STANDARD OPERATING METHOD (SOM)
FOR GLEN TARRAN
ANALYSIS AND QUANTIFICATION OF NANO- AND PICOPLANKTON FROM STATION L4 BY FLOW CYTOMETRY (BD Accuri C6 flow cytometer)

Sample Collection

Triplicate seawater samples from the surface, 10, 25 and 50 m are collected from the rosette bottle sampler aboard RV Plymouth Quest into 0.25L square polycarbonate bottles after first rinsing the bottles with seawater. The bottles are then stored in a coolbox and transported back to the laboratory for analysis. The time taken between sample collection and the beginning of analysis is between 3.5 to 5.5 hours, depending on the state of the tide and access through the lock gates of Sutton Harbour, Plymouth.

Sample preservation for analysis of bacteria and heterotrophic nanoflagellates (HNAN)

Samples are taken to a fume cupboard (room 212) along with sample tubes, tube lids, tube rack, pipettors, pipettor tips, gloves and 50% glutaraldehyde solution (Sigma TEM grade in 10 mL bottles) taken from the walk-in freezer on the first floor.
Put on gloves.
Place triplicate 5mL flow cytometry sample tubes for each sample depth into the tube rack and put into fume cupboard.
Pipette 15 µL glutaraldehyde solution slowly into each tube (this will provide approx. 0.5% glataraldehyde final concentration after sample addition).
Carefully wash off pipettor tip inside and out using the sink at the back of the fume cupboard whilst still attached to the pipettor.
Place pipettor on its side in the fume cupboard.
Take a seawater sample bottle and carefully invert 4 times.
Take a 1-5 mL pipettor, remove 1.5 mL of sample and place in the appropriate flow cytometry sample tube.
Repeat this process for all sample tubes.
Place lids on sample tubes.
Hand vortex (by flicking bottom of tube with finger) all tubes to ensure even mixing of glutaraldehyde into the samples. *Very cold glutaraldehyde may take up to 15 vortexes before it mixes.*
Remove tube rack from fume cupboard.
Return to the fume cupboard, remove the glutaraldehyde pipettor tip from the pipettor with gloved hand and remove glove, turning it inside out. You should now have the tip inside the glove.
Hold the glove in the remaining gloved hand and remove the remaining glove from your hand, again, turning it inside out. The first glove should now be in the second and the tip is now inside 2 gloves.
Dispose of gloves in the bin.
Take live and preserved samples to the flow cytometry facility, room 114.
Place preserved samples in a refrigerator for 30 minutes.

Flow cytometric analysis of live nano- and picophytoplankton using the BD Accuri C6 flow cytometer

Check the sheath fluid bottle is full and the waste bottle is empty.
If not, empty the waste into the white plastic sink.
To fill the sheath tank, fill the upturned 1 L beaker with Elga water from the 10 L aspirator and pour into the sheath bottle.
Turn on the flow cytometer and computer.
While the C6 goes through a startup cycle double click on the C-flow plus icon on the desktop and allow the programme to start.
Go to File on the main menu and select Open C-flow file or template.
Select the Flow_cytometry directory, Templates and then 1L4PPLK.
Remove the sample of Milli-Q/Elga water from the SIP (Sample input port) and replace with the empty tube marked Backflush in the polystyrene tube holder.
Click the Backflush 'button' on the screen. Wait until Backflush has completed.
Remove the tube, discard the fluid into the sink and put a fresh tube of Milli-Q/Elga water on the SIP.

Look at the representation of the 96 well in the top left corner of the screen and check to see that cell H1 is selected. Check that the time limit is set at 2 minutes and then select 'RUN'.
In the Save window that appears, go up one level in the directory structure, select Users, Glen, L4 and then create a new folder with today's date in the format yymmdd.
Open the folder and click Save. The C6 will start up automatically and analyse the sample.
Whilst the water sample is running, prepare a tube rack with 12 flow cytometer samples tubes.
When the water sample has been analysed, remove the sample tube.
Take sample bottle A1 (surface), invert it carefully 4 times, remove a 2mL sample with a pipettor and place in a clean sample tube.
Hand vortex the sample and discard down the sink.
Place another 2 mL sample in the tube and place in the SIP of the flow cytometer.
Select cell A01 and see that the filename in the line above is P021
Change the analysis time limit from 2 to 5 minutes, check that the flow rate is set at 100 µL per minute and the core size is 15 µm and then begin acquisition. NOTE: do not trust the flow rate to be accurate.
The sample will be analysed for 5 minutes at high flow rate.
After approximately 4 minutes, prepare the next sample.
After 5 minutes the flow cytometer will stop analysing.
Remove the sample and replace it with the new one.
Go to the next cell, see the new filename and then begin acquisition.
Repeat this process for the remaining samples. When all live phytoplankton samples have been analysed, run samples of Beckman Coulter Flowset fluorospheres to calibrate the flow rate as outlined in the calibration of flow cytometer flow rate section below.

Staining of preserved samples for bacteria and HNAN analysis
When samples have been fixed for at least 30 minutes, put on a pair of gloves, go to the walk-in freezer next to room 114 and collect a 2 µL aliquot of Sybr Green I DNA dye.
The aliquots are kept in 0.5 mL microcentrifuge tubes in blue pipettor tip racks.
Take a flow cytometry sample tube and half fill it with 300 mM potassium citrate buffer (0.1 µm filtered and kept in the refrigerator). Remove 2 mL with a pipettor, discard the fluid remaining in the tube and then pipette the 2 mL back into the tube.
Using a different pipettor, take out 150 µL of the buffer from the 2 mL and add it to the 2 µL Sybr Green I aliquot.
Use the pipettor to mix the Sybr and buffer.
Pipette the Sybr/buffer mix from the microcentrifuge tube into the 1.35 mL remaining buffer.
Use the pipettor to mix the buffer and Sybr.
Take the samples out of the refrigerator and take the lids off them.
Add 150 µL of buffer/Sybr Green mix to each sample, replace the lids and hand vortex the tubes.
Place the samples in a drawer (in the dark at room temperature) for at least 1 hour.

Calibration of flow cytometer flow rate – whilst samples are staining
The flow rate of the flow cytometer is calibrated by analysing samples of fluorescent microspheres (beads) at a known concentration for a set length of time. The beads used are Beckman Coulter Flowset fluorescent microspheres. These beads are used because they are of a similar size to the nano- and picoplankton (they are 3.6 µm in diameter) and their fluorescence emission is in the same range so that instrument settings do not need to be altered. Bead stock concentration is determined using a Beckman Coulter Multisizer 3. This determination is covered by a separate SOM.

High flow rate calibration
Take 3 flow cytometry sample tubes and place in a tube rack.
Fill one with Elga/Milli-Q (de-ionised) water.
Take the bottle of beads out of the refrigerator and invert the bottle to ensure the beads are evenly suspended for at least 1 minute.
While inverting the beads, pipette 900 µL of water from the full tube to one of the remaining empty tubes.
When the bottle of beads has been inverted for 1 minute, dispense 4-5 drops of bead suspension into the remaining empty tube.

Remove 100 µL of bead suspension from the tube and add to the 900 µL of Milli-Q water. You now have a 10x dilution of the bead suspension.
Hand vortex the diluted bead suspension.
Insert the tube into SIP of the flow cytometer, select an empty cell, type in the filename Flowset1 and analyse on high flow rate for 2 minutes.
When the analysis is complete, remove the tube.
Hand vortex it, tap it a couple of times on the bench top, reinsert the tube into the SIP of the flow cytometer, select an empty cell, type in the filename Flowset2 and analyse as before.
Repeat these steps for a third time (Flowset3).
Create a bivariate plot of orange vs. red fluorescence.
The beads should appear as a line, with single beads in the bottom left of the line, then doublets (showing up as a cluster with approximately double the fluorescence of the single beads), then triplets and above. Draw separate regions around the single, double and triple beads and record the numbers for each region.
Repeat for the remaining replicates.
Input the numbers into a spreadsheet. Add columns to multiply the doublets by 2 and the triplets by 3. Sum these 2 new numbers, along with the singles number to get a total number of beads analysed.
You can now calculate the flow rate as microlitres per minute as follows:

$$\text{Flow rate } (\mu L\ min^{-1}) = \frac{\frac{\text{Total count}}{\text{Diluted bead concentration}} \times 1000}{2}$$

Flow cytometric analysis of preserved bacteria, stained with Sybr Green I
Collect a stained sample from the drawer and hand vortex the sample.
Place the sample in the SIP of the flow cytometer
Go to File on the main menu and select Open C-flow file or template.
Select Yes when prompted to Save Changes of the previous analyses (phytoplankton).
Select the Flow_cytometry directory, Templates and then 1L4BAC.
Look at the representation of the 96 well plate in the top left corner of the screen and check to see that cell A01 is selected and see that the filename in the line above is B021.
Check that the time limit is set at 2 minutes, that the flow rate is set to FAST (nominally 66 µL per minute and the core size is 15 µm. NOTE: do not trust the flow rate to be accurate.
Select 'RUN'.
In the Save window that appears, go up one level in the directory structure, select Users, Glen, L4 and then the folder previously created for the phytoplankton.
Open the folder and click Save. The C6 will start up automatically and analyse the sample.
After 2 minutes the flow cytometer will finish.
Remove the sample, save it for heterotrophic nanoflagellate analysis and replace it with a new one.
Select the next cell and then begin acquisition.
Repeat this process for the remaining samples.

Flow cytometric analysis of heterotrophic nanoflagellates, stained with Sybr Green I
Collect a stained sample from the drawer and hand vortex the sample.
Place the sample in the flow cytometer
Go to File on the main menu and select Open C-flow file or template.
Select Yes when prompted to Save Changes of the previous analyses (bacteria).
Select the Flow_cytometry directory, Templates and then 1L4HNAN.
Look at the representation of the 96 well plate
in the top left corner of the screen and check to see that cell A01 is selected and see that the filename in the line above is H021.
Check that the time limit is set at 7 minutes, that the flow rate is set at 100 µL per minute and the core size is 15 µm. NOTE: do not trust the flow rate to be accurate.
Select 'RUN'.

In the Save window that appears, go up one level in the directory structure, select Users, Glen, L4 and then the folder previously created for the phytoplankton and bacteria.
Open the folder and click Save. The C6 will start up automatically and analyse the sample.
After 7 minutes the flow cytometer will finish.
Remove the sample and replace it with a new one.
Select the next cell and then begin acquisition.
Repeat this process for the remaining samples.

SHUT DOWN FLOW CYTOMETER

When all samples have been analysed, remove the last sample tube and replace it with a tube containing cleaner fluid (detergent).
Select an empty cell.
Change the sample run time to 4 minutes.
Select RUN.
After 4 minutes replace the cleaner fluid with a tube of decontamination fluid (0.5% bleach).
Select an empty cell.
Select RUN.
After 4 minutes replace the cleaner fluid with a tube of Elga/Milli-Q water.
Select an empty cell.
Select RUN.
After 4 minutes, leave the water sample in the flow cytometer.
Insert a USB stick into the computer.
Download the data from the experiment.
Eject the USB stick.
Shut down the computer and monitor.
Press the C6 power button.
Observe the blue light flashing on the front of the C6 and hear the instrument running – the C6 is beginning its cleaning cycle and you can leave it alone.

ANALYSE DATA FILES

Calculate cell abundance as cells per millilitre

Live phytoplankton

$$\text{Cells per mL} = \text{cell count} \times \frac{1000}{\text{Flow rate} \times \text{Analysis time (minutes)}}$$

Preserved plankton

$$\text{Cells per mL} = \text{cell count} \times \frac{1000}{\text{Flow rate} \times \text{Analysis time (minutes)}} \times \frac{1665}{1500}$$