

STANDARD OPERATING METHOD (SOM)

ANALYSIS AND QUANTIFICATION OF NANO- AND PICOPLANKTON FROM STATION L4 BY FLOW CYTOMETRY

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 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.

Put on gloves.

Place duplicate 5mL flow cytometry sample tubes for each sample depth into the tube rack and put into fume cupboard.

Pipette 40 μ L glutaraldehyde solution slowly into each tube (this will provide approx. 1% glutaraldehyde 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 2 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 all tubes to ensure even mixing of glutaraldehyde into the samples.

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.

Place preserved samples in a refrigerator for 30 minutes.

Flow cytometric analysis of live nano- and picophytoplankton

Turn on the flow cytometer and computer (instrument startup is covered by a separate SOM)

Check the sheath fluid tank is full and the waste tank is empty.

Pressurise the system and turn the sample flow on.

When the computer has finished booting connect the flow cytometer and computer within the software.

Open the 1WCOPPLK protocol, the instrument control windows and open the 1WCOPPLK instrument settings.

Define a data destination folder.

Prepare a tube rack with 12 flow cytometer samples tubes.

When the flow cytometer is ready, turn it to its highest flow rate.

Take a sample bottle, invert it carefully 4 times, remove a 2mL sample with a pipettor and place in a sample tube.

Hand vortex the sample and discard down the sink.

Place another 2 mL sample in the tube and place in the flow cytometer.

Type in an appropriate filename, allow the flow rate to stabilise and then begin acquisition.

The sample will be analysed for 4 minutes at high flow rate.

After approximately 3 minutes prepare the next sample.
After 4 minutes the flow cytometer will make a noise to indicate that the analysis has finished.
Remove the sample and replace it with the new one.
Type in a new filename, allow the flow rate to stabilize and then begin acquisition.
Repeat this process for the remaining samples.

Staining of preserved samples for bacteria and HNAAN analysis

When acquisition on the 5th live sample has begun, put on a pair of gloves, go to the walk-in freezer and collect a 2 µL aliquot of Sybr Green I DNA dye.

The aliquots are kept in 0.5 mL microcentrifuge tubes in 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 200 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 1.8 mL remaining buffer.

Use the pipettor to mix the buffer and Sybr.

Place this mixture in the refrigerator.

When the preserved samples have been fixed for 30 minutes, take them out of the refrigerator and take the lids off them.

Add 200 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 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 1.62 mL of Milli-Q water from the full tube to one of the remaining empty tubes.

When the bottle of beads has been inverted for 1 minute, dispense 8 drops of bead suspension into the remaining empty tube.

Remove 180 µL of bead suspension from the tube and add to the 1.62 mL of Milli-Q water. You now have a 10x dilution of the bead suspension.

Hand vortex the diluted bead suspension.

Insert the tube into the flow cytometer, 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 flow cytometer, 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:

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

Medium and low flow rate calibration

As above except pipette 900 μL of Milli-Q water , dispense 4 drops of bead suspension and remove 100 μL of bead suspension from the tube and add to the 900 μL of Milli-Q water.

Analyse 3 replicates as above on either medium or low flow rate.

Flow cytometric analysis of preserved bacteria, stained with Sybr Green I

Open the 1WCOBAC protocol, and open the 1WCOBAC instrument settings.

Define a data destination folder – the same as for the phytoplankton.

Check that the flow cytometer is set to medium flow rate.

Collect a stained sample from the drawer and hand vortex the sample.

Type in an appropriate filename, allow the flow rate to stabilize and then begin acquisition.

The sample will be analysed for 1 minute at medium flow rate.

If the count rate per second is >1000 either increase the threshold/trigger (green fluorescence) OR change the flow rate to a lower one.

After 1 minute the flow cytometer will make a noise to indicate that the analysis has finished.

Remove the sample, save it for heterotrophic nanoflagellate analysis and replace it with a new one.

Type in a new filename, allow the flow rate to stabilise and then begin acquisition.

Repeat this process for the remaining samples.

Flow cytometric analysis of heterotrophic nanoflagellates, stained with Sybr Green I

Open the 1WCOHFLG protocol, and open the 1WCOHFLG instrument settings.

Define a data destination folder – the same as for the phytoplankton.

Check that the flow cytometer is set to high flow rate.

Collect a stained sample from the drawer and hand vortex the sample.

Type in an appropriate filename, allow the flow rate to stabilise and then begin acquisition.

The sample will be analysed for 7 minutes at high flow rate.

If the count rate per second is >700, either increase the threshold/trigger (green fluorescence) OR change the threshold/trigger to red fluorescence.

After 7 minutes the flow cytometer will make a noise to indicate that the analysis has finished.

Remove the sample and replace it with a new one.

Type in a new filename, allow the flow rate to stabilize and then begin acquisition.

Repeat this process for the remaining samples.

SHUT DOWN FLOW CYTOMETER

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

$$\text{Cells per mL} = \text{cell count} \times \frac{1000}{\text{Flow rate} \times \text{Analysis time (minutes)}} \times \frac{2200}{2000}$$