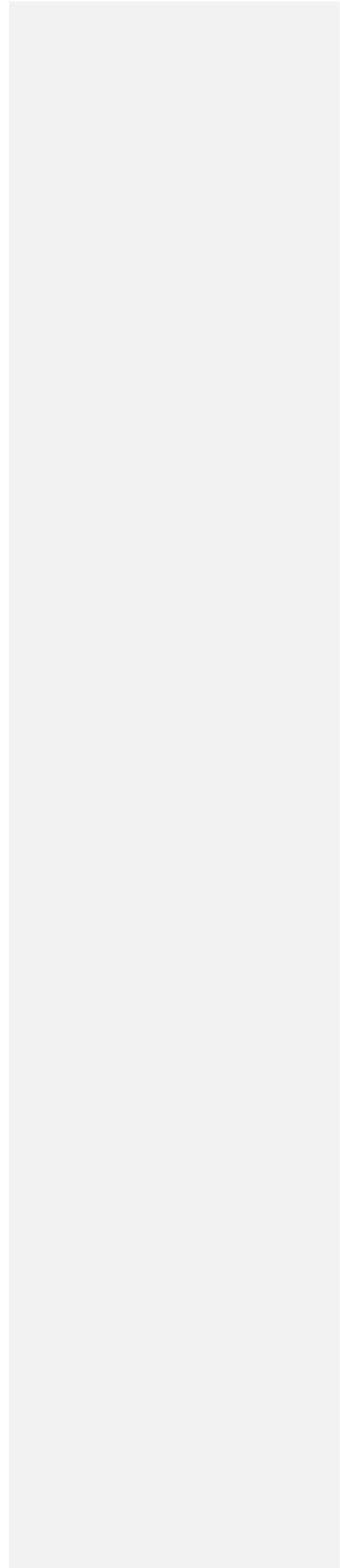
BIOMARKS SAMPLING PROTOCOLS

MICROSCOPY



Flow Cytometry

I- Introduction
Determine small phytoplankton and small cryptophytes cells abundances based on natural fluorescence of the plastids. Used with a fluorescent dye enumeration of bacteria and viruses is also possible.
Analysis performed on whole (unfiltered) sea water

II – Major Peculiarities

III – Material and Instruments
Pipettes 10µl and 1000µl + Pipettes tips
Cryovials 2ml
Liquid Nitrogen

IV – Chemicals and buffers
Paraformaldehyde (PFA) 10X
Glutaralhdehyde 25%

V – Material preparation

VI – Sampling and conditioning
Seawater is fixed with a mix of paraformaldehyde (1% final conc.) and glutaraldehyde (0.25% final con). In 2ml cryovials (3 replicates):
 1.5 ml SW
 150 µl PFA 10%
 15 µl Gluta 25%
Flash freeze in Liquid Nitrogen

VII – Storage and shipping conditions
Store at -80°C
Ship on dry-ice

VIII – Chemicals and buffers preparation before the sampling
PFA 10X preparation:
- Heat 65 ml of ddH_2O to 60°C
- Add 10g of paraformaldehyde
- Add few (1-3) drops of 2M NaOH solution and stir rapidly until the solution has nearly clarified (should take 1-5 min.).
- Remove from heat source and add 33 ml of PBS 3X
- Adjust pH to 7.2 with HCl.
- Filter solution through 0.2 µm
- Quickly cool down to 4°C and store it at 4°C for few weeks (2-3 max) or freeze it to –20°C for longer preservation. Once defreeze it has to be store at 4°C

IX – Disposal of trials and waste
Plastic material used with fixatives has to be treated following a special procedure

X - Bibliography

PFA preparation: Amann RI (1995) *In situ* identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In: Molecular Microbial Ecology Manual. Kluwer Academic, The Netherland, p 1-15

XI - Annexes

Store fixative frozen or at 4°C when thawed

FISH Liquid

I- Introduction
For identification of protists in the micro- and mesoplankton size group which cannot be identified by microscopy or where the genetic characterization not has been done previously.

II – Major Peculiarities

III – Material and Instruments
Bongo nets
Sieve nets 20 µm
Metallic Sieve 20 µm
Filtration unit
3 µm filters
Plastic pipettes
50ml falcons

IV – Chemicals and buffers
Formol (Formaldehyde 37% neutralized with $CaCO_3$)
PBS 1X
Ethanol

V – Material preparation
The material from the plankton net can be concentrate using 20 µm mesh filter prior fixation.
The totality of the material should be sampled to be used also for counting,

VI – Sampling and conditioning

Sub-surface and DCM, 20-1000 µm (2 replicate)
- From 20µm plankton tow, fix 45 ml of SW with 5 ml of formol (3.7% final conc.)
- Let it incubate for 1 hour in the dark.
- Rinse with ca 50 ml of PBS 1X, onto a 20 µm sieve (dedicated to fix samples)
- Store in a mix of PBS/Ethanol (1:1) in a 50 ml falcon tube.

Sub-surface and DCM > 3 < 20 µm (2 replicate)
- Prefilter water on a 20 µm net. If possible concentrate material on filtration units with filter 3µm PC filter. Take the water remaining on the 3µm filter. If concentration is not possible use water filtered on a 20 µm net.
- Fix 45 ml of SW with 5 ml of formol (3.7% final conc.) put into a rack.
- Let it incubate and sediment for 1 hour in the dark.
- take gently the supernatant
- Rinse with ca 50 ml of PBS 1X
- Store in a new 50 ml falcon with a mix of PBS/Ethanol (1:1).

Sub-surface and DCM < 3 µm (2 replicate)
- Collect water passing through the 3µm PC filter of a filtration unit.
- Fix 45 ml of SW with 5 ml of formol (3.7% final conc.) put into a rack.

- Let it incubate and sediment for 1 hour in the dark.
- Take gently the supernatant
- Rinse with ca 50 ml of PBS 1X
- Store in a new 50 ml falcon with a mix of PBS/Ethanol (1:1).

VII – Storage and shipping conditions
Store at -20°C on board and ship at 4°C max
In the lab store at -20°C if samples are going to be analyzed in few weeks, otherwise store at -80°C

VIII – Chemicals and buffers preparation

IX – Disposal of trials and waste

X - Bibliography

XI - Annexes
Ethanol can be removed in storage buffer to see chlorophyll autofluorescence

FISH-FCM & HTM

I- Introduction
For further FISHing of specific groups and followed by Flow cytometry sorting of these groups.
Samples to perform tests of High throughput microscopy development
HTM stands for High Throughput Microscopy

II – Major Peculiarities

III – Material and Instruments
50ml falcons

IV – Chemicals and buffers
Formaldehyde 37% (Formaldehyde 37% neutralized with $CaCO_3$)
10% Pluronic F-68

V – Material preparation

VI – Sampling and conditioning

For FISH-FCM
2 replicates
Collect 35 ml of SW from Niskin bottles
Fix with 4 ml of 37 neutralized formalin (3.7% final conc.)
Add 400 µl of 10% Pluronic F-68
Store tubes at -80°C

For HTM
2 replicates
Collect 35 ml of SW from Niskin bottles
Fix with 4 ml of 37 neutralized formalin (3.7% final conc.)
Add 400 µl of 10% Pluronic F-68
Store tubes at -80°C

VII – Storage and shipping conditions
Store at -20°C on board and ship on dry ice

VIII – Chemicals and buffers preparation

IX – Disposal of trials and waste

X - Bibliography
Biégala et al. 2003. Quantitative Assessment of Picoeukaryotes in the Natural Environment by Using Taxon-Specific Oligonucleotide Probes in Association with Tyramide Signal Amplification-Fluorescence In Situ Hybridization and Flow Cytometry. AEM p. 5519–5529 Vol. 69, No. 9

XI - Annexes

SEM of coccolithophores

I- Introduction
This is a simple method for SEM examination that does not require dehydration. Large coccolithophores may loose their shape at times, but they will be still recognizable by their coccoliths. Sub-surface and DCM, 3-20 µm. 20-1000

II – Major Peculiarities
Samples are prepared from live material on board.
The prefilter (PC 3µm pore size) allows a better distribution of the material on the filter

III – Material and Instruments
If available, a filtration unit for multiple (6-10) filters with diameter 13 mm or 10 or 50 ml plastic syringes (the 50 ml is more difficult to handle) and Swinnex Filter Holder, 13 mm (Millipore SX0001300)
Hand pump (Nalgene, Mityvac)
Polycarbonate filters, 13 mm diameter, 0.8 µm pore size (Millipore Isopore ATTPPO1300)
Polycarbonate filters, 13 mm diameter, 3µm pore size (Millipore Isopore TSTPO1300)
SEM stubs with double-face tape on it
Stub-holder boxes

IV – Chemicals and buffers
Alkaline mineral water (non-sparkling)

V – Material preparation

VI – Sampling and conditioning

Sub-surface and DCM, 20-1000 µm
5 replicates
Collect 500 ml seawater (SW) from the Niskin bottle in a plastic bottle passing it through a 1000 µm-sieve. Process the sample as soon as possible on board

Sub-surface and DCM, 3-20 µm
5 replicates
Collect 350 ml seawater (SW) from the Niskin bottle in a plastic bottle passing it through a 20 µm-mesh net. Process the sample as soon as possible on board

With filtration unit:
1. Place the filters on the filtration unit (3 x 2 filters). Gently homogenize sample and add 50-150 mL of SW to the filtration funnels until the filters start to clog (note the volume). Use a hand pump connected to the filtration unit to get vacuum.
2. Wash the filters with 3 x 10 mL alkaline water

With syringes:
1. Open the Swinnex, place one prefilter (3µm pore size PC filter), the o-ring and then the 0.8 µm filter, and screw up the Swinnex.

2. Gently shake the samples to homogenize it.
3. Wash the syringe with the sample.
4. Fill the syringe with 10 ml of sample
5. Connect the syringe to the Swinnex and filter the sample
6. Detach the Swinnex from the syringe and fill it with another aliquot of sample

7. Repeat these step several times until the filter clogs (50 ml are enough in surface coastal waters, 100-150 ml at DCM)
8. Rinse by filling the syringe with 10 ml of drinkable water
9. Open the lo Swinnex, remove the filter with a tweezer and place it onto the stub

For both procedures, after filtering:
- Put the stub with filter in the box
- Leave the box open to let it dry, or use a dessicator

VII – Storage and shipping conditions

The stubs should be preserved preferably in a dry vacuum container.

VIII – Chemicals and buffers preparation

IX – Disposal of trials and waste

X - Bibliography

Cros L, Fortuno J-M (2002) Atlas of northwestern Mediterranean coccolithophores. Sci Mar 66:7-182

XI - Annexes

Lugol (Neutral)

I- Introduction
Lugol's fixed samples will be used to determine the diversity and abundance of an array of planktonic species using optical microscopy.
Seawater will be harvested at sub-surface, DCM, and benthos and 3 size fractions 0.6-3, 3-20, 20-1000 µm will be separated. Samples will be used for optical microscopy and eventually SEM.

II – Major Peculiarities
As compared to other fixatives, Lugol's solution is less toxic. In addition, it is possible to amplify DNA from Lugol fixed material. The acid solution preserves small flagellates and naked forms in general better than formalin, but it is not suitable for calcareous phytoplankton, since Lugol is acid and coccoliths will be dissolved. In addition, this fixation is not suitable to preserve material for a long time.

III – Material and Instruments
Low quality 250 and 500 ml dark glass bottles
Polycarbonate filters 3 µm and 0.8 µm
Filtration unit
Vacuum pump

IV – Chemicals and buffers
Neutral Lugol
Acid lugol stock solution (see chemicals and buffer preparation section).

V – Material preparation

VI – Sampling and conditioning

For quantitative analysis
2 replicates
At each depth. Collect 500 ml of total sea water (no pre-filtration at all) from niskin bottles and fix with 5 ml of Lugol (1% final conc.). Store at RT in the dark on board and 4°C in the lab in the dark.

For qualitative analysis :
Sub-surface and DCM – 20-1000 µm (3 replicates of 250ml)
Fix 245 ml of material concentrated with net sample (20µm) with 2.5 ml of Lugol. Store at RT in the dark on board and 4°C in the lab in the dark.

Sub-surface and DCM – <20 µm (3 replicates of 250ml)
Prefilter water on a 20 µm net. If possible concentrate material on filtration units with filter 3µm PC filter. Fix 245 ml of concentrated material (water ling on the filter of 3µm) with 2.5 ml of Lugol. Store at RT in the dark on board and 4°C in the lab in the dark.

Sub-surface and DCM - <3 µm (3 replicates of 250ml)
Pre-filter of sea water by gravity through a 3µm PC filter. If possible concentrate material on filtration units with filter 0.8µm PC filter. Fix 245 ml of concentrated material (water ling on the

filter of 0.8µm) with 2.5 ml of Lugol. Store at RT in the dark on board and 4°C in the lab in the dark.

VII – Storage and shipping condition
All fixed samples have to be stored at room temperature in the dark

VIII – Chemicals and buffers preparation

IX – Disposal of trials and waste

X - Bibliography
Stoecker, D. K., Gifford, D. J. and Putt, M. (1994a) Preservation of marine planktonic ciliates: losses and cell shrinkage during fixation. Mar. Ecol. Prog. Ser., 110, 293–299.

XI - Annexes
The colour of the organisms may be too intense and hamper identification. In this case the sample can be treated with Sodium thiosulphate (gives one drop after another to the Lugol-fixed sample until the brownish color disappears)

Lugol (Acid) for planktonic ciliate

Acid Lugol solution is a very versatile fixative for ciliate plankton enabling an array of downstream-applications.

Procedure:
Microplankton samples should be fixed immediately to avoid loss of cells. Samples should also always be rapidly added to the concentrated fixative, diluting them to the final concentration. For our samples we used a final concentration of 2 to 5 % Lugol's iodine (vol:vol). Both the bottles should be plastic if possible, as they need to be later shipped to Germany. If glass is only available at the time of collection, the fixed sample can later be transferred to plastic bottles.

Fixed material can be stored for months at room temperature (in the dark).

Sub-surface and DCM – <20 µm (1 replicate of 1000 ml)
Prefilter water on a 20 µm net. If possible concentrate material on filtration units with filter 3µm PC filter. Fix 950 ml of concentrated material (water ling on the filter of 3µm) with 50ml of Acid Lugol. Store at RT in the dark on board and 4°C in the lab in the dark.

Sub-surface and DCM – <1000 µm (1 replicate of 1000 ml)
Fix 945 ml of material concentrated with net sample (20µm) with 50ml of Acid Lugol. Store at RT in the dark on board and 4°C in the lab in the dark.

Lugol + Glutaraldehyde

I- Introduction
Used for SEM analysis Seawater will be harvested at sub-surface, DCM and 3 size fractions 0.6-3, 3-20, 20-1000 µm will be separated.

II – Major Peculiarities
Glutaraldehyde should be added on board as soon as possible.

III – Material and Instruments
Low quality 250 and 500 ml dark glass bottles
Polycarbonate filters 3 µm and 0.8 µm
Filtration unit
Vacuum pump

IV – Chemicals and buffers
Acidi Lugol solution (SIGMA 62650)
Glutaraldehyde 25%

V – Material preparation

VI – Sampling and conditioning
For quantitative analysis
2 replicates
At each depth. Collect 500 ml of total sea water (no pre-filtration at all) from Niskin bottles and fix with 5 ml of Lugol (1% final conc.) and 5 ml Glutaraldehyde 25%. Store at RT in the dark on board and 4°C in the lab in the dark.

For qualitative analysis :

Sub-surface and DCM – 20-1000 µm (3 replicates of 250ml)
Fix 245 ml of material concentrated with net sample (20µm) with 2.5 ml of Lugol and 2.5 ml of Glutaraldehyde. Store at RT in the dark on board and 4°C in the lab in the dark.

Sub-surface and DCM – <20 µm (3 replicates of 250ml)
Prefilter water on a 20 µm net. If possible concentrate material on filtration units with filter 3µm PC filter. Fix 245 ml of concentrated material (water ling on the filter of 3µm) with 2.5 ml of Lugol and 2.5 ml of Glutaraldehyde. Store at RT in the dark on board and 4°C in the lab in the dark.

Sub-surface and DCM - <3 µm (3 replicates of 250ml)
Pre-filter of sea water by gravity through a 3µm PC filter. If possible concentrate material on filtration units with filter 0.8µm PC filter. Fix 245 ml of concentrated material (water ling on the filter of 0.8µm) with 2.5 ml of Lugol and 2.5 ml of Glutaraldehyde. Store at RT in the dark on board and 4°C in the lab in the dark.

VII – Storage and shipping conditions

All fixed samples have to be stored at room temperature in the dark

VIII – Chemicals and buffers preparation

IX – Disposal of trials and waste

X - Bibliography

XI - Annexes

Formol

I- Introduction
Formalin fixed samples will be used to determine the diversity and abundance of an array of planktonic species using optical microscopy and SEM observations (see analyses parts of the protocol). Seawater will be harvested at sub-surface, DCM, and 3 size fractions <3, >3-<20, >20-<1000 µm will be separated.

II – Major Peculiarities
Formalin is relatively toxic but preserves the material in a more effective way and for a long time.

III – Material and Instruments
Low quality 250 and 500 ml dark glass bottles
Polycarbonate filters 3 µm and 0.8 µm
Filtration unit
Vacuum pump

IV – Chemicals and buffers
Formol (Neutral Formaldehyde 37% neutralized with $CaCO_3$

V – Material preparation
Samples should be fixed immediately. To avoid handling and measuring formalin on board, it is advisable to put the formalin in each bottle while preparing the material on land.

VI – Sampling and conditioning
For quantitative analysis
2 replicates
At each depth. Collect 500 ml of total sea water (no pre-filtration at all) from Niskin bottles and fix with 40 ml (3.7% final conc.). Store at RT in the dark on board and 4°C in the lab in the dark.

For qualitative analysis :

Sub-surface and DCM – 20-1000 µm (3 replicates of 250ml)
Fix 240 ml of material concentrated with net sample (20µm) with 20 ml of Formol.Store at RT in the dark on board and 4°C in the lab in the dark.

Sub-surface and DCM – <20 µm (3 replicates of 250ml)
Prefilter water on a 20 µm net. If possible concentrate material on filtration units with filter 3µm PC filter. Fix 240 ml of concentrated material (water ling on the filter of 3µm) with 20 ml of Formol. Store at RT in the dark on board and 4°C in the lab in the dark.

Sub-surface and DCM - <3 µm (3 replicates of 250ml)
Pre-filter of sea water by gravity through a 3µm PC filter. If possible concentrate material on filtration units with filter 0.8µm PC filter. Fix 240 ml of concentrated material (water ling on the filter of 0.8µm) with 20 ml of Formol. Store at RT in the dark on board and 4°C in the lab in the dark.

VII – Storage and shipping conditions

In the lab samples should be kept at 4°C in the dark.

VIII – Chemicals and buffers preparation

Calcium carbonate in excess (100g per liter of formalin) is normally used to neutralise formalin. To this end, calcium carbonate is added to formalin and the bottle is shaken gently. The excess calcium carbonate is allowed to settle before using the supernatant as fixative.

IX – Disposal of trials and waste

Formalin has to be disposed of among toxic liquids. The bottles can be washed and recycled for other samples.

X - Bibliography

XI - Annexes

Live for TEM and SEM

I- Introduction
Live samples to be processed back in the lab for different purposes.
Require extremely toxic fixative and long procedures not feasible on board.
Collect samples from each size fraction

II – Major Peculiarities
These samples should be collected at the end of the sampling day to minimize the time between collection of the samples and the various treatments to perform in the lab.
Samples to be processed as soon as possible in the lab. The quantity of water to collect depends strictly on the sampling site and on the period of the year.

III – Material and Instruments
6 x 500 ml plastic or glass bottles
Filtration unit
3 µm PC filters
Plastic pipettes

IV – Chemicals and buffers
None

V – Material preparation
VI – Sampling and conditioning

Sub-surface and DCM – 20-1000 µm
(1 replicate of 500ml)
Collect material concentrated with net sample (20µm) in a plastic or glass bottles. Wrap with wet paper and store at 4°C.

Sub-surface and DCM – >3 µm <20 µm (1 replicate of 500ml)
Prefilter water on a 20 µm net. Concentrate material on filtration units with filter 3µm PC filter. Collect concentrated material (water ling on the 3µm filter) in a plastic or glass bottles. Wrap with wet paper and store at 4°C.

Sub-surface and DCM - <3 µm (1 replicate 500ml)
Pre-filter of sea water by gravity through a 3µm PC filter.Collect water passing through the filter in a plastic or glass bottles. Wrap with wet paper and store at 4°C.

VII – Storage and shipping conditions
Protect the samples with wet paper (e.g. kitchen roll kind) for transfer to the lab.
Samples to be processed as soon as possible in the lab

VIII – Chemicals and buffers preparation
None
IX – Disposal of trials and waste
X - Bibliography

XI - Annexes

Samples of each size fraction will be divided for live observation, TEM and SEM analysis.
Concentration of material of fraction >3 µm <20 µm takes long time

FISH euk

I- Introduction

FISH allows a visualization of target cells under epifluorescence microscopy. It is fundamental to identify the cells harboring novel sequences detected in the environment, and to estimate cell abundance. Filters are collected, kept at -80°C, and then cut in small pieces for independent hybridizations (a 25 mm filter can be used for 8 hybridizations).

There are two main protocols, regular FISH where the oligonucleotide probe is labeled with a fluorescent dye, and TSA-FISH (or CARD-FISH), where the probe is labeled with an enzyme that after hybridization catalyzes an extra reaction, increasing significantly the fluorescence signal. For very small protists, CARD-FISH is mandatory, whereas FISH works generally fine for protists larger than 3 µm, and it is easier and more reliable. Sample preparation is the same for both protocols

Samples will be collected at sub-surface, DCM, and 3 size fractions 0.6-3, 3-20 will be prepared.

II – Major Peculiarities

There are a number of probes at different taxonomic levels available.

Repeated freeze/defreezing steps may introduce noise when doing CARD-FISH.

III – Material and Instruments

Vacuum pump

Filtration Manifold (DHI, EQU-FM-10x20-SET). This is a filtration device with 10 towers of 20 mm diameter.

Filtration unit/device for 47 mm for toxic samples

Hood to place the Filtration Manifold and prepare the filters

Petrislides boxes (Millipore PDMA04700 - 47mm)

Cellulose acetate, 25 mm diameter, 0.8 µm pore size (Millipore, AAWP02500)

Filters for prefiltering the sample

- 3 µm PC (Polycarbonate) filter 47 mm (Whatman 111112 - 3µm, 47mm)
- 1000 µm mesh size “filter”

Filters for FISH

- 0.6 µm PC filter 25 mm
- 3 µm PC filter 25 mm
- 20 µm PC filter 47 mm[F1]

IV – Chemicals and buffers

Formaldehyde 37% (Sigma F 1635)

Neutralized the formaldehyde by adding ca 50 ml (solid) of calcium carbonate in 1L of formol. Filter the neutralized formaldehyde before use.

V – Material preparation

VI – Sampling and conditioning

<u>Sub-surface and DCM - 0.6-3 µm</u>

- Pre-filter 900 ml of sea water through 3 µm PC filter 47 mm
- Collect the filtrate and fix it with neutralized formol 3.7% (final conc. i.e. add 100 ml of formol 37%). Keep 1 hour at 4°C in the dark (not more than 24 hrs).

- Place a 0.8 µm cellulose acetate filter in the tower filtration system and add some water drops in the prefilter.
- Place the 0.6 µm polycarbonate filter above the 0.8 µm filter (shinny side of filter upwards)
- Add 100 ml of the sample. Filter and rinse with ca. 10 ml filtered (by 0.2 µm) MilliQ water[F2]
- Dry filter, write a small number on the edge with pencil. Put in PetriSlide, blue paper between filters and write explanation of numbers (data, sample, volume filtered) on top of the PetriSlide. **10 replicates**

Sub-surface and DCM – 3-20 µm

- Pre-filter 2.250 L of sea water through 20 µm PC filter 47 mm.
- Collect the filtrate and fix it with neutralized formol 3.7% (final conc. i.e. add 250 ml of formol 37%). Keep 1 hour at 4°C in the dark (not more than 24 hrs)
- Place a 0.8 µm cellulose acetate filter in the tower filtration system and add some water drops in the prefilter.
- Place the 3 µm polycarbonate filter above the 0.8 µm filter (shinny side of filter upwards)
- Add 500 ml of the sample. Filter and rinse with ca. 10 ml filtered (by 0.2 µm) MilliQ water
- Dry filter, put small number on edge with pencil. Put in PetriSlide, blue paper between filters, write explanation of numbers (data, sample, volume filtered) on top of the PetriSlide.

8 replicates (4 X 200 ml + 4 X 100 ml)

Sub-surface and DCM – 20-1000 µm

Collect 90 ml of water from net tow samples. Sieve onto 1000µm. Fix it with neutralized formol 3.7% (final conc. i.e. add 10 ml of formol 37%). Keep 1 hour at 4°C. Filter on a 20 [F3]µm PC filter 47 mm. **4 replicates (1 X 50ml; 1 X 25 ml; 2 x 10 ml)**

VII – Storage and shipping conditions

Store petrislides with filters at -80 C
Ship on dry ice.

VIII – Chemicals and buffers preparation

See point V

IX – Disposal of trials and waste

Formaldehyde is TOXIC
After filtering the sample, the filtrate waste should be collected in a special waste contained, to be collected by a specialized company
Tips and tubes in contact with fixative should be thrown in a contained for contaminated plastic.

X - Bibliography

Amann, R. and B.M. Fuchs. 2008. Single-cell identification in microbial communities by improved fluorescence in situ hybridization techniques. Nature Rev. Microb. 6: 339-348

Massana, R., Guillou, L., Díez, B., and Pedrós-Alió, C. (2002) Unveiling the organisms behind novel eukaryotic ribosomal DNA sequences from the ocean. *Appl Environ Microbiol* **68:** 4554-4558.

Massana, R., R. Terrado, I. Forn, C. Lovejoy, and C. Pedrós-Alió. 2006. Distribution and abundance of uncultured heterotrophic flagellates in the world oceans. Environ. Microbiol. 8: 1515 – 1522

Not, F., N. Simon, I.C. Biegala and D. Vaulot. 2002. Application of fluorescent in situ hybridization coupled with tyramide signal amplification (FISH-TSA) to assess eukaryotic picoplankton composition. Aquat. Microb. Ecol. 28: 157-166

Pernthaler, A., Pernthaler, J., and Amann, R. (2002) Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. *Appl Environ Microbiol* **68:** 3094-3101.

XI - Annexes

DAPI

I- Introduction

The smallest microorganisms (from 0.2 to 5 µm) are not visible by phase contrast microscopy and need epifluorescence to be optimally visualized. Microbes are attached to a flat membrane (polycarbonate filter), stained with a fluorescent dye, excited by a short band light, and the fluorescence emitted is observed under the microscope. This allows counting and a rough morphological characterization (size, shape, presence of chloroplasts and flagella) of the smallest microbes: viruses, bacteria (including archaea), picoeukaryotes (phototrophic and heterotrophic cells, normally flagellated) and the smallest nanoplankton (up to 5-10 µm).
Two samples: Sub-surface and DCM[F4]. Each sample will have 2 size fractions (>0.6 µm and >2 µm). Three replicates for each filter. TOTAL: 12 filters

II – Major Peculiarities

Preparing the samples is easy. Observing and counting is time consuming and requires patience and some training, since many different cells should be counted simultaneously. Also, "fading" is a problem: by observing a field, the fluorescence signal is burnt and lost. So counts in a given filed should be reasonably fast.

III – Material and Instruments

• Filtration Manifold (DHI, EQU-FM-10x20-SET). This is a filtration device with 10 towers of 20 mm diameter.
• Vacuum pump
• Filters
    Cellulose acetate, 25 mm diameter, 0.8 µm pore size (Millipore, AAWP02500)
    Polycarbonate, 25 mm diameter, 0.2 µm pore size, black (DHI, STO-FIL-0.2-25-BL)
    Polycarbonate, 25 mm diameter, 0.6 µm pore size, black (DHI, STO-FIL-0.6-25-BL)
    Polycarbonate, 25 mm diameter, 2.0 µm pore size, black (DHI, STO-FIL-2.0-25-BL)
• Hood to place the Filtration Manifold and prepare the filters
• Gloves, Automate pipettes (5 ml and 50 µl) and tips (Ramon)

IV – Chemicals and buffers

• Glutaraldehyde 25% (Merck, 8.20603.1000)
• DAPI (4',6-diamidino-2-phenylindole) (SIGMA, D 9542)
• Low-Fluorescence immersion Oil (Olympus, IMMOIL-F30cc)

V – Material preparation

Prepare working solutions of glutaraldehyde and DAPI

Glutaraldehyde 10%

    Add 40 ml of Glutaraldehyde 25% to 60 ml of MQ water.
    Filter by 0.2 µm and keep at 4ºC.

DAPI 0.5 mg/ml

    Add 1 ml of MQ water to the 10 mg of DAPI (make it into the DAPI commercial bottle).
    Shake it and transfer to a new tube, rinse the bottle with 1 ml of MQ water until 20 ml in total are added (1 by 1 ml).
    Filter by 0.2 µm, aliquote in 0.5 ml tubes and keep at –20ºC in the dark. Avoid freezing and defreezing many times.

VI – Sampling and conditioning

1. Fix the sample

a. Fix 288 ml of sample with 32 ml of 10% glutaraldehyde (1% final concentration).
b. Incubate samples for one hour (not more than 24h) at 4ºC in the dark.

2. Filter the sample for picoeukaryotes

a. Place a 0.8 µm cellulose acetate filter in the tower filtration system and add some water drops in the prefilter.
b. Place the 0.6 µm polycarbonate black filter above the 0.8 µm filter.
c. Add 20 ml of the sample. Filter about 15 ml and stop filtration. Add 50 µl of DAPI (0.5 mg/ml) to the remaining 5 ml. Wait for 5 minutes (in the dark) and filter until the filter is dry.
d. Put the filter in a slide, add one drop of immersion oil and cover it with a cover-slip

3. Filter the sample for nanoeukaryotes

The protocol is the same that the one described for picoeukaryotes, but changing the volume of the sample and the filter pore size.

Use a 2 µm polycarbonate black filter (25 mm).
Add 80ml of fixed sample. Filter about 75 ml stop filtration and process as before.

4. Filter the sample for bacteria

The protocol is the same that the one described for picoeukaryotes, but changing the volume of the sample and the filter pore size.

Use a 0.2 µm polycarbonate black filter (25 mm).
Add 5ml of fixed sample.

FILTERS PREPARED: Put the Bacteria filter (5 ml in 0.2 µm) and the Picoeuk filter (20 ml in 0.6 µm) in the same slide. Put the Nanoeuk filter (80 ml in 2 µm) in a separate slide. Prepare 3 replicates per size fraction
Total: 2 samples (Subsurface and DCM) x 2 slides (3 filters) x 3 replicates. **Prepare 12 slides (18 filters)**. Label them and keep at -20°C until processed.

VII – Storage and shipping conditions

Filters can be stored at -20°C for several weeks. Ideally, they should be counted soon (the first week) to better preserve the autofluorescence.
Shipping can be done in dry ice

VIII – Chemicals and buffers preparation

See point V

IX – Disposal of trials and waste

Glutaraldehyde is TOXIC and irritant. Should be handled with caution because related aldehydes are carcinogens, mutagens and teratogens. DAPI is irritant
After filtering the sample, the filtrate waste should be collected in a special waste contained, to be collected by a specialized company

Tips and tubes in contact with fixative or DAPI should be thrown in a contained for contaminated plastic.

X - Bibliography

Porter, K. G., and Y. S. Feig. 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. 25:943-948.

Sherr, E.B. and B.F. Sherr. 1993. Preservation and storage of samples for enumeration of heterotrophic protists. In: P.F. Kemp, B.F. Sherr, E.B. Sherr and J.J. Cole (eds.), Handbook of Methods in Aquatic Microbial Ecology, Lewis Publishers, Boca Raton. pp: 207-212.

XI - Annexes

FISH prok

I- Introduction
FISH allows a visualization of target cells under epifluorescence microscopy. It is fundamental to identify the cells harboring novel sequences detected in the environment, and to estimate cell abundance. Filters are collected, kept at -80°C, and then cut in small pieces for independent hybridizations (a 25 mm filter can be used for 8 hybridizations).
Samples will be collected at sub-surface, DCM, and 1 size fractions will be prepared. Use water pre-filtered through 20µm sieve

II – Major Peculiarities
There are a number of probes at different taxonomic levels available.
Repeated freeze/defreezing steps may introduce noise when doing CARD-FISH.

III – Material and Instruments
• Filtration Manifold (DHI, EQU-FM-10x20-SET). This is a filtration device with 10 towers of 20 mm diameter.
• Vacuum pump
• Epifluorescence microscope with a UV-transparent 100x objective and filters for excitations at UV radiation, Blue light and Green light
• Filters
    Cellulose acetate, 25 mm diameter, 0.8 µm pore size (Millipore, AAWP02500)
    0.2µm PC filter 25 mm
• Hood to place the Filtration Manifold and prepare the filters
• Gloves, Automate pipettes (5 ml and 50 µl) and tips (Ramon)
* Petrislides boxes (Millipore PDMA04700 - 47mm)

IV – Chemicals and buffers
Formaldehyde 37% (Sigma F 1635)

V – Material preparation

VI – Sampling and conditioning
At sub-surface and DCM
45 ml of unfiltered SW are fixed with 5 ml of formol 37%. Keep 1 hour at 4°C in the dark (not more than 24 hrs)
- Place a 0.8 µm cellulose acetate filter in the tower filtration system and add some water drops in the prefilter.
- Place the 0.2 µm polycarbonate filter above the 0.8 µm filter (shinny side of filter upwards)
Then 10 ml of fixed water filtered through a 0.2 µm PC filter and rinse with ca. 5 ml filtered (by 0.2 µm) MilliQ water.
Dry filter, write a small number on the edge with pencil. Put in PetriSlide, blue paper between filters and write explanation of numbers (data, sample, volume filtered) on top of the PetriSlide.
5 replicates

VII – Storage and shipping conditions
Store petrislides with filters at -80 C

Ship on dry ice.

VIII – Chemicals and buffers preparation

See point V

IX – Disposal of trials and waste

Formaldehyde is TOXIC

After filtering the sample, the filtrate waste should be collected in a special waste contained, to be collected by a specialized company

Tips and tubes in contact with fixative should be thrown in a contained for contaminated plastic.

X - Bibliography

Amann, R. and B.M. Fuchs. 2008. Single-cell identification in microbial communities by improved fluorescence in situ hybridization techniques. Nature Rev. Microb. 6: 339-348

Pernthaler, A., Pernthaler, J., and Amann, R. (2002) Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. *Appl Environ Microbiol* **68:** 3094-3101.

XI - Annexes

Culture

I- Introduction

The aim is to establish cultures of protists that can be examined in more detail genetically and morphologically. Unialgal strains can be established by serial dilution series (fine for small, common taxa) or by single cell capillary isolation (better for large, rare taxa). Here is described the serial dilution method for isolation of phytoplankton taxa, but see also Andersen (2005) for other methods.

II – Major Peculiarities

Protists have different requirements and different media and growth conditions should be tried in parallel. It is well known, and we must accept it, that many protists are still resisting cultivations with all different attempted conditions.

III – Material and Instruments

Cell culturing flasks 50 mL -1 L (non-toxic material either of borosilicate glass or non-toxic plastic, such as Nalgene cell culturing flasks).

- Multiwell cell culturing plates (24 wells, Nunclon), or 15 mL autoclaved culturing borosilicate tubes with caps
- Automate pipettes, 5 mL and 1 mL (or 200 µL) with tips
- Culture room or climate chamber with white illumination (for autotrophs) (Bente)

IV – Chemicals and buffers

Sterile algal medium of suitable salinity similar to the natural conditions. E.g. IMR ½ (Eppley et al 1967), L-medium, K-medium for oceanic phytoplankton or ES for some dinoflagellates.

V – Material preparation

Distribute 1.8 mL temperature adjusted medium to each well in a multiwell plate (or 9 mL in 24 or more borosilicate tubes, which can be standing for a longer period and are easier to transport).

VI – Sampling and conditioning

Collect 200-500 ML SW from subsurface and DCM in a non-toxic bottle (e.g. polyethylene or borosilicate glass) onboard and protect the bottle from rapid changes in temperature and strong light. Wrapping the samples with humid paper (e.g. kitchen roll kind) keeps the temperature close to the original one.

Serial dilution cultures (SDC)

In the lab, add 200 µL/1 mL fresh SW sample to the first well/tube in a row (e.g. 4 rows per sample). Mix and transfer 200 µL/ 1 mL of this sample to the next well/tube in the row for 6 times to dilute the sample $10\text{-}10^6$ times.
Incubate the plate/tubes (plates put in a transparent bag to reduce evaporation) in a culture room for 2-4 weeks and then examine the results under the microscope.

VII – Storage and shipping conditions

If shipping, fill the bottle completely with culture/medium to avoid destroying air bubbles in the flask. Send by courier and cooled.

VIII – Chemicals and buffers preparation

IX – Disposal of trials and waste

Among other thing, please mention if it has to be recycled, treated.

X - Bibliography

Andersen, R. (red.) (2005). Algal culturing techniques. Elsevier Academic Press, Amsterdam, 578 p.
Eppley, R.W., R.W. Holmes & J.D.H. Strickland (1967). J. exp. mar. Biol. Ecol. 1:191-208.
Sournia, A. (red.) (1978) Phytoplankton Manual. UNESCO.

XI – Annexes

Girus culture

I- Introduction
Girus small filtration is for Virus culture in lab. All of Girus samples are filters.

II – Major Peculiarities
Two storage conditions. Do not freeze any filter!

III – Material and Instruments
GF/A 150 mm/1.6µm Filters
Millipore 47mm diameter Polycarbonate 0.22µm pore filters
Petrislides
5ml cryovial
Parafilm
200µm sieve or mesh
20µm sieve or mesh
Filtration holder
Peristaltic pump (I/P 2 channels)
Tubing
Tweezer

IV – Chemicals and buffer
V – Material preparation

VI – Sampling and conditioning
8 x 1L volumes of water for isolation of viable viruses on 47mm membranes Polycarbonate.
For each station, we require 8 Polycarbonate 47mm membranes. 1L of water should be passed through each membrane. When the membrane becomes clogged, the filtration is finished for the membrane. Polycarbonate filter has low porosity, the filtration will be slow.
Dry samples: 2 replicates for sub-surface and 2 replicates for deep chlorophyll max.
Wet samples: 2 replicates for sub-surface and 2 replicates for deep chlorophyll max.

1. Pre-filter each 1L of seawater through 20µm
2. Set the filters on the filter holder on a available filtration ramp carefully avoiding air bubbles.
3. Full the funnel with the sample
4. Start the pump and stop it as soon as 1 liter has passed through the filter membrane.
5. Remove the membrane using tweezers
6. **Dry samples:** put the filter membrane face up in a sterile petrislide and encircle the joint with parafilm.
7. **Wet samples:** roll up the filter membrane with the tweezers and put it in 5ml cryovial (70mm). Fill with filtered seawater (filtrate of 0.22µm). Screw down the cap firmly and encircle the joint with parafilm.

VII – Storage and shipping conditions
Dry and Wet samples at 4°C. Filters to be stocked wet should never be allowed to dry out.
Delivery address: Laboratoire Information Génomique et Structurale
Parc Scientifique de Luminy
163 Avenue de Luminy

13009 Marseille - France

VIII – Chemicals and buffers preparation
IX – Disposal of trials and waste

X - Bibliography

XI – Annexes

Live Imaging

I- Introduction
Obtain taxonomic information based on cells motility/behavior and on cells that do not preserve well in usual fixatives.

Sub-surface, 3-20 µm DCM, 3-20 µm Sub-surface, 0.6-3 µm DCM, 0.6-3 µm
Sub-surface, 20 -1000 µm DCM, 20-1000 µm

II – Major Peculiarities
Time consuming. Need to be performed as soon as possible after sampling.
It requires a lot of microscopic and taxonomical expertise, so not all scientists are capable to do so.

III – Material and Instruments
Inverted microscope equipped with camera
Stereomicroscopes equipped with camera
Compound microscope with camera
Petridishes
Glass slides and cover slips
Pasteur pipettes

IV – Chemicals and buffers

V – Material preparation

VI – Sampling and conditioning
From each size fraction considered. Collect a small amount of sample, dilute if too concentrated, place it in a Petridish or on a slide. Set the microscope and record 10 random fields for 2 min each.

VII – Storage and shipping conditions
Hard drive or USB drive

VIII – Chemicals and buffers preparation
None

IX – Disposal of trials and waste
Remove glass material in an appropriate manner (see lab local rules)

X - Bibliography

XI - Annexes

Work To Be Performed Back In The Lab On Live Material

SEM on live material

1- ***Osmium***
Add 2% osmium teroxide in 0.1 M cacodylat buffer (pH ca 7.8) for ca 15-30 min (longer if you wish).
Rinse 3 times in 0.1 M cacodylat buffer.
Dehydrate in ethanol series; once for 5 min in 70, 90 and 96 % then 4 times in 100%.
Critical point dry the sample or conclude with 10 min in a 50:50 mixture of 100% ethanol and hexamethyldisilizan and two subsequent rinses in only hexamethyldisilizan

2-
Glutaraldehyde and osmium
Fix in 1-4 % glutaraldehyde final concentration. Concentrate on filter or centrifuge to form pellet.
Rinse 2 times in growth medium and 2 times in 0.1M cacodylat buffer (ca 15-30 min)
Fix in 1-2% osmium tetroxide in 0.1 cacodylat buffer.
Rinse 3 times in 0.1 cacodylat buffer.
Dehydrate in ethanol series; once for 5 min in 70, 90 and 96 % then 4 times in 100%.
Critical point dry the sample or conclude with 10 min in a 50:50 mixture of 100% ethanol and hexamethyldisilizan and two subsequent rinses in only hexamethyldisilizan

3-
Osmium
Add 0.01-1 ml of 1-2 % osmium teroxide (in 0.1 M cacodylat buffer or distilled water to a concentrated sample (1ml-100 ml, concentrated either by centrifugation or filtration) for 15- 30 min (or longer).
Rinse 3 times in 0.1 cacodylat buffer.
Dehydrate in ethanol series; once for 5 min in 70, 90 and 96 % then 4 times in 100%.
Critical point dry the sample or conclude with 10 min in a 50:50 mixture of 100% ethanol and hexamethyldisilizan and two subsequent rinses in only hexamethyldisilizan

TEM on live material

Thin sections
Fix in 1-4 % glutaraldehyde final concentration (ca 60 min). Centrifuge to form a pellet.
Rinse 2 times in growth medium and 2 times in 0.1M cacodylat buffer (ca 30 min)
Fix in 1-2% osmium tetroxide and 1-2 % ferricyanide in 0.1 cacodylat buffer (ca 60 min).
Rinse 3 times in 0.1 cacodylat buffer.
Stain in 1 % aqueous uranyle acetate over night (or some hours)
Dehydrate in ethanol series; once for 10 min in 30, 40, 50, 70, 90 and 96 % then 4 times in 100%.
Rinse twice for 5 min in propylenoxide. Leave over night (or ca 6 hours) in 50:50 mixture of propylene oxide and Epon’s embedding resin. Change twice to fresh Epon’s and polymerize at 50˚C for 12 hours.

Whole-mounts
1. ***Osmium vapor***
Concentrate sample live by filtration or centrifugation (if the sample is very dense this step is not required). When volume is small enough (ca one drop) place on grid and fix in the vapour of 1-3 % osmium tetroxide.

2. ***Glutaraldehyde and (acidic) Lugol's solution***
Add 1 ml of (acidic) Lugol's solution and 1 ml 25 % GA (or 0.5 ml 50 %, EM grade) to the sample. Leave sample to settle, remove supernatant and transfer settled material to grid. Rinse in distilled water. Shadow-cast at 30° using Au/Pd, Cr or platinum.

***** All the following procedures can be done when processing samples *****

SEM on Lugol fixed samples
SEM on Formol fixed samples
SEM and TEM on Lugol + Gluta fixed samples
Concentrate on filter or leave sample to settle and mount on glass (use poly-lysin).
Rinse 3 times in growth medium (15-30 min altogether).

Analysis of lugol fixed samples for ciliates
- simple counts of Lugol-fixed cell (settling chambers)
- filtration of fixed cells followed by DAPI staining for cell counts
- quantitative protargol staining (identification AND counts). Therefore, Lugol-fixed materials needs to be postfixed in the lab with Bouin's fixative prior to silver staining.
- SEM. Osmium postfix of Lugol-fixed material recommend prior to dehydration in lab (but not essential).
- Single cell analyses: individual cells can be photographed, identified and then subjected to destaining with Na-thiosulfate. Afterwards, genes can be amplified from same individual cell using PCR.