MaCuMBA

Marine Microorganisms: Cultivation Methods for Improving their Biotechnological Applications

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Protocols for samples preservation prior to microalgae cultivation

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Dissemination Level	
PU Public	**X**
PP Restricted to other programme participants (including the Commission Services)	
RE Restricted to a group specified by the consortium (including the Commission Services)	
CO Confidential, only for members of the consortium (including the Commission Services)	

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Issue	Date	Implemented by
v.1		

Indicate any document related to this deliverable (report, website, ppt etc) and give file name

** Please attach deliverable documents and any additional material if needed.*

Contents

Summary

It is not always possible to isolate microalgae immediately after sample collection, for example on long cruises where no proper conditions are available (lack of culture chamber, sterile hoods) or on trips to remote location. Therefore it is desirable to be able to preserve samples in such a way that microalgae can be isolated once the samples are brought back to the laboratory. One possible strategy is to use cryopreservation that is currently used to preserve live cultures of microalgae.

We are presenting protocols that have been successfully used on marine samples. They are relying on the use of DMSO as cryoprotectant and a range of freezing techniques that depend on what is available in the field.

Partner(s) involved in Deliverable production: CNRS (16).

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1. Sample pre-processing

Prior to preservation samples can be pre-processed to increase chances of getting target organisms. Two possibilities have been tested

- Pre-filtration through 2 or 3 µm. This allows to remove large cells such as diatoms that may become dominant in the unfrozen samples (see Appendix 1)
- Tangential flow filtration. This will concentrate the phytoplankton community about 100-fold and will therefore result in more cells being preserved and therefore higher recovery rates (see Appendix 2).

2. Sample freezing

The samples are preserved in 2 mL Cryotube with DMSO added (5% final concentration).

Then they are frozen according to one of three different protocols

- Freezing into liquid nitrogen. This is often the only method available in the field.
- Using Mr Frosty box in a -80°C freezer. This allows progressive freezing but the rate of freezing is not precisely controlled.
- Progressive freezing at a rate of -1°C per minute. This requires the availability of special equipment which is both expensive (around 20 000 €) and difficult to transport to the field (See Appendix 3)

3. Sample recovery

Samples are quickly unfrozen in a water bath at 25°C and then diluted into algal growth media in 50 mL flasks. The flasks are covered with aluminum for 24°C to avoid light shock and put in a culture cabinet using a temperature close to the one recorded at the sampling site (e.g. 4°C for polar waters, 15°C for temperate waters, 22°C for tropical waters).

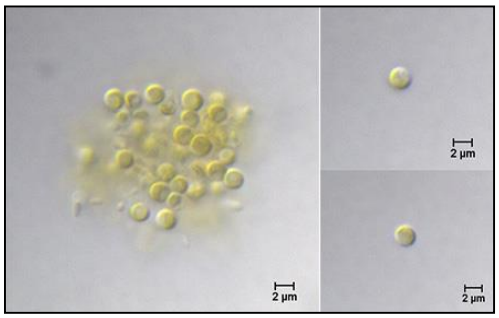
Several media can be used: K for eukaryotes and PCRS11 for cyanobacteria (media composition is available from http://roscoff-culture-collection.org/protocols/media-recipes). Samples can be incubated either in full concentration medium or in diluted medium.

4. Protocol validation

We validated the protocols by comparing a number of conditions on samples from the English Channel off Roscoff. The following tables show the number of successful cultures according to the different conditions tested. Cultures were incubated into different media (K for eukaryotes and PCR S11 for cyanobacteria).

Conditions		Successful/Total
	Concentrated	9/24
	Not concentrated	1/18
	Filtered < 3µm	4/24
	Unfiltered	6/18
Freezing	Progressive	4/12
	Mr. Frosty	4/12
	Liquid nitrogen	1/12
Medium	K/2	5/12
	K/100	1/12
	PCRS11/2	1/8
	PCRS11/100	0/8

The highest recovery is obtained on concentrated samples using either progressive or Mr Frosty freezing and with full concentration K/2.



A culture of picoplankton obtained from a Roscoff sample preserved with 5% DMSO and progressive freezing and grown in K/2

We tested also the protocol for samples obtained from a cruise on the continental shelf of Brazil (CARBOM, Nov 2013). Because of the material available on the cruise, samples were spiked with DMSO 5% and preserved directly in liquid nitrogen. Still we were able to isolate five strains of *Synechococcus*.

Code	Type	Sample date	Station	Depth (m)	Lat	Long
CARBOM 02 Syn 01	*Synechococcus*	18/11/2013	96	5	27°23.660	47°48.962
CARBOM 02 Syn 02	*Synechococcus*	18/11/2013	96	30	27°23.660	47°48.962
CARBOM 02 Syn 03	*Synechococcus*	18/11/2013	96	50	27°23.660	47°48.962
CARBOM 02 Syn 04	*Synechococcus* IIa	18/11/2013	98	5	27°35.137	47°23.242
CARBOM 02 Syn 05	*Synechococcus*	19/11/2013	106	60	28°07.00	46°10.47

Appendix 1 – Pre-filtration

Equipment

- Filtration tower (see image below - Nalgene N300-4000 available from Thermo Scientific http://www.thermoscientific.com/en/product/nalgene-reusable-filter-holders-receiver.html).
- Nuclepore Filter 47 mm 2 or 3 µm

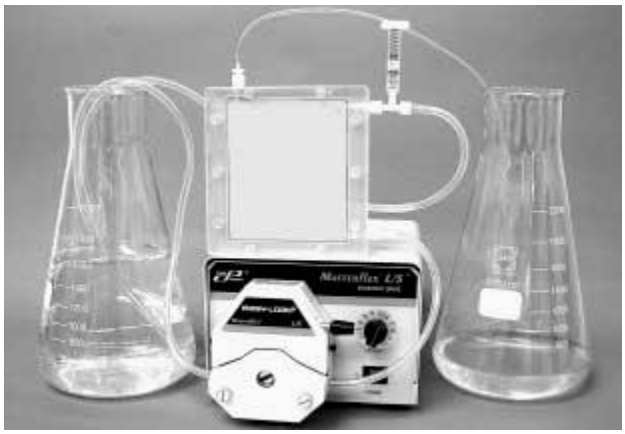
Protocol

- Install two filters on top of each other in the filtration tower to ensure maximum filtration efficiency
- Filter 200 mL of sample by gravity without exercising any depression.

- Collect the filtered sample.
- Rince very thoroughly filtration tower before proceeding to the next sample

Appendix 2 - Sample concentration by tangential flow filtration

Equipment



- Vivaflow Cartridge 0.2 µm (PES) VF20P7
- Masterflex Pump 6-600 rpm (ref Bioblock F39671)
- Rotor 3 “cylinders” (ref F39110)
- 1 high throughput head (ref F40103) (can be replaced with quick load head)
- Replace tube provided by stronger tube with two connectors (see picture)
- Bottle 6 L
- Bottle 1 L
- Conical tube 50 mL
- Masterflex Tygon tubing size 16
- Plastique pipettes (1 mL)
- Clamps with screw (to control retentate[1] speed)
- Clamps

Solutions

- Rinsing solution for regenerated cellulose membranes: 250 ml of 0.1 M NaOH
- Storage solution: Deionised water with 10 % ethanol.
- Deionized water
- Filtered sea water

Protocole

- Get Vivaflow cartridge out of storage
- Mount as in Fig. 1 (TFF Figures, courtesy of C. Brussaard, NIOZ)

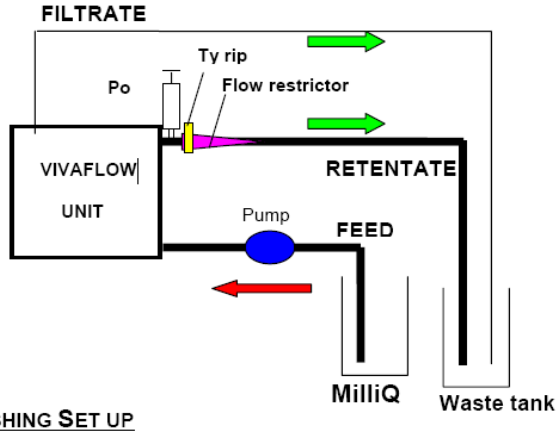


FIG 1. FLUSHING SET UP

[1] Retentate : the portion of the feed solution that does not pass through a cross flow membrane filter.

- Remove the clamps
- Set the pump to maximum speed. Manometer should be at about 2.5 bars (with a new cassette sometimes the manometer get stuck, if the value is too low there is a leak in the system)
- Rinse the cartridge with about 250 mL of distilled water (longer if cartridge has been stored in ethanol)
- Replace MilliQ water by sample in 6L bottle
- Rinse cartridge with about 250 mL of sample
- Put the retention line into the sample bottle (Fig. 2)
- Put a screwing clamp on the retention line to increase filtrate flow so that Manometer gets up to 2.5 bars.

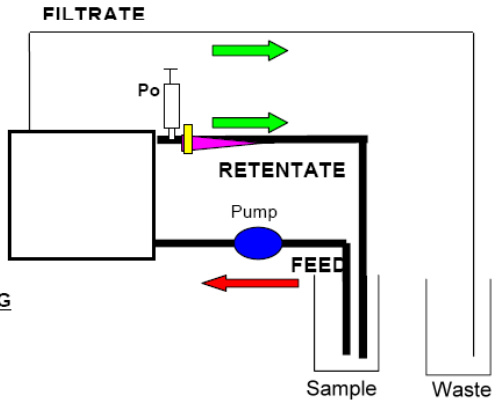
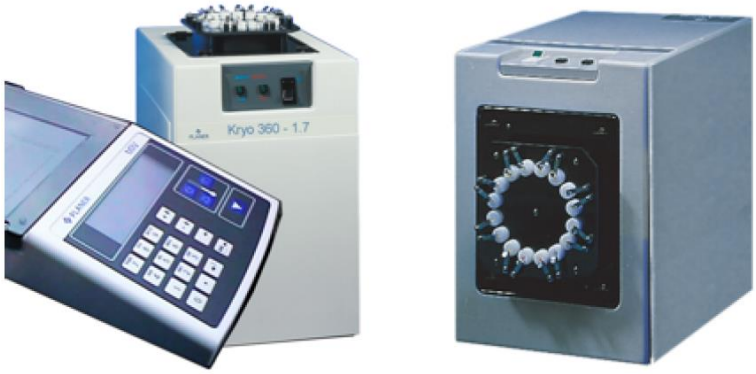


FIG 2 CONCENTRATING

- Concentrate sample until about 250 mL (6 L takes about one hour)
- Transfer to a smaller container (first to a 250 mL bottle then finally to a 50 mL tube)
- Continue to concentrate being very careful and lowering the pump speed
- When final volume is about 10 mL, clamp filtrate tube and recirculate slowly (no change of volume should take place)
- leaving the filtrate tube clamped, get the feed line out of the sample in order to get back the total volume of concentrated sample
- Store concentrated sample (measure concentration by flow cytometry and process as needed: sorting, cryopreservation etc.).
- Go back to Fig. 1 configuration
- Rinse 1 min with filtered sea water
- Rinse 1 min with distilled water
- Rinse with 50 mL NaOH 0.1 M
- Put all three tubes (feed, retention, filtrate) in bottle containing NaOH 0.1 M
- Recirculate for 20 min (to get rid of everything on the cartridge filter)
- Rinse with 250 mL of distilled water (Fig. 1)
- Stop the pump and clamp all three tubes
- Store at 4°C
- For a storage longer than 1 day, store with 10% ethanol

Appendix 3 - Progressive freezing protocol

Equipment and chemicals



- Progressive Freezer Planer (Kryo 360-3.3 - http://www.planer.com/products/cryo-freezers/small-crf/kryo-360.html)
- Isothermal box (to contain liquid nitrogen during transport)
- Liquid nitrogen tank and/or -150°C freezer

- Brady IP™ Printer - 300 DPI Standard - # BP-IP300 (http://www.bradyid.com/bradyid/pdpv/BP-IP300.html)
- Dimethylsulfoxyde (DMSO)
- Liquid nitrogen
- 2 mL Cryogenic tubes (Simport, T311-2)
- Brady Labels (THT-181-492-3, ref VWR: BRDY805919)
- Cryobox (Nalgène, 5026-0909)
- Gloves, glasses and lab coat

Protocol

- Prepare cryo-resistant labels with sample references
- Add **5** % DMSO (Dimethylsulfoxyde) final to 1**.5** mL of sample and transfer to a 2mL cryogenic tube with label.
    - Start the Progressive Freezer Planer and run the following program:
    - Start at 20°C
    - Decrease temperature by 1°C per minute until -40°C
- Wait 10 min at -40°C
- When the cycle is finished, protect yourself with gloves, glasses, and lab coat. Plunge the cryogenic tubes into liquid nitrogen
- Store cryogenic tubes in cryoboxes and store in a -150°C freezer and/or in a liquid nitrogen container for long-term storage.