COLLECTION STANDARD OPERATING PROCEDURE
OMICS SIZE FRACTIONATION

I. Sample collection

- Sample seawater from the selected depth using Niskin bottles previously thoroughly rinsed with tap water;
- A total of 10 L seawater are collected from the Niskin bottle after sieving through 200 and 20 µm mesh size nylon sieves, sequentially;
- The 200 µm sieve (Fig. 1) is placed on top of a 20 µm net inside a funnel (Fig. 2)
- The sieved seawater is collected into an acid-cleaned graduated tank with a tap (named *IN-tank*)
- Make sure that the tap of the IN-tank is closed and turned up, before collecting the water;
- After sieving 10 L seawater, place the 20 µm sieve (from the funnel) in a beaker with some filtered seawater to keep it humid;
- Carry the IN-tank to the bench to start filtration;

II. Sample filtration

A. Size fractions 0.2-3 and 3-20 micron

The filtration apparatus is composed by two 142 mm filter holders (tripods), connected with appropriate silicon tubes, an EZ-Stream Merck Millipore vacuum pump, and a collecting tank, named *OUT-tank* , with a tap (Fig. 1).
Filters are Millipore Isopore TSTP 14250 (3 µm) and Millipore Isopore GTTP14250 (0.22 µm)

Warnings:

- It is crucial that the tank with ingoing water (called *IN-tank*) is placed higher than the first tripod which, in turn, is placed higher than the second tripod;
- It is important that the pump is placed at a safe distance from the tripods, to avoid contact between water and sockets;
- Correct placement of the pump is shown in Figure 1, where the pump is located on a support (in this case a chair), and behind the workbench;
- The pump is then connected through a tube to the tank of the outgoing water (*OUT-tank*), placed on the floor;

Figure 1

Figure 2

1. Filtration setup

a. Always wear gloves;
b. Place clean paper on the benchtop;
c. Clean tweezers with ethanol;
d. Open the tripods on the benchtop, removing the top and placing it upside to avoid contact with surfaces;
e. Place the 3 µm membrane filter in the higher tripod and the 0.22 µm filter in the lower one. Keep the same orientation as they are in the box and discard the blue paper inter-filters;
f. Filters are to be placed on the blue plastic support of the tripod;
g. Once filters are placed inside each tripod, close them, tighten the hand knobs gradually and simultaneously (this facilitates the correct sealing of the tripod, avoiding spillovers);
h. Connect the inlet and outlet tubes;

i. Place the tripods as indicated in point I and shown in Fig.1 and set up the connections among the different components of the filtration apparatus.

2. Filtration (reduce the filtration time to a max of 30')

a) Before starting filtration, make sure to mark the cryovials appropriately;
b) MC is the code for MareChiara, S for Sarno river, C for Capri stations
c) The code for the fraction 0.22-3 µm (Free Bacteria) is "NRFB+CODE OF STATION+SEQUENTIAL NUMBER OF SAMPLING AT THE SPECIFIC STATION SITE+SAMPLE NUMBER" (e.g. NRFB C5 #82)
d) The code for the fraction 3-20 µm (NanoEukaryotes) is "NRNE+ CODE OF STATION+SEQUENTIAL NUMBER" (e.g. NRNE C5 #82)
e) Connect the *IN-tank* to the higher tripod with a tube and open the tap, allowing the complete filling of the first connecting tube;
f) Make sure that the pump is connected with a tube to the *OUT-tank*;
g) See the scheme below

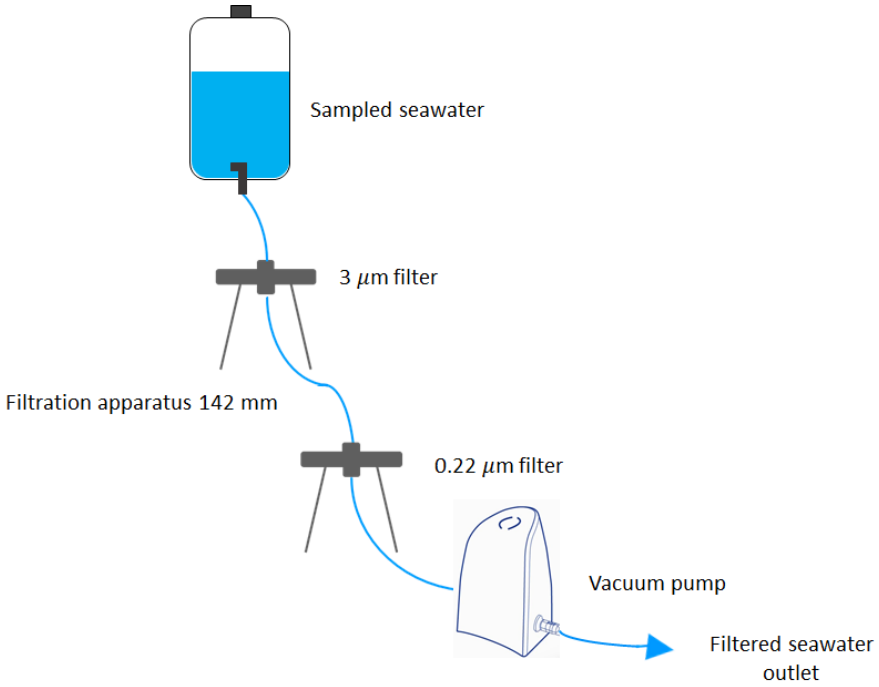

Figure 3 Filtration set up

h) Turn the pump on;
i) Always initiate the filtration with the filter holder vents open. Wait until air bubbles are expulsed and close the vents;
j) Filter 5 L, then close the tap of the *IN-tank* and let all the remaining water to be filtered through;
k) Once no more water is left in the tubes, turn the pump off;
l) Disconnect the tripods and put them on the bench;

m) Open the higher tripod, remove the top and position it upside down on the bench, avoiding the contact with surfaces;
n) Use the tweezers to handle the filter;
o) Fold the filter in half repeatedly by folding in a cylinder to fit in a vial;
p) Carefully place the filter in a 5 mL cryovial and gently push it to the bottom;
q) Close the cryovial and quickly place it in the liquid nitrogen container;
r) This is the ii fraction (3-20 µm)
s) The code for the fraction 3-20 µm (NanoEukaryotes) is "NRNE+ CODE OF STATION+SEQUENTIAL NUMBER" (e.g. NRNE C5 #82)
t) Repeat points i to m for the lower tripod
u) This is the i fraction (0.2 – 3 µm)
v) The code for the fraction 0.22-3 µm (Free Bacteria) is "NRFB+CODE OF STATION+SEQUENTIAL NUMBER OF SAMPLING AT THE SPECIFIC STATION SITE+SAMPLE NUMBER" (e.g. NRFB C5 #82)
w) Repeat points a) to r) for the remaining 5 L (second replicate).
x) Store the OUT-tank in the shade; the 0.22 µm filtered seawater will be used for virus-DNA (link to the protocol)

B. Size fraction 20-200 m

This is done after completing the process for 0.2-3 and 3-20 micron fractions.

a. Always wear gloves
b. Clean the workbench and replace the paper;
c. Wash the *IN-tank* and the tripods with DW;
d. Use only one tripod
e. Clean tweezers with ethanol;
f. Place a 3 µm filter as described in A-1;
g. Place the tripod on the stool;
h. Take the beaker with the 20 µm sieve from the initial collection (Water sampling, f);
i. Rinse the 20 µm nylon sieve using the filtered water from the OUT-tank;
j. Pour the remaining filtered water from the OUT-tank into the IN-tank, together with the water used to rinse the nylon sieve in h);
k. Connect the tripod to the pump and to the tubes;
l. Make sure that the pump is connected with a tube to the *OUT-tank*;
m. Turn the pump on;
n. Always initiate the filtration with the filter holder vents open. Wait until air bubbles are expulsed and close the vents;
o. Filter 5 L, then close the tap of the *IN-tank* and let all the remaining water to be filtered through;
p. Once no more water is left in the tubes, turn the pump off;
q. Disconnect the tripod and put it on the bench;
r. Open the tripod, remove the top and position it upside down on the bench, avoiding the contact with surfaces;

s. Use the clean tweezers to handle the filter.
t. Fold the filter in half repeatedly by folding in a cylinder;
u. Carefully place the filter in a 5 mL cryovial and gently push it to the bottom;
v. Close the cryovial and quickly place it in the liquid nitrogen container;
w. This is the fraction 3-20 micron
x. THE CODE FOR THIS FRACTION IS NRME+CODE OF STATION+SEQUENTIAL NUMBER
y. Repeat points I to u for the remaining 5L (second replicate)

Once in the lab, the cryovials are stored at -80 °C

The OUT-tank, containing filtered seawater is used to collect samples for viral diversity (see appropriate SOP)

III. List of materials, equipment and supplies

- FILTRATION APPARATUS (2 TRIPODS + PUMP + POWER SUPPLY)
- STOOL AND WOOD BASE
- SILICON TUBES WITH DIFFERENT SECTIONS FOR PUMP-FILTRATION APPARATUS-TANK
- 10 L GRADUATED TANK 10 WITH TAP (IN-TANK)
- 10 L TANK WITH A TAP (OUT-TANK)
- 20 µm mesh size NYLON SIEVE
- PLASTIC FUNNEL
- 200 µm mesh size SIEVE MOUNTED ON A PLASTIC CONTAINER (Fig. 1)
- 2 TWEEZERS
- POLYCARBONATE FILTERS 142 mm DIAMETER 0.22 Millipore Isopore GTTP14250) AND 3 µm (Millipore Isopore TSTP 14250) PORE SIZE
- DISTILLED WATER
- ETHANOL
- 2L PLASTIC BEAKERS
- GLOVES
- TABLE PAPER
- SIX 5 ml CRYOVIALS (DUPLICATE VIALS FOR EACH OF THE THREE SIZE FRACTION)
- MARKER PEN RESISTANT TO LIQUID NITROGEN (Sharpie or similar)
- LIQUID NITROGEN