BIOMARKS SAMPLING PROTOCOLS

SPITZBERGEN

Seawater sampling

I- Introduction
Seawater collection for total analysis

II – Material and Instruments
For each mesocosm
on the boat:
1x 15L for carboy for SW
Back on the pontoon:
Sieve 20µ
Big funnel
1*5L carboy for DNA [<20µ]
2*5L or 1*10L carboy for RNA [<20µ]
1x2.5L carboy for [>20µ] filled with 0.2µm filtered SW *[prepare before sampling 2.5L filtered SW at 0.2µ for each mesocosm → for 10 mesocosms prepare one carboy of 25L before sampling !]*

III – Sampling and conditioning
For each mesocosm, proceed on the same way:
- Sample 15L of SW for each mesocosm (write down on the carboy the number of the sampled mesocosm)

If you are also doing the morphological protocol, plan to deduct 1502 mL for fixation (500mL for Lugol&Gluta, 2 mL for GB, 1L for Syraco) from the total SW fraction.
- Back on the pontoon, prefill on a 20µ sieve (use the big funnel) into the 15L carboys.
- Dispatch the 15 L into 3x5L carboys (2 for RNA, 1 for DNA) or 1x10L (RNA → 1 carboy but connected on two filtration units) and 1x5L (for DNA) :

If you are also doing the morphological protocol, plan to deduct 357 mL for fixation (200mL for Lugol&Gluta, 2 mL for GB, 355mL for FISH) from the <20 µm fraction.
- Rinse the funnel
- Collect the >20µ fraction concentrate on the sieve and dilute it with 2.5 L filtered SW in a carboy (XX L)

If you are also doing the morphological protocol, plan to deduct 357 mL for fixation (200mL for Lugol&Gluta, 2 mL for GB, 355mL for FISH) from the >20µm fraction .
Rinse the 15L carboys with tap water if you are using the same carboys on the next sampled mesocosms.

RNA and DNA

I- Introduction

Filtration on different size-fractions to analyze the protists biodiversity.

II – Material and Instruments

Peristaltic pump (L/S 6 channels)
6*3 in line Filtration units 47 mm
Tubing and connectors
Decontaminated tweezers
PC filters 47mm 12µ
PC filters 47mm 3µ
PC filters 47mm 0.2µ
TFF filters
1*5L carboy for DNA
2*5L carboy for RNA or 1*10L carboy
1*2,5L carboy for (2RNA+1DNA) for the >20µm fraction
Cryovials 5mL and 2mL and racks
EtOH 70% and distilled water squizzes.

III – Chemicals and buffers

RNA Later or Liquid Nitrogen

IV – Material preparation

Set up the pump, tubing and filter. Annotate the tubes.

V – Sampling and conditioning

Fractions <20µm:

Set up and start the RNA filtration (2 lines of filtration), then the DNA filtration (1 line of filtration). Run filtration in parallel.

- Put the PC filters 47mm 3µm, 0.2µm and TFF filters in line setup of the filter holders;
- Collect SW with sieve it onto 20µm before to fill up :
  1x5L for DNA } for 1 mesocosm
  2x5L or 1x10L for RNA }
- Filter the <20µm SW onto a 3 µm polycarbonate filter 47mm followed by a 0.2 µm polycarbonate filter 47mm, followed by a TFF filter. Use the 6 channels of the pump separately, with 3 independent set up. (you can filter SW from 1 carboy with 2 channels)

If you are also doing the morphological protocol, plan to deduct 357 mL from the <3 µm fraction for fixation (200mL for Lugol&Gluta, 2 mL for GB, 355mL for FISH).

- Once filtration is done, collect the filter with clean tweezers, transfer the two filters in separate 2ml cryovials and flash freeze the tubes in liquid nitrogen;
- If there is no liquid nitrogen, cover the filter with RNA Later (ca 1 ml) and store the tubes in -20°C ;
- If there is lots of pression with the filtration, change the 3µm or 0.2µm filters and pool them in one single cryovial.
- Label the tubes accordingly to the size fractions: Mesocosm_number_XX_RNA/DNA_ [size fraction]_date_ Volume_filtered.

Fraction >20µm (→use the 2.5L re-concentrated):

- Put the PC filters 47mm 12µm filters in line setup of the filter holders (you can run 6 mesocosms in parallel with the peristaltic pump)
- Filter the >20µm SW onto a 12 µm polycarbonate filter 47mm
- Once filtration is done, collect the filter with clean tweezers, transfer the two filters in separate 2ml cryovials and flash freeze the tubes in liquid nitrogen;
- If there is no liquid nitrogen, cover the filter with RNA Later (ca 1 ml) and store the tubes in -20°C ;
- If there is lots of pression with the filtration, you can change the 3µm or 0.2µm filters and pool them in one single cryovial.
- Label the tubes accordingly to the size fractions: Mesocosm_number_XX_RNA or DNA_ [size fraction]_date_Volume_filtered.

VI – Storage and shipping conditions

After having flash freezed the tubes in liquid nitrogen and store the tube whatever possible at -80°C or at -20°C. Ship on dry ice.

Lugol + Glutaraldehyde

I- Introduction
Used for SEM analysis Seawater will be harvested at 3 size fractions [<3], [<20], [>20].

II – Major Peculiarities
Glutaraldehyde should be added <u>as soon as possible.</u>

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

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

V – Material preparation
Annotate the bottles. Fill with Lugol and Gluta before the return of the boat and the water.

VI – Sampling and conditioning
For quantitative analysis

2 replicates with SW

Collect 250 ml of total sea water (no pre-filtration at all) from Niskin bottles and fix with 2.5 ml of Lugol (1% final conc.) and 2.5 ml Glutaraldehyde 25%. Store at RT in the dark on board and 4°C in the lab in the dark.

For qualitative analysis :

<u>>20µm</u> (2 replicates of 250ml) (→ retrieve from the 2.5L re-concentrated)

Fix 245 ml of material concentrated with sieve 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.

<u>[<20]</u> (2 replicates of 100ml)

Prefilter water on a 20 µm sieve. If possible concentrate material on filtration units with filter 3µm PC filter. Fix 100 ml of concentrated material (water ling on the filter of 3µm) with 1 ml of Lugol and 1 ml of Glutaraldehyde. Store at RT in the dark on board and 4°C in the lab in the dark.

<u>[<3]</u> (2 replicates of 100ml)

Prefilter water on a 3 µm PC filter. On the line filtration 47mm, fix 100 ml of material with 1 ml of Lugol and 1 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

Sample cryopreservation using Glycine Betaine

I- Introduction

The method was found to work well on diverse marine and freshwater samples; both the numbers and the optical properties of prokaryote cells were well preserved and the downstream single cell FACS-MIDA-16SPCR success rate was slightly better than for fresh samples.

II - Material and Instruments

- P100 and P1000 and tips
- cryovials 2mL

III- Chemicals and buffers

- Betaine stock 48%
- Liquid Nitrogen

IV – Chemical preparation

Betaine stock 48% :

Betaine anhydrous Fisher AC20424-1000

- Dissolve 48g betaine in 40 mL of deionized water, bring volume up to 100 mL
- Pass through 0.2µm filter
- Store at 4°C, re-filter every month

V – Sampling and conditioning

→ **2 replicates** for 3 size fraction : total SW // <20 // <3

- Transfer 143 µL betaine stock and 1 mL unfiltered sample to a sterile cryovial. Mix.
- Store in liquid Nitrogen or at -80°C.

VI – Storage and shipping conditions

Store at -80°C. Ship on dry ice.

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. 2 size fractions 0.2-3, 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 2*3 towers of 25 mm diameter.

Hood to place the Filtration Manifold and prepare the filters

Petrislides boxes (Millipore PDMA04700 - 47mm)

Filters for prefiltering the sample

- 20 µm sieve
- 3 µm PC (Polycarbonate) filter 47 mm (Whatman 111112 - 3µm, 47mm)

Filters for FISH

- 3 µm PC filter 25 mm
- 0.2 µm PC filter 25 mm
- 0.8 µm cellulose acetate filter 25 mm (Nitrocellulose filters, 25 mm diameter, 0.8 µm pore size (Millipore, AAWP02500))

Bottles for FISH (plan to use 20 bottles of 500mL, 2bottles / mesocosm)

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

[0.2-3 µm] (1 bottle → **4 filters / pro mesocosm)**

- Pre-filter 355 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 45 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)

– Add small volume (ie 20 ml of the sample). Filter and rinse with ca. 10 ml filtered (by 0.2 µm) MilliQ water

(For this filtration and for the next ones it is better to start with one replicate in order to check if the volume is correct or if one need to reduce it (check out the filtration time and/or the color of the filter to figure this out)

– 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.

<u>[3-20 µm] **(1 bottle → 4 filters / pro mesocosm)**</u>

- Pre-filter 355 mL of sea water through 20 µm sieve.
- Collect the filtrate and fix it with neutralized formol 3.7% (final conc. i.e. add 45 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 100 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.

VII – Storage and shipping conditions

Store petrislides with filters at -20 C. Ship on dry ice.

VIII – 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

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Syraco

I – Material and Instruments

Vacuum pump + Ramp filtration
Filtration unit/device for 47 mm
Petrislides boxes (Millipore PDMA04700 - 47mm)
0.8 µm nitrocellulose filter 47 mm

II – Sampling and conditioning

[Total SW] : **(2 replicates per mesocosm)**
Filter **500ml** onto a **47mm** 0.8um NITROCELLULOSE membrane.
Place filters on Petrislides and allow to air dry. Store at 4°C.

III – Storage and shipping conditions
Store and ship at 4°C.

Ethanol

I – Material and Instruments
Sieve 20µm
Squeeze filled with 95% Ethanol
Falcon 50mL

II – Sampling and conditioning

[>20µ] (2 replicates per mesocosm)
Pass 200ml of non-filtered SW through the 20µm sieve.
Rinsed with 95% Ethanol molecular grade (squeeze bottle).
Recover the plankton from the sieve into a 50ml falcon tube, using the Ethanol from the squeeze bottle. Fill up to 40 ml. Store at -20°C and repeat the whole process 12 hours later with Fresh ethanol. Store at -20°C.

III – Storage and shipping conditions

Store at -20°C. Ship on dry ice.

Cultures

I – Material and Instruments

II – Sampling and conditioning

(????waiting for Ian's Probert instruction, maybe in the second part of the sampling with Sarah ...)