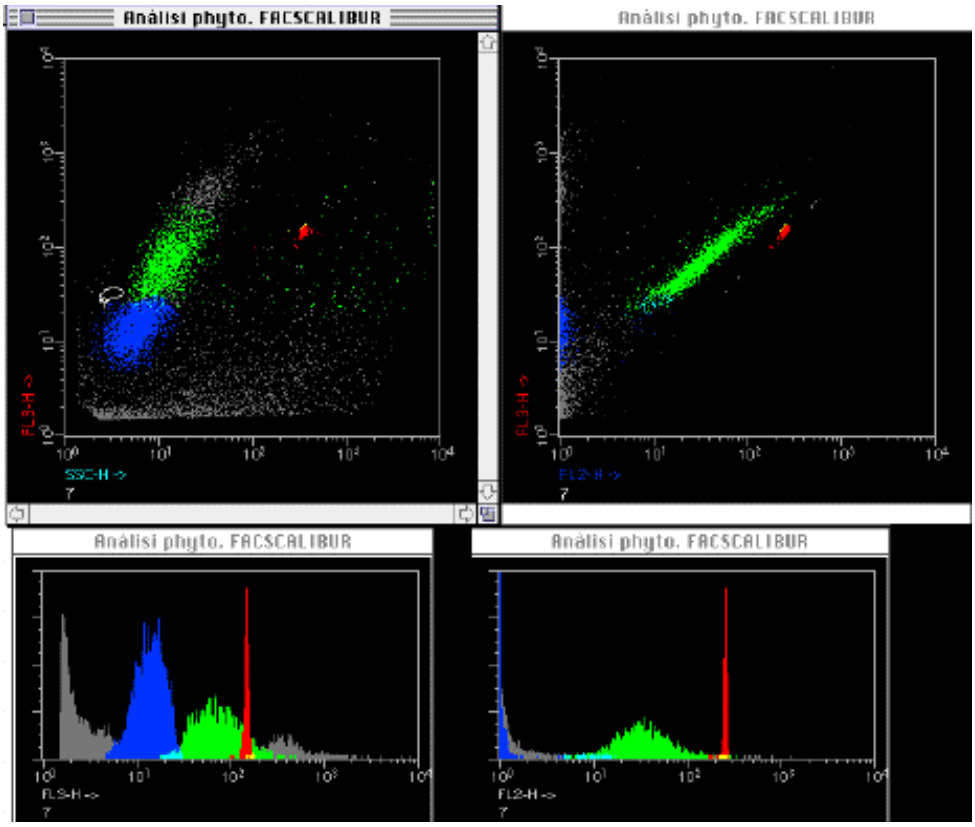
- Select the green color (menu **Paint**), and paint the Synechococcus as in the right panel of the graph.



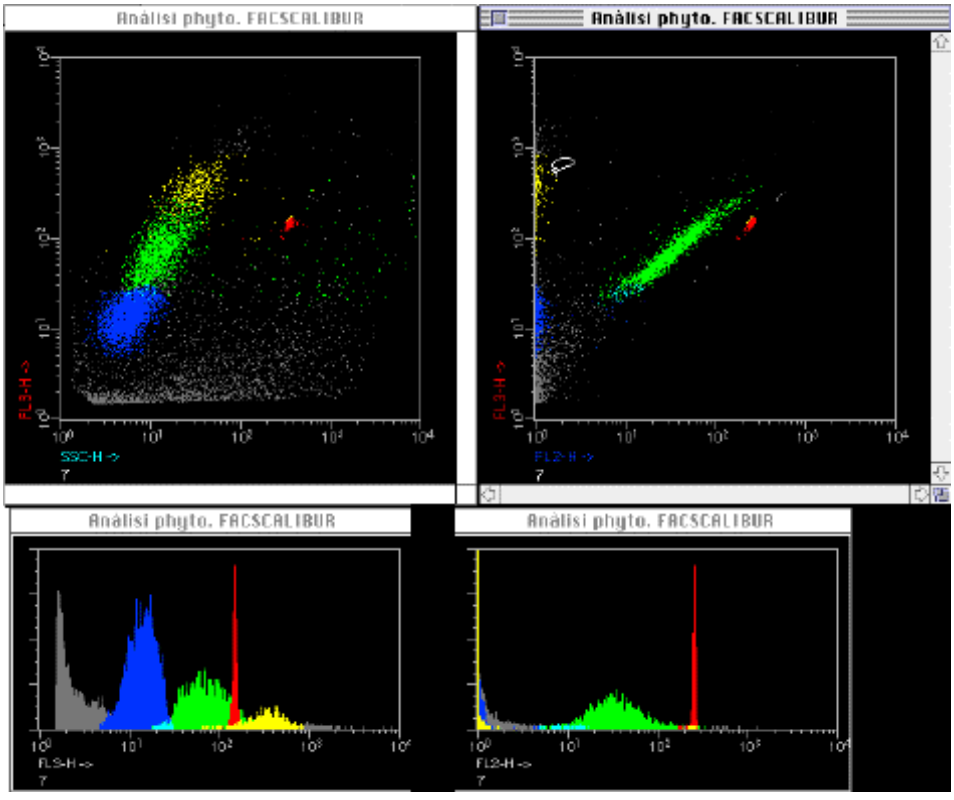
- Select the blue color (menu **Paint**), and paint the Prochlorococcus as in the right panel of the graph, OR



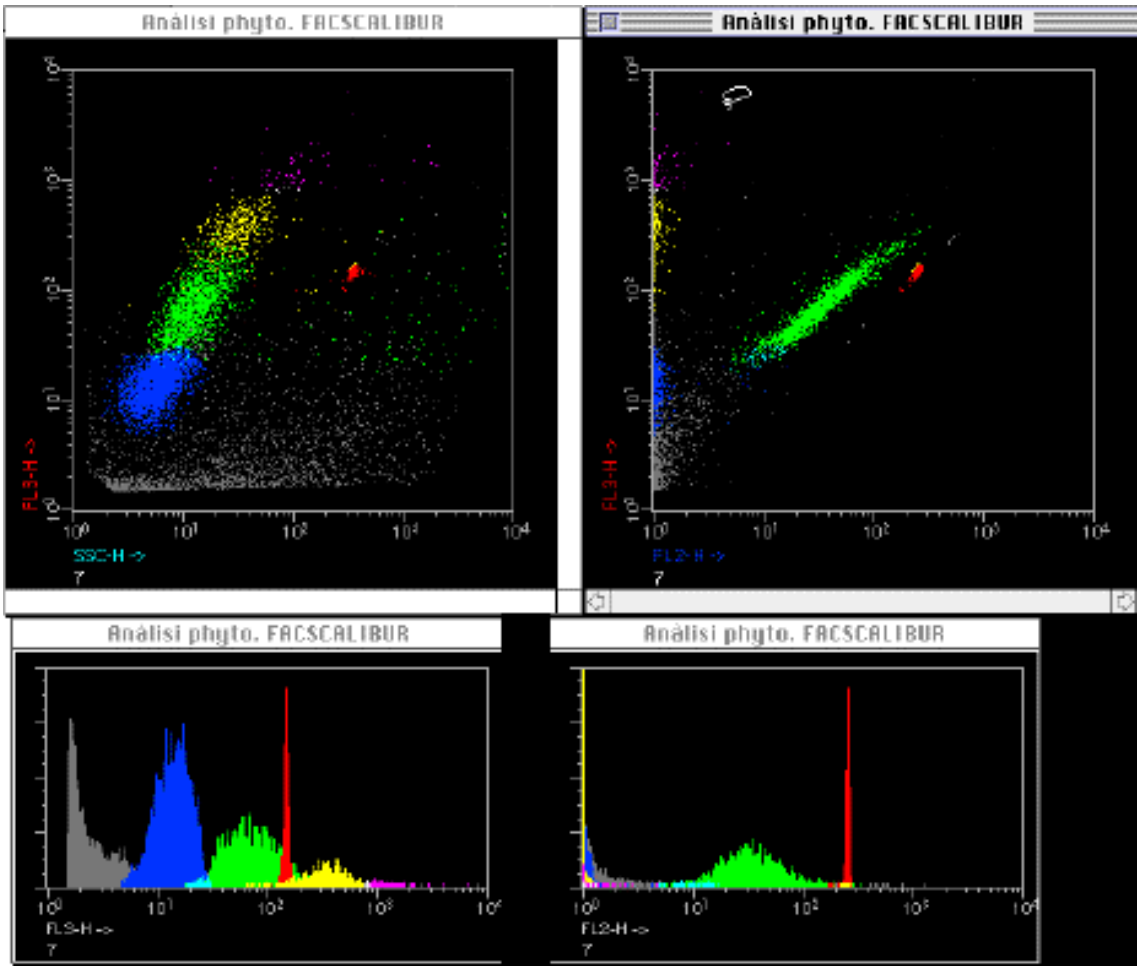
... as in the left graph. Choice between these two options depend on the type of sample, and the amount of noise.



- Select the yellow color (menu **Paint**), and paint the picoeukaryotes as in the right panel of the graph.



- Select the purple color (menu **Paint**), and paint the nanoeukaryotes as in the right panel of the graph.



- Now the data has been collected. Send the data to your file. To do so, you have first to go to menu **Report**, and "Open new spreadsheet". Give a name to the file.
- Now you can **Report**, "Send data to spreadsheet".
- Your data is now in the spreadsheet. Now you can open Excel, select the data of interest and paste it, for example, in a spreadsheet like this one:

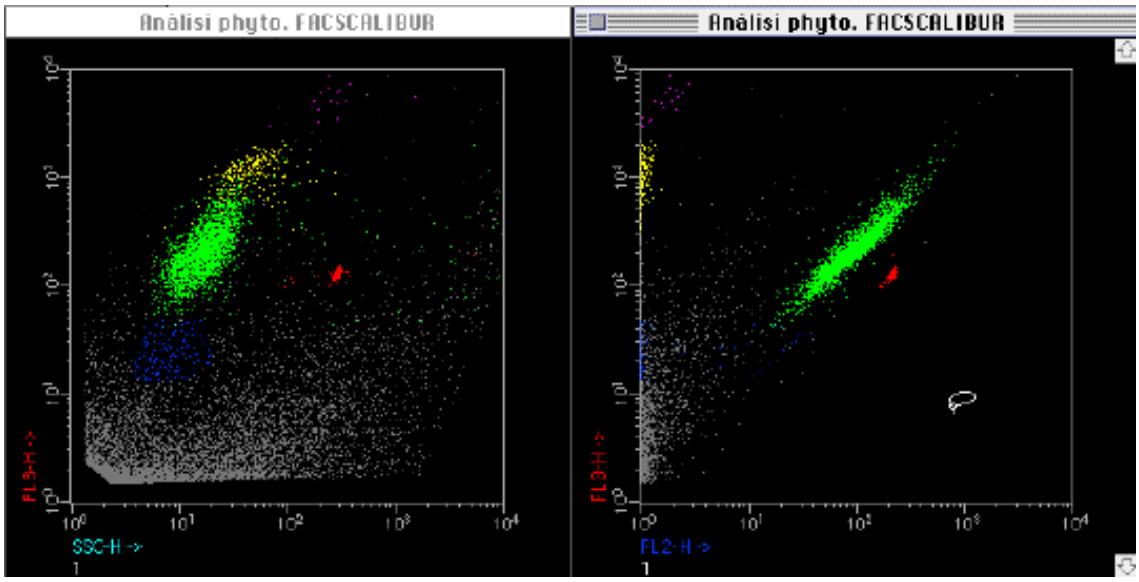
Fitoex.xls

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1				Speed	Speed	TIME O	TIME F	TIME (S)	VOL	Factor	Vol	Vol	Conc	Beads		
2					µl min-1				(µL)	dilució	passat	beads	beads	#	FL2	FL3
3	T150	0.8 A	0	HI	67.3	14:09:10	14:10:40	90.00	100.95	0.9091	0.6	10	1.00E+05	1718	634	536.64
4	T151	0.8 B	0	ME	32.9	14:09:10	14:10:40	90.00	49.35	0.9091	0.6	10	1.00E+05	1592	636.09	537.91
5	T152	150 A	0	LO	20.7	14:09:10	14:10:40	90.00	31.05	0.9091	0.6	10	1.00E+05	595	647.81	547.05
6																
7																

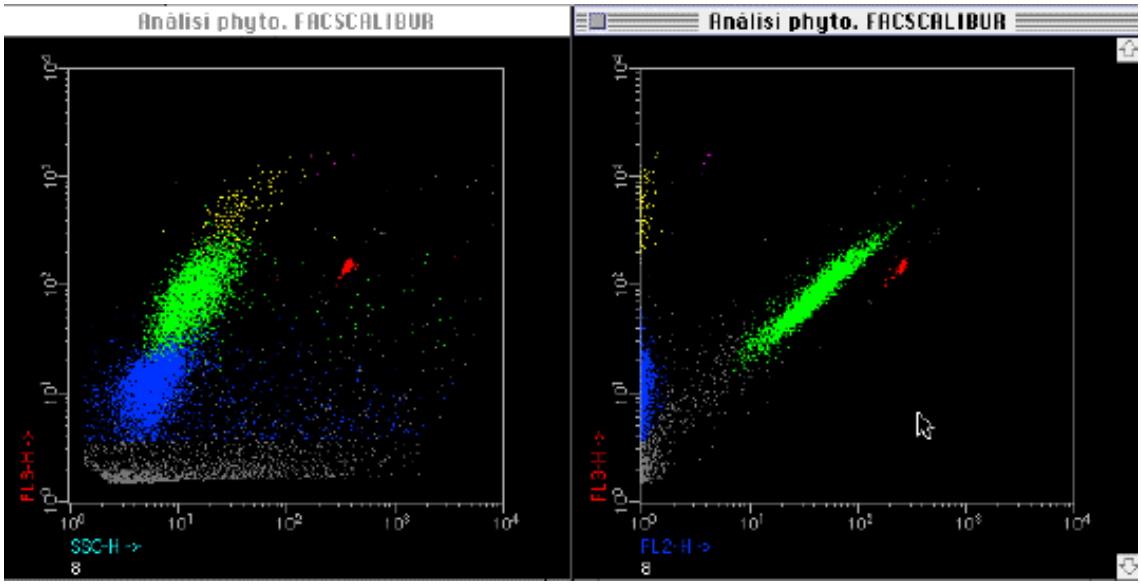
Fitoex.xls

- Picoalgal samples are less homogenous than bacterial samples. Here follow some very different examples:

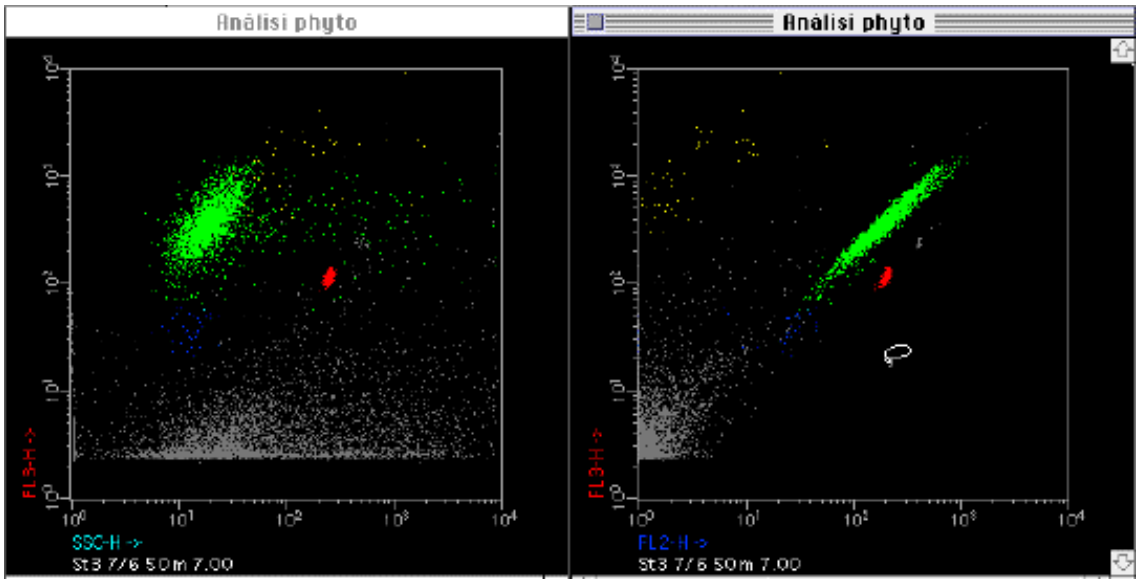
1.- This is a sample from an Atlantic estuary (cruise Incocéano'97). Note that there are no Prochlorophytes.



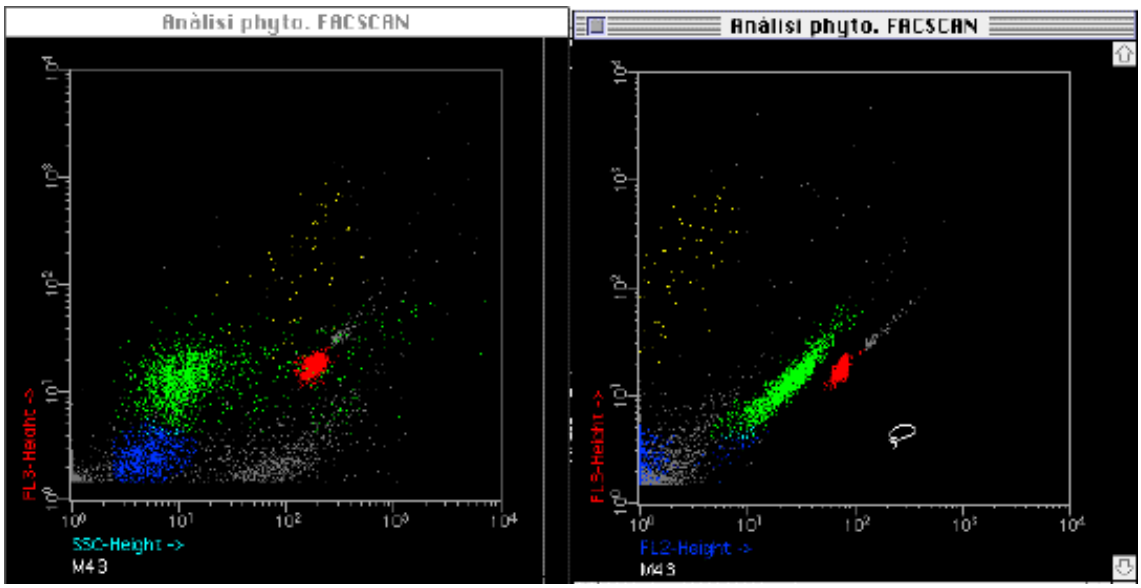
2.- A few milles offshore of the same estuary (cruise Incocéano'97). Note that there lots of Prochlorophytes.



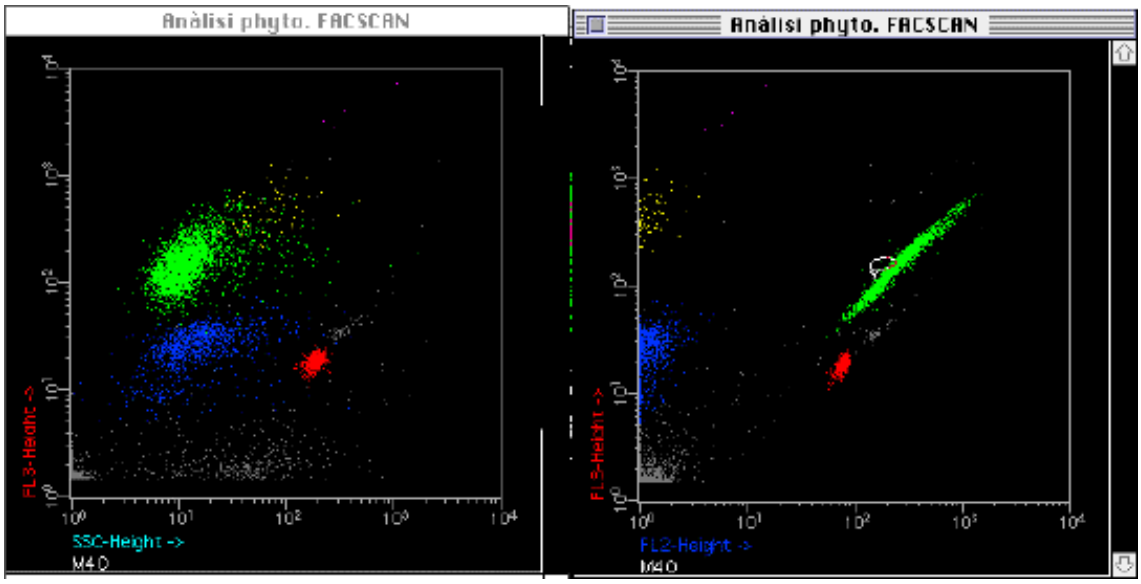
3.- This is a Mediterranean sample (cruise Varimed'95). Note that there are no Prochlorophytes.



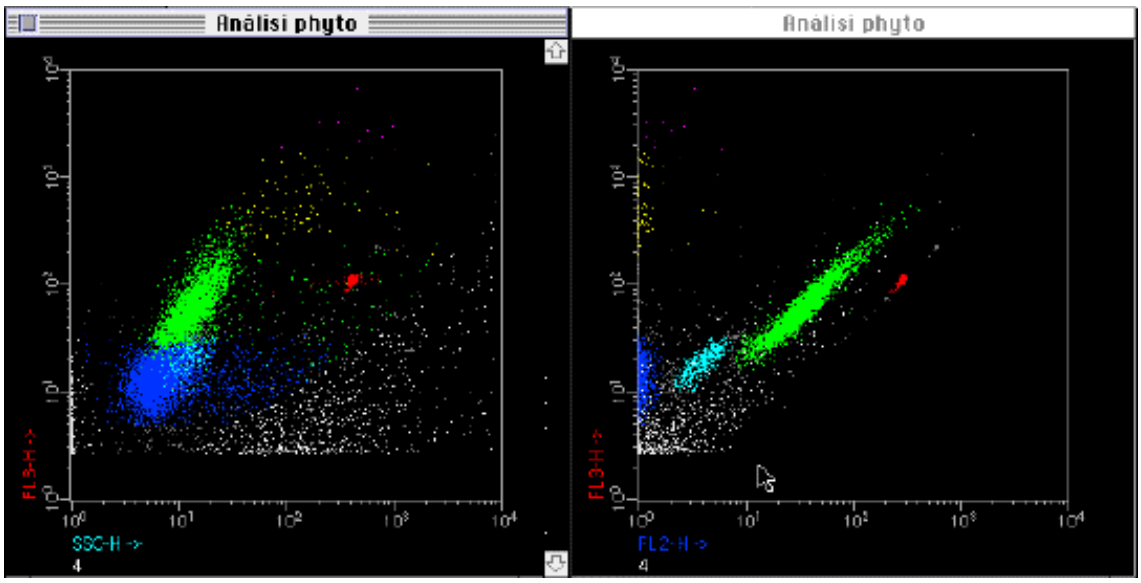
4.- Same Mediterranean area (cruise Varimed'96) a year later. Note that there this is very different. Note also the fluorescence of the different populations at the surface...



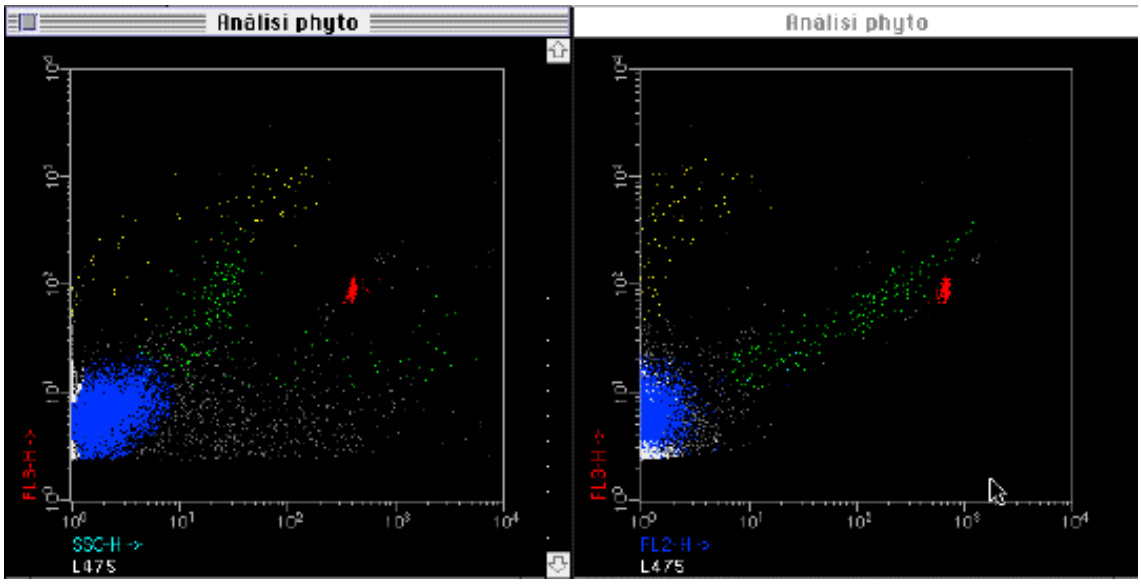
5.- ... and compare to this deep sample.



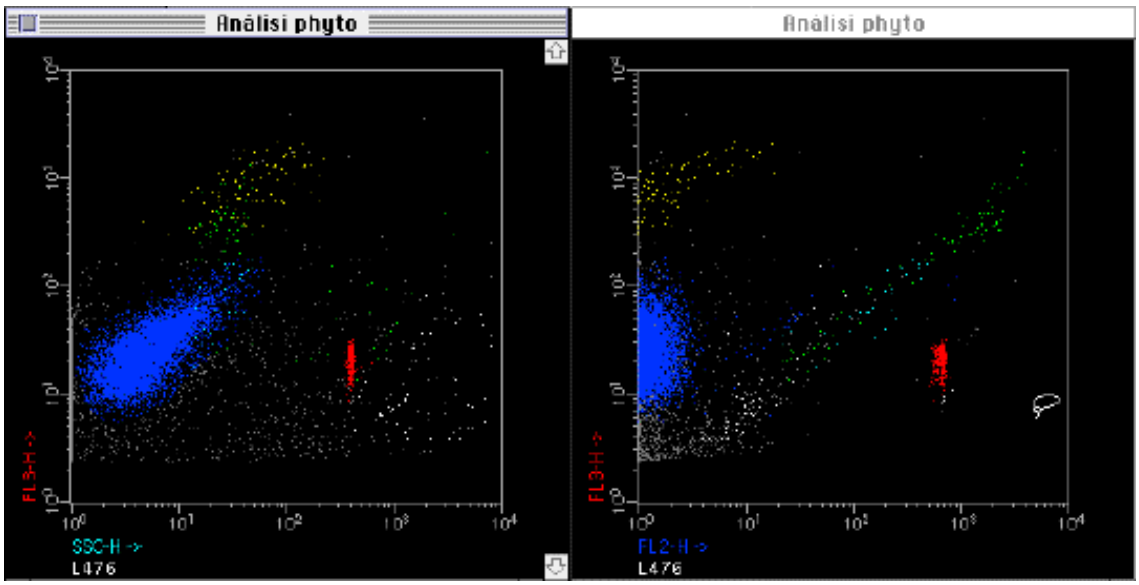
6.- Again in the Mediterranean (cruise Omega'97). Note that there is a new population, half the way between *Prochlorococcus* and *Synechococcus*.



7.- And finally, this is a sample from surface waters in the Equator (Atlantic, cruise Latitud-II, 1995). Note the large number of Prochlorophytes and their very low fluorescence.

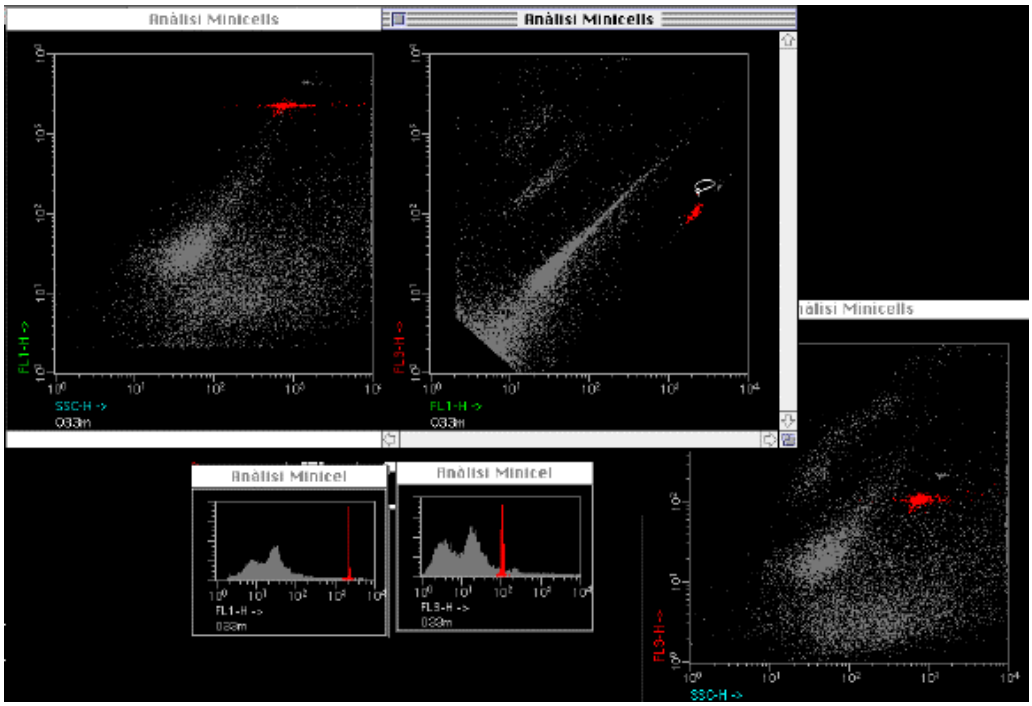


8.- And some meters below the surface. Note the increased number of picoeukaryotes and the higher *Prochlorococcus* fluorescence.

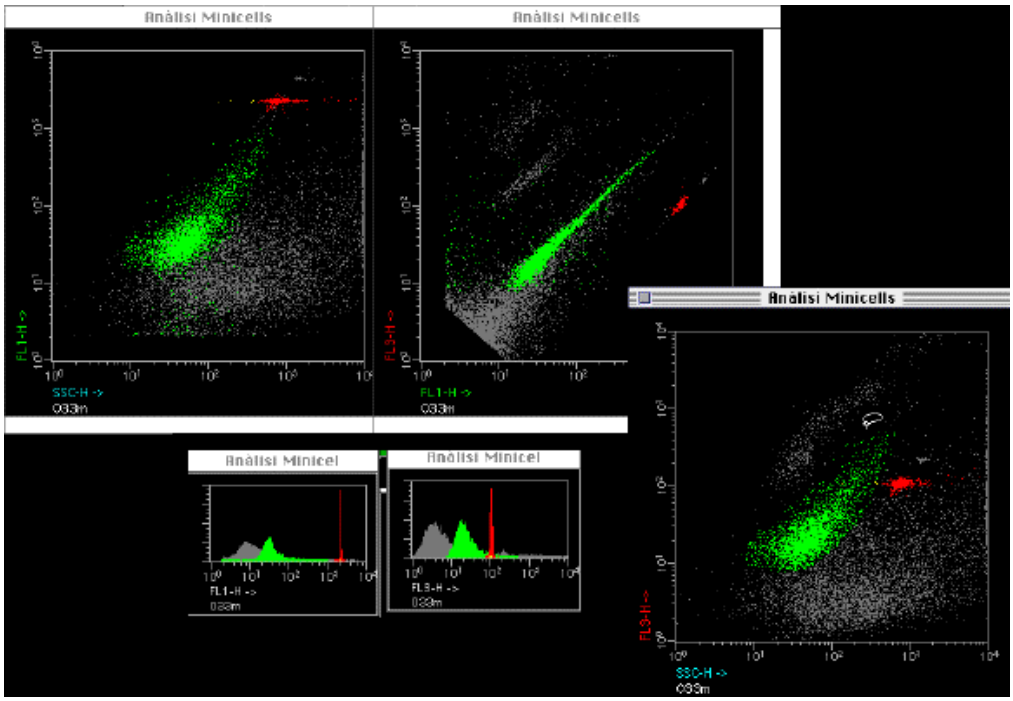


c.- <u>minicells</u>

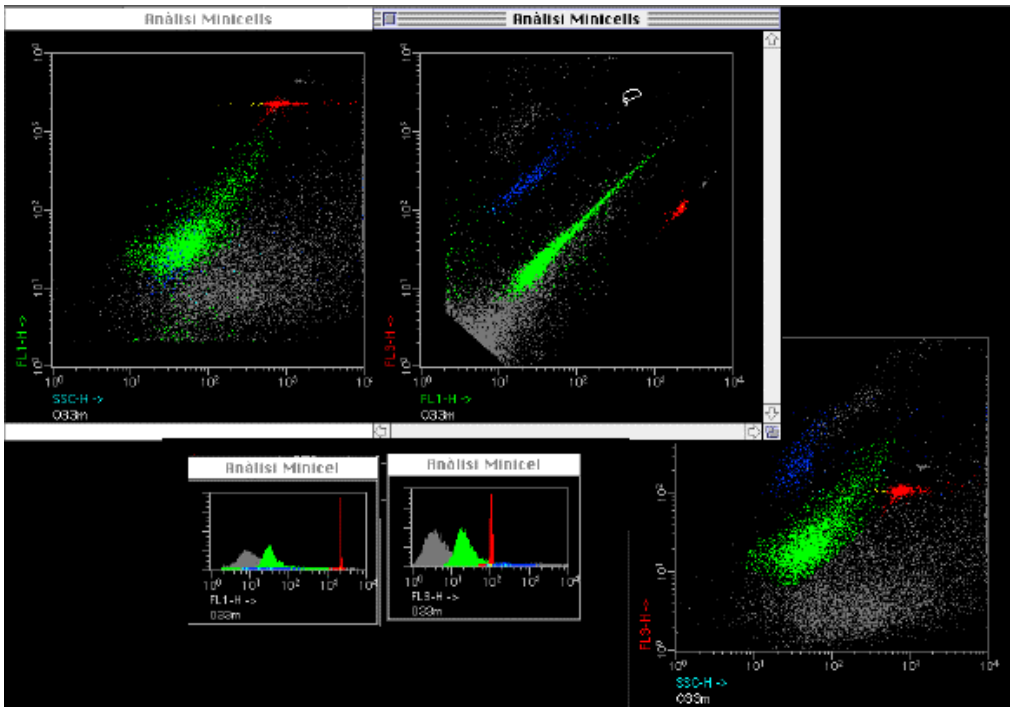
- Open Paint-A-Gate vs. 3 (Apple menu).
- Go to the menu **File**, under **Open PAG set** and select in the Users folder, the file "Análisis Minis". Nothing will happen. Go to the menu **Process** and select **Load FCS file**. Select one data file of this kind. A window like the one below will appear.
- Select the red color (menu **Paint**), and paint the beads as in the right panel of the graph.



- Select the green color (menu **Paint**), and paint the minicells as in the left panel of the graph.



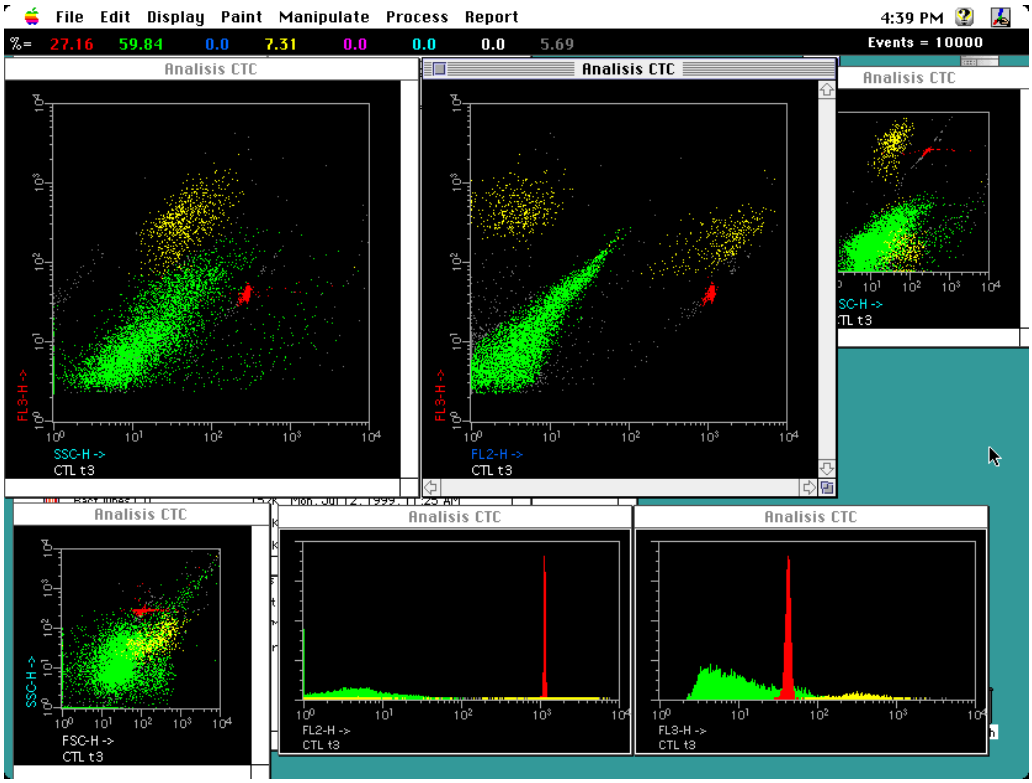
- Select the blue color (menu **Paint**), and paint the Synechooccus as in the right panel of the graph.



- and proceed as described above.

d.- A word about CTC analysis

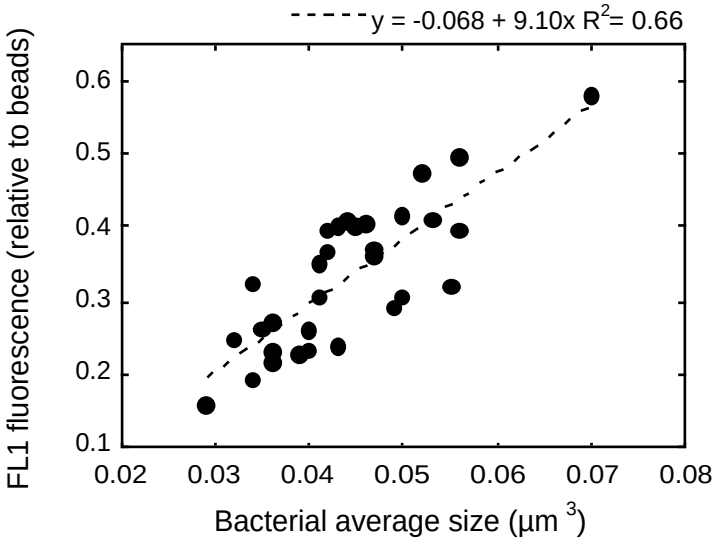
- This is how a CTC sample, which also has algae, looks like. The best way of separating the CTC+ cells (in green) is in the FL3-FL2 plot



P. Bacterial size

Literature presents conflicting results on the relationship between cell size and light scattering (see discussion in Gasol & del Giorgio, 2000). Most current bench top cytometers are equipped with a photodiode to capture the light scattered in the forward direction, which is less sensitive than the photomultiplier tubes typically used to collect side scatter and fluorescence. It is our experience that in both FACSCalibur and Coulter, the dispersion of the reference beads is much greater in forward scatter than it is in any other parameter. Cytograms included in recent published papers also often show the same large dispersion of beads as well as of target cells in forward scatter. This limits the usefulness of forward scatter and possibly weakens any relationship with cell size. Some instruments, however, have been equipped with photomultiplier tubes protected by screens to capture light scattered in forward angles, and this probably greatly increases the sensitivity of this parameter. On the other hand, the range of bacterial cell sizes used to establish an empirical relationship between cell size and scatter is also critical. The evidence to date is that forward (and perhaps side) scatter is a good index of bacterial cell volume for larger, typically cultured bacteria, but there is still no convincing evidence that forward scatter can be used to estimate the size of natural bacterioplankton cells in the 0.03 to 0.1 µm3 range.

An alternative to using scattered light as an index of bacterial size, is the use of the fluorescence of DNA-bound stains (Steen & Boye, 1981). Veldhuis et al. (1997) have found that DNA content, as estimated with PicoGreen, varies with cellular C and N content, at least for pico- and nanoalgae. We have also found that filters which are known to be size-selective (Glass fiber and cellulose ester filters) remove a large portion of the Syto-stained cells with the strongest green fluorescence (Gasol & Morán, 1999), offering indirect support to the relationship found by Veldhuis et al. (1997). Troussellier et al. (1999) also found cell size to be related equally to SSC and to DNA fluorescence. We recently found a very good relationship between image analysis measurements of planktonic bacterial size (in the range 0.03 – 0.09 μm^3) and the average green Syto13 fluorescence per cell (with data from Prairie et al., submitted) suggesting that indeed, DNA-related fluorescence can be used as a surrogate of bacterial size, although some calibration is needed.



We have been using this relationship in a wide variety of systems and have found very reasonable estimates of bacterial size except in the most eutrophic environments where

long bacterial filaments were abundant (J.M. Gasol and K. Simek, unpublished). Calibration with bacteria of known sizes is required also if we are using DNA-related fluorescence as a means of measuring bacterial size.

The formula is implemented in the excel file used for caculation, and it refers always to 1 µm YG Polysciences beads.

Q. Where to buy...

This is just a compilation of codes, names and fax numbers to speed up buying the things.

a) stains

Molecular probes Fax 31715233419

Syto13 Cat# S7575 (250 µl)

b) beads

Polysciences Fax 496221764620

Fluoresbrite 0.2 µm YG Cat# 17151

c) tubes

Falcon tubes for cytometry

1000 units	sterile	Code 2052	10200 pta
1000 units	not sterile	Codi 2008	7000 pta

In EMB (93 412 3721, fax 93 412 2192)

e) calling the B&D service Fax: 91 8488104

R. How to cite the methods in a paper

Flow cytometry determination of bacterial abundance and biomass. 1.5 ml samples are fixed with 1% paraformaldehyde + 0.05% glutaraldehyde (final), let 10 minuts to fix in the dark, deep frozen in liquid nitrogen and then stored frozen at -70° C. The samples are then unfrozen, stained with Syto13 (Molecular Probes) at 1.6 - 5 µM (diluted in DMS), let 15 minutes to stain in the dark and run through a flow cytometer. We use a bench machine FACScalibur of Becton&Dickinson with a laser emitting at 488 nm. Samples are run at Low speed (approx. 18 µl min^{-1}) and data are adquired in log mode until around 10000 events have been adquired. We dilute the samples when the sample acquisition rate is higher than 800 cells s^{-1}. We usually add 10 µl per 200 µl sample of a 10^6 ml^{-1} solution of yellow-green 0.92 µm Polysciences latex beads as an internal standard. Bacteria are detected by their signature in a plot of Side scatter (SSC) vs. FL1 (green fluorescence). If we adjust the settings so that the beads fall in channels ~ 10^3 for SSC and FL1, bacteria fall in the channels around 10^1 for SSC and 10^2 for FL1 parameters. Algae are told apart by spotting a plot of FL1 vs. FL3. In this graph, beads fall in a line, bacteria in another one, and noise in a third one (respectively with more FL3 than FL1). Phototrophic bacteria (*Prochlorococcus* and *Synechococcus*) fall in between noise and bacteria. This method is based in that published by del Giorgio et al. (1996) as discussed in Gasol and del Giorgio (2000). The average fluorescence of the bacterial population, as normalized to that of the beads, is a rough approximation of bacterial size. We use the reference line of Paririe et al. (submitted) as a way of estimating average bacterial size. This is, however, calibrated by comparison to image analysis of epifluorescence preparations processed following Massana et al. (1997).

Flow cytometry determination of pico- and nanoalgal concentration. 1.5 ml samples are fixed with 1% paraformaldehyde + 0.05% glutaraldehyde (final), let fix for 10 min., deep frozen in liquid nitrogen and stored frozen at -70° C. The samples are then unfrozen and run through a flow cytometer. We use a bench machine FACScalibur of Becton&Dickinson with a laser emitting at 488 nm. Samples are run at High speed (approx. 60 µl min^{-1}) and data are adquired in log mode until around 10000 events have been adquired. We usually add 10 µl per 600 µl sample of a 10^5 ml^{-1} solution of yellow-green 0.92 µm Polysciences latex beads as an internal standard. *Synechococcus* are detected by their signature in a plot of orange fluorescence (FL2) vs. red fluorescence (FL3). *Prochlorococcus* have a lower FL3 signal and no FL2 signal. Eukaryotic picoplankton have higher FL3 signals and no FL2 signals. Nanoeukaryotes have much higher FL3 signals. If *Cryptomonas* are present, they have much higher FL2 and FL3 signals. The settings are adapted for each sample as the populations and their fluorescence may change from sample to sample. With standard settings, beads fall at SSC: 3E2 and FL3: 1E2, and then *Prochlorococcus* fall at SSC: 1E1 and FL3: 3E1; *Synechococcus* fall at SSC: 3E1 and FL3: 2E2 and picoeukaryotes at SSC: 1E2 and FL3: > 1E3. Further details can be obtained from Olson et al. (1993, pp. 175-186, In: Kemp et al. (eds.) Handbook of Methods in Aquatic Microbial Ecology, Lewis Publ.).

S. For learning more (absolutely all the references you always wanted to read...)

I put together all the references in George Dubelaar's web page (), which deal mainly on technical aspects and on the analysis of phytoplankton, but are slightly outdated (1997), and all the references in a recent review on the application of flow cytometry to count bacteria in plankton (Gasol, J.M. and P.A. del Giorgio. 2000. Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. Scientia Marina, in press). If you read them all, you'll learn a lot !

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