The EMO BON handbook

updated version 2024

EMO BON
EUROPEAN
MARINE OMICS
BIODIVERSITY
NETWORK

European Marine Omics Biodiversity Observation Network (EMO BON) – Handbook

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Version 2.0
March 2024

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How to cite:

Ioulia Santi, Raffaella Casotti, Thierry Comtet, Michael Cunliffe, Yolanda Koulouri, Lara Macheriotou, Fabrice Not, Matthias Obst, Christina Pavloudi, Sarah Romac, Eric Thiebaut, Jan Vanaverbeke, Nicolas Pade. European Marine Omics Biodiversity Observation Network (EMO BON) – Handbook. Version 2.0, 2024.

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The European Marine Omics Biodiversity Observation Network (EMO BON) is a European initiative from the European Marine Biological Resource Centre (EMBRC) to establish a coordinated, long-term biodiversity observatory. Currently there are many ongoing genomic observation stations in Europe. The goal for EMO BON is to support the individual marine biodiversity observatories within EMBRC and connect them under one centrally coordinated network, with shared protocols, data, and metadata standards. EMBRC provides the context and opportunity for partner institutions to participate and complement EMO BON by initiating biodiversity observation stations. EMO BON includes marine stations from Polar regions to the Red Sea that will sample for genomic marine biodiversity at frequent intervals. This network will contribute to the United Nations Decade of Ocean Science for Sustainable Development and aims to be an important European component to the global ocean observation networks.

Collection of marine water, sediment and organisms will take place at the EMBRC participating observatory stations according to the protocols described in this document – the EMO BON Handbook – setting a minimum standard for participation to the network. DNA extraction and sequencing will be performed at a centralised facility to reduce biases and ensure consistency in the high-quality of sequencing. The data generated within this initiative will follow the FAIR data principles. The life cycle of the EMO BON data will be described in detail in the EMO BON Data Management Plan. Overall, EMBRC aims to build a long-term genomic observatory, generating cost-effective, high-quality, baseline genomic biodiversity data that will be produced in the long term.

This Handbook contains all the guidelines and procedures from sampling to sequencing that will be followed within EMO BON. The purpose of this document is not only to ensure the rigorous adhesion to the appropriate protocols within EMO BON, but also to provide all the necessary information to potential external participants from the wider scientific community.

Introduction

Marine ecosystems are rich and complex, hosting a diverse array of life from the smallest microorganisms to the largest mammals. Together, this biodiversity sustains marine ecosystems and provides services to human society as well as an inherent natural beauty. Restoring and protecting biodiversity is critical today as the wellbeing of humans is tightly linked to fauna, flora, and ecosystem health. The health and status of marine biodiversity is becoming of increasing concern because of a range of impacts, including climate change and pollution. The marine environment and its biodiversity are proving to be even more susceptible than previously believed, with increasing losses of biodiversity observed as temperature increases (Smale et al., 2019; Antão et al., 2020). Eventually, holistic knowledge on marine biodiversity is essential for fully understanding the ocean (Muller-Karger et al., 2018).

Across the European Union, the political will to allay environmental damage while protecting and restoring natural ecosystems is growing, with promising and ambitious plans proposed to reduce environmental impact and mitigate climate change (European Green Deal), and with pledges to restore degraded ecosystems and biodiversity (European Biodiversity Strategy for 2030). Policy makers require focused, sustained, consistent, quality-controlled, and transparent information with regional coverage to rapidly detect the impact of their management and policy decisions on biodiversity. Nowadays, at the European level, data on marine biodiversity are fragmented and targeted at specific research questions. Furthermore, with the increased need to understand and minimize anthropogenic impact on the oceans, policy makers need biological monitoring to provide operational input to their decision-making processes.

To move towards a sustainable and environmentally conscious society, it is urgent that we rapidly develop and organize marine biodiversity observing capabilities to complement the well-organised physio-chemical observation networks (Heymans et al., 2020). There is a need for regional coverage of established long-term biodiversity series, where traditional methods and emerging techniques are combined (Claudet et al., 2020). Finally, biological data need to adopt a rigorous approach to protocols and quality control to ensure the replicability of experiments and models.

Omics biodiversity defined as the collective identification of the species composition of an ecosystem by using molecular biology omics approaches such as metagenomics and metabarcoding, is a global point of discussion. Recognizing the need to continuously assess biodiversity, many organisations and research infrastructures are expressing interest in establishing such an observation network.

The core mission of EMBRC is to provide access to marine biodiversity for research and innovation purposes. As a multi-domain research infrastructure, supporting both environmental and biological research, EMBRC can pave the path to biological observation in the marine environment. Therefore, as a contribution to the global omics observation, in 2021 EMBRC launches the first coordinated, long-term European Marine Omics Biodiversity Observation Network (EMO BON). EMO-BON will provide long-term baseline genomic biodiversity data, supporting biodiversity and EOVs (Essential Ocean Variables) and EBVs (Essential Biological Variables) monitoring, and ensuring continuity between the many current short-term genomics observatory projects. EMO BON will build on robust methodologies and produce cross-checked and quality-controlled datasets following appropriate data and metadata standards. By establishing EMO BON, EMBRC will mobilise a substantial network and bring transformation to the marine

biological science landscape. Long-term observation will provide the backbone of large research projects and initiatives, from ocean science, and climate change to microbiome research and bioprospecting.

Several EMBRC nodes are already carrying out genomic observation and have brought considerable expertise on the subject. EMO BON will support the individual marine biodiversity observatories present in EMBRC and connect them under one centrally coordinated network, thus creating one of the few operational genomics observatory networks in the world. By widening the distribution of the observatories along the European coastline, EMBRC can ensure a unique and broad coverage of habitats and longitudes from northern Norway to tropical Israel. In addition, EMBRC is involved in the international community, which has been heavily engaged in the conceptualization and development of genomic observation. Therefore, EMBRC observatory will be operational in Europe, but relevant in a global context drawing users of the planned outputs from around the planet.

To launch the initiative, 2021 and 2022 will serve as pilot years to establish and improve operational procedures, create, and apply a data management plan based on FAIR principles and, build the tools required to publish the produced data in a useful manner to all end users. These two years will serve as the baseline to establish the future operation of EMO BON and its position in the global marine observation roadmap.

Participation to EMO BON

EMO BON will initiate in 2021 and operate as a pilot until December 2022. For this period, selected EMBRC partner institutions are invited to establish operational observatories. The selection of partners was done to obtain a broad geographic distribution and to include representatives from each EMBRC node. After the pilot phase, the geographical coverage will be expanded by including additional observatory stations. Observatories are invited to select their sampling sites and provide information to EMBRC by filling in the EMO BON registration form.

Three different marine habitats will be sampled within the EMO BON: water column, soft substrates, and hard substrates. The observatories are expected to participate by sampling the water column as a minimum and they may also select whether to sample soft and/or hard substrates. Therefore, they may select a water column, a soft substrate, and a hard substrate site to include to their EMO BON observatory. It is recommended that observatories select sampling sites that have been studied in the past and that are well characterized (i.e., include other marine variables such as seawater chemistry and/or biological components). Prior knowledge on the system will help putting EMO BON data in a broader context and will result into an integrated study of the sites.
Therefore, in case EMBRC partner institutions run long term time series of biological data, it is recommended that EMO BON sampling is carried out at the same sites. In addition, it is suggested that the sampling sites of the different habitats are located near each other or at least in the same marine area, if possible.

Participating institutions should decide on a name and a short ID for their observatory that will be maintained for the duration of EMO BON and will characterize all samples collected at this observatory. A contact person should be appointed who will be responsible for any future communication. This person should hold all information regarding sampling campaigns, samples collection, laboratory analyses, shipping, environmental variable measurements and will be the link between EMO BON coordinators and the observatory. The contact person should be reachable for an extended period after sampling.

An EMO BON Operating Committee (OpCo) will be established and comprise of the EMBRC executive director, the EMBRC EMO BON science officer, one representative from each EMBRC node, and one representative from the e-Infrastructures EMBRC working group. The OpCo will ensure connection and communication with all its national observatories. A member of the EMBRC General Assembly will also follow the OpCo during the pilot phase. The OpCo will be the connection between EMO BON coordinators and the observatories and will be meeting monthly to tackle technical and operational issues.

Sampling Protocols

Three different marine habitats will be sampled within the EMO BON: water column, soft substrates, and hard substrates. Considering the diverse interests, experience, and equipment within EMBRC, this handbook contains different sampling modules ranging from simplified to more complex and time demanding. The basic water column procedure is the only prerequisite for participating in the observatories network. All other protocols are included to help guide the decision on future protocols that observatories may wish to add depending on their local interests.

All modules are based on robust methodologies that generate reliable and versatile data and have been priorly tested either in the EMBRC operating marine stations or through other EMBRC activities [ASSEMBLE Plus: Ocean Sampling Day (OSD) and Autonomous Reef Monitoring Structures (ARMS)]. Considerable effort has been put into making the methodologies as flexible and adaptable as possible to accommodate all the observatories chosen for the pilot phase. The standardization of the sampling procedures is important to avoid biases and to produce comparable results; thus, EMO BON participating observatory stations should adhere to the Standardized Operating Procedures (SOPs) described in this Handbook. Using the protocols in this Handbook will allow for interoperability and comparability between observatory stations. In addition, EMBRC will organize training workshops for its members and beyond, to support users during the sampling and laboratory procedures and to ensure consistency in the implementation of the procedures.

During the pilot phase in 2021 and 2022, EMO BON will focus on creating and maintaining the network of observatories, applying and testing methodologies and procedures, producing high quality datasets, and generate a robust data management plan based on FAIR principles. In this initial phase it is important to give perspective to this network and make it operational for the ongoing decade. For a successful and cost-effective implementation phase EMO BON will have to concentrate the efforts to a broad geographic coverage and the sampling of diverse habitats rather than the thorough sampling of fewer sites. Therefore, EMO BON will cover the sampling based on one SOP per sampled habitat and a fixed number of ARMS deployments. Particularly for the water column, **Water Column Standard Operating Procedures 1 (WaSOP 1 basic)** will be covered as a prerequisite for participation to the network. In addition, each observatory may select one of the **Soft Substrate Standard Operating Procedures (SoSOPs)** to sample the communities of choice (microorganisms and/or meiobenthos and/or macrobenthos). For **Hard Substrates Standard Operating Procedures (HaSOP)**, it will be possible to deploy ARMS three times in one year. The additional SOPs are included in the Handbook not only as future approaches but also to help standardization for any observatory who wishes to establish further operational procedures. Observatories are welcome to follow them and either perform in-house analyses or biobank the samples. This approach will be re-evaluated by the EMO BON OpCo and the EMBRC General Assembly at the end of the pilot phase.

The different habitats will be sampled at different time intervals; this information is summarized in Table 1. Water column sampling events will take place at the participating operating stations once every two months during daytime hours. There will be three modules for genomic biodiversity sampling of water column. **Water Column Standard Operating Procedures 1 (WaSOP 1 basic)** will represent the basic

module for water column sampling within EMO BON. It includes sequential filtration for the collection of different sized microbial plankton organisms. With equipping the appropriate filtration apparatuses, seawater filtration becomes simplified while ensuring adequate biomass collection at minimum processing time. This protocol will constitute the EMBRC contribution to European marine genomics observation and is the prerequisite for participating in the network. All EMO BON participating stations are expected to follow this basic protocol.

In addition to the basic water column sampling (**WaSOP 1 basic**), EMOBON participating stations may wish to optionally perform **WaSOP 2** and/or **WaSOP 3. WaSOP 2** and **WaSOP 3** target plankton organisms of larger sizes (micro-, meso- and macro-plankton) and include the use of plankton nets. These protocols are included to assist observatories that are interested in sampling larger in size plankton but cannot be covered by EMO BON during the pilot phase. After the pilot phase, the possibilities of covering the costs of further SOPs will be re-evaluated.

Soft substrate sampling will be based on different modules, depending on the sampling capacities of each operating station. Participating stations that have access to intertidal sediments are encouraged to sample such suitable sites using the **Soft Substrate Standard Operating Procedures 1** for intertidal sediments (**SoSOP 1**). If access to intertidal sediments is limited or unfeasible, operating stations may choose between **SoSOP 2** for sampling by diving or **SoSOP 3** for sampling onboard a research vessel. Operating stations are to select one or more modules for soft substrate sampling and maintain it for the duration of EMO BON. Each of the **Soft Substrate SOPs** describes the collection of samples to access the microbial community, the meiobenthos and the macrobenthos. Participating stations may sample any of the communities they are interested in.

Sampling intervals will be different for the various soft substrate communities since the dynamics of each community are different (Table 1). Sampling for sediment microbial communities will take place once every two months, sampling for meiobenthos once every 4 months while macrobenthos sampling will take place twice a year.

Table 1: Sampling intervals for the different modules and sampled communities

Water Column	Once every two months (February, April, June, August, October, December)
Soft substrate - Microbial community	Once every two months (February, April, June, August, October, December)
Soft substrate - Meiobenthos community	Once every four months (April, June, October)
Soft substrate - Macrobenthos community	Twice a year (October, April)
ARMS deployment / retrieval	Flexible timeframe for operating stations

The **Hard Substrates Standard Operating Procedures (HaSOP)** describes the passive sampling of hardbottom communities based on the Autonomous Reef Monitoring Structures Marine Biodiversity Observation Network (ARMS-MBON). This module requires the purchase of ARMS units from the Smithsonian Institution (https://www.oceanarms.org/) and their deployment in the sea. The procedures described here have been continuously and successfully employed during the ASSEMBLE Plus ARMS-MBON project (Obst et al., 2020). Operating stations may adopt different sampling strategies for the deployment and retrieval of ARMS for the hard substrate samplings. However, it is important that the sampling variations are well documented and linked to the dataset as metadata.

The deployment and recovery of ARMS may be challenging for inexperienced users. Therefore, observatories not familiar with ARMS protocols need to be trained in the deployment, recovery, and data collection. This will be done by participating in a hard substrate sampling in an experienced observatory before being able to apply HaSOP in EMO BON. EMO BON may help bring new and experienced observatories in contact. EMBRC plans to host training events within the pilot phase of EMO BON for additional training on sampling and laboratory procedures.

Water Column

Water Column Standard Operating Procedures 1 – WaSOP 1 (basic)

Summary

Subsurface seawater (0-3 m depth) will be collected from the water column sampling site of each observatory. The exact water sampling depth is not specified as observatories may not be able to sample at a specific depth. In case an observatory can sample at specified seawater depth, then sampling at 1 m depth is recommended. Plankton community will be prefiltered to screen out organisms >200 µm and then concentrated by sequential filtration on polycarbonate (PC) membrane filters. Initially seawater volumes will be filtered through 3 µm polycarbonate membrane filters (142 mm in diameter); the outflow seawater will be sequentially filtered through 0.2 µm polycarbonate membrane filters (142 mm in diameter). This will result in collecting 2 different fractions of plankton: 3-200 µm and 0.2-3 µm organisms.

The required seawater volume to ensure adequate biomass collection may vary between geographic locations. The observatories may decide on the filtration volume based on the characteristics of their sampling site and on previous filtration experience in the area. As an indication, in the oligotrophic open-sea, filtration volumes may reach 20 l, while in nearshore coastal areas filtration of 5 l may be adequate. However, it is important that operating stations filter the same seawater volume in every sampling event to obtain consistency among samples from each location. EMBRC can provide further guidance on selecting filtration volumes upon request.

After filtration, each filter membrane will be cut into two pieces by using a sterile scalpel and each piece will represent one replicate. In total 4 replicates should be collected (more information in Replication and Biobanking section, a diagram representation is available in Figure 11); that is, 2 separate sequential filtrations will take place and the cutting of each filter into two will result in 4 replicates. Filtration should be performed by delicate pumping either using a peristaltic pump or by manual hand pumping. After filtration, membranes should be left to dry, carefully folded, placed in individual containers with the preservative DNA/RNA shield, flash frozen in liquid nitrogen and stored at -80°C until shipping for further analysis.

Filtration may initiate in the field immediately after sampling or following the transportation of the sampled seawater to the laboratory. Seawater should remain in a cool dark place during transportation and until filtration. It is important to be consistent to when the filtration takes place among the different sampling events, therefore observatories are expected to either always filter in the field or always transport water and filter in the lab. For a diagram representation of the procedures see Appendix 2.

Protocol

A. **Materials, equipment, and supplies**

Before starting, ensure all materials equipment, and supplies are available by checking the following table (Table 2).

Table 2: List of materials, equipment, and supplies for WaSOP 1.
10% HCl
0.2 µm prefiltered seawater
Purified water (Milli-Q or Nanopure)
Gloves
50 ml screw cap tubes
Aluminium foil
Wash bottles
Forceps
Scalpel
Peristaltic pump and pump heads / manual hand pump [1]
Appropriate silicone tubes (adjustable to the pump and the filtration apparatuses)
Stainless steel filtration apparatuses (tripods), 142 mm (see Appendix 1)
Polycarbonate hydrophilic membranes, 3 µm pore size, 142 mm in diameter (see Appendix 1)
Polycarbonate hydrophilic membranes, 0.2 µm pore size, 142 mm in diameter (see Appendix 1)
5 ml cryotubes
Permanent marker
Transparent tape
Niskin bottle / bucket / hand pump [2]
200 µm mesh
Sample containers of appropriate volume (carboys or Nalgene-type bottles, depending on the seawater sampling volume at each observatory)
Funnel
Parafilm or shrink film
DNA/RNA Shield
Pipette (1000-5000 µl) and suitable tips
Access to liquid N_2
Access to -80°C

[1] The filtration may take place by a peristaltic pump or a manual hand pump. However, filtration using a manual hand pump is feasible only at small seawater volumes. At seawater volumes larger than 2 l, it is highly recommended to use a peristaltic pump.

[2] For the collection of seawater it is recommended to equip Niskin bottles. However, depending on the equipment availability at each observatory, it may also take place using a bucket of a hand pump.

B. Preparation and Cleaning

- Always wear gloves.
- Prepare 10% HCl.
- Clean the workspace using 10% HCl.
- Filter seawater at 0.2 µm to prepare 0.2-prefiltered seawater. Ideally this should be seawater from the sampling site.
- Prepare a wash bottle filled with 0.2-prefiltered seawater.
- Prepare a 50 ml screw cap tube filled with 10% HCl and a 50 ml screw cap tube filled with 0.2-prefiltered seawater. Soak forceps and scalpel in the HCl tube and then in the 0.2-prefiltered seawater tube before every use. Store forceps in the 10% HCl tube after use.
- Follow the manufracturer's instructions for proper cleaning and maintenance of the filtration apparatuses and the peristaltic pump. Clean the peristaltic pump with paper towel humidified with tapwater before and after use.
- Filtration apparatuses and tubes are cleaned at the end of each sampling event and if allowed to dry and stored properly (i.e., tube ends covered, tripod tube inlets/outlets covered, interior of the tripod does not touch other surfaces, all parts are left to dry) there is no need to clean again. It is recommended to rinse all parts of the filtration apparatuses using 70% ethanol after use and allow them to dry overnight.
- After each filtration sequence, the system is cleaned by passing purified water (Milli-Q or Nanopure) through.
- Never touch the filter (not even with gloves on).
- Avoid touching the inside of the filtration apparatuses.
- Prepare the appropriate volume of purified water (Milli-Q or Nanopure) to be used as negative control (see below, Negative Controls section).
- Clean sampling equipment (Niskin bottle and/or buckets) using tap water. Rinse vigorously and make sure material from previous samplings is washed away. Cleaning of sampling equipment (Niskin bottle and/or buckets) may also take place at the end of every sampling event. Make sure that equipment was cleaned and stored properly before using it.
- Rinse the 200 µm mesh and funnels vigorously with tap water and purified water (Milli-Q or Nanopure). Make sure material from previous samplings is washed away. Cleaning of the 200 µm mesh and funnels may also take place at the and of every sampling event. Make sure that materials were cleaned and stored properly before using them.
- Clean the sample containers (carboys or or Nalgene-type bottles) by incubating overnight with 10% HCl. The following day wash twice using distilled water and twice using purified water (Milli-Q or Nanopure). Rinse vigorously to ensure all interior surfaces are cleaned and acid is washed away. Cleaning and decontamination of the sample containers (carboys or or Nalgene-type bottles) may also take place at the and of every sampling event. Make sure they were cleaned and stored properly before using them.

C. Setting up

Follow the manufacturer's instructions for fully setting up the filtration apparatuses and the pump. Here, only a brief description is included, considering that a peristaltic pump is used. The equipment set up is described in the following sketch (Figure 1). In addition, a filtration workspace is depicted in Figure 2.

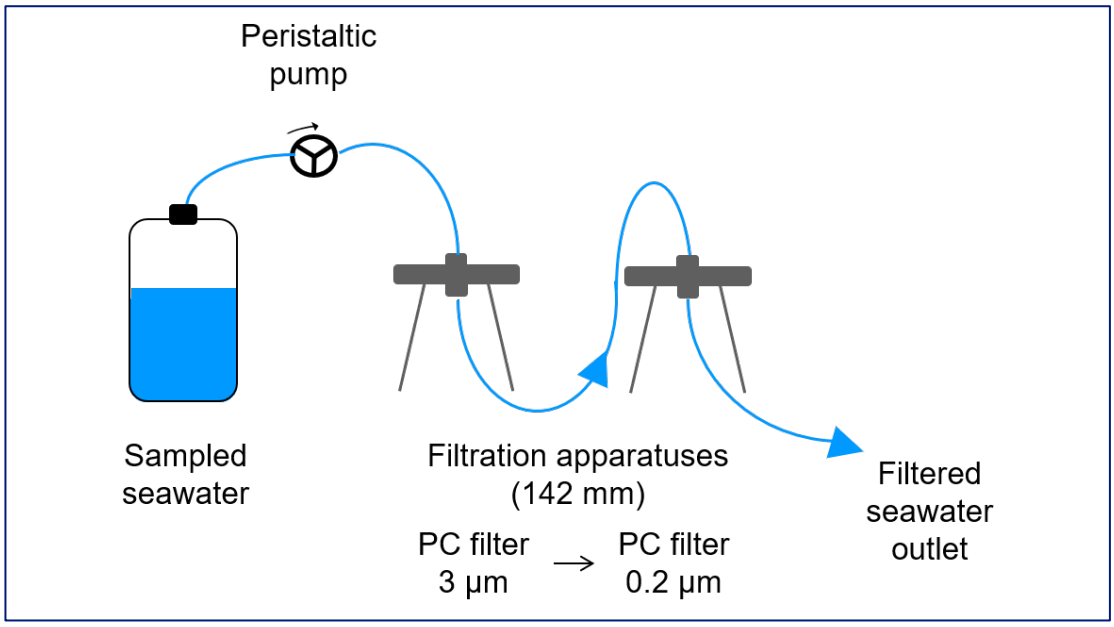

Figure 1: Set up of the water column filtration equipment. The sampled seawater is collected in a container. A peristaltic pump is passing the seawater through the system. Seawater is first passing through a 142 mm filtration apparatus equipped with a 3 µm PC filter and subsequently through a 142 mm filtration apparatus equipped with a 0.2 µm PC filter. The seawater is transported through appropriate tubes that connect the seawater container to the peristaltic pump and the filtration apparatuses.

Set up the filtration apparatuses (tripods) as described below:

1. Open the tripod filter holders. Avoid touching the inside of the filter holders.
2. Humidify filter holder area of filtration tripods using 0.2 µm filtered seawater (Figure 3).
3. Soak forceps in the HCl tube and then in the 0.2-prefiltered seawater tube before every use. Handle the filter with the forceps not by hand.
4. Place a PC filter membrane (3 µm pore size; 142 mm diameter) on the first tripod and a 0.2 µm pore size PC filter membrane (142 mm diameter) on the second tripod using forceps (Figure 4). Each PC filter is separated by 2 blue inter-filters in the filter boxes. Make sure to remove the blue inter-filters or the water will not go through. Filters are polarized and both sides can be used.
5. Close the tripods (Figure 5), tighten the hand knobs and connect the inlet and outlet tubes.
6. Connect the inlet tube of the first filtration tripod (3 µm filter membrane) to the peristaltic pump and cover it using aluminium foil until filtration.
7. Connect the outlet tube of the first filtration tripod (3 µm filter membrane) to the inlet of the second filtration tripod (0.2 µm filter membrane).
8. Connect the outlet of the second filtration tripod (0.2 µm filter membrane) to a tube and place the tube in a waste container or in the sink.

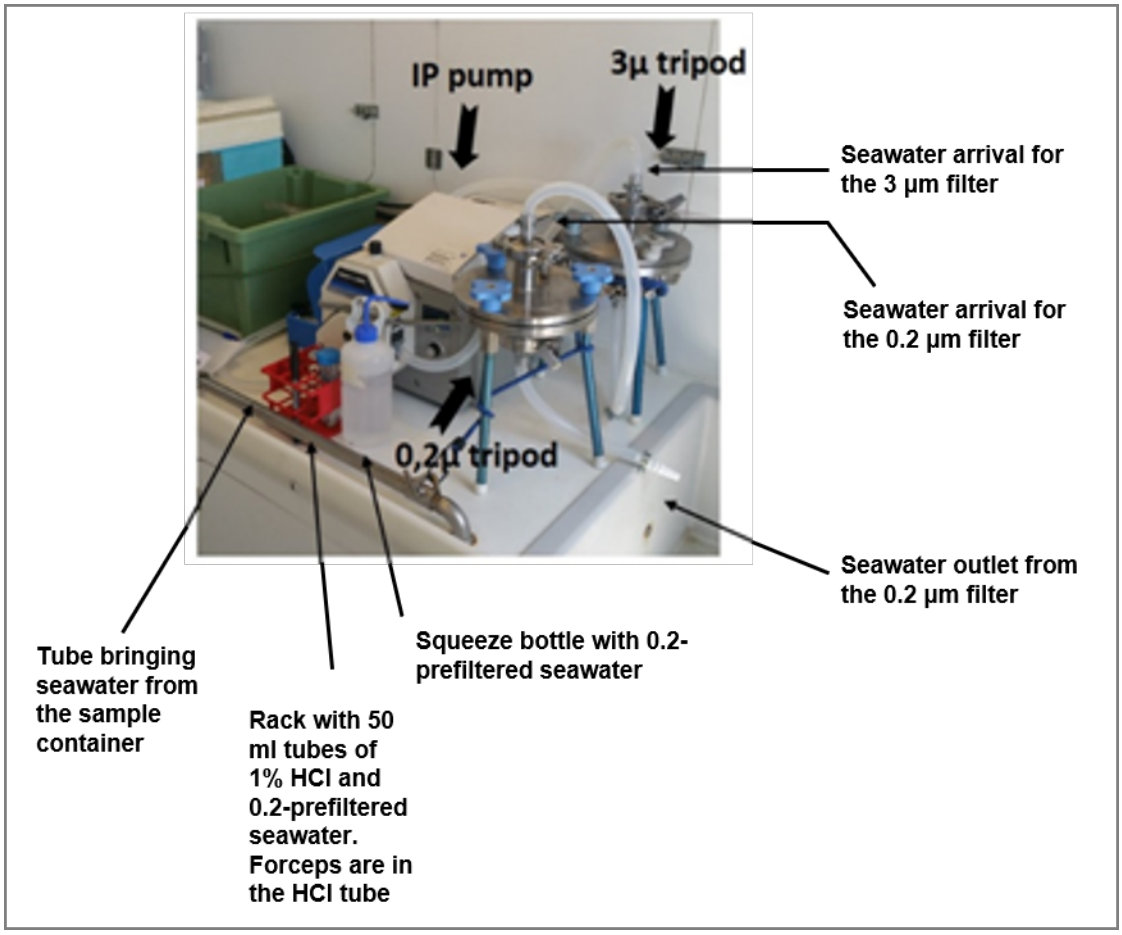

Figure 2: Set up of the water column filtration equipment (peristaltic pump, filtration apparatuses (tripods), tubing system, seawater collection carboys) on the laboratory bench.

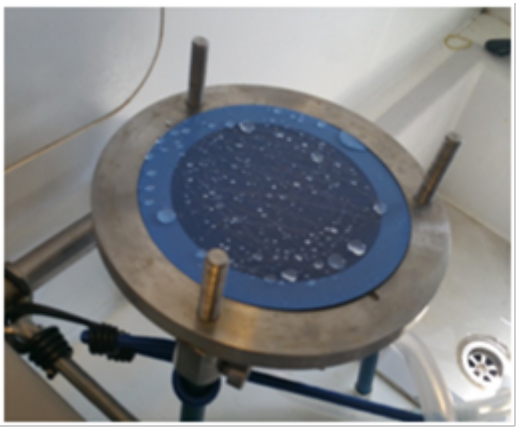

Figure 3: Open filtration tripod and humidified filter holder.

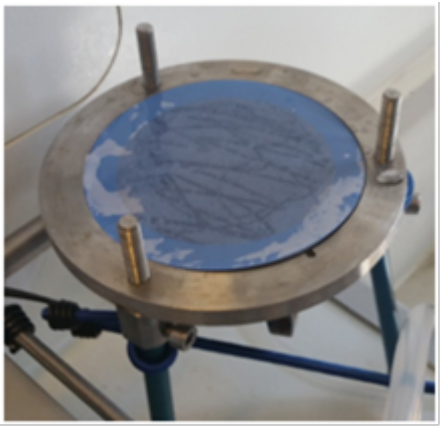

Figure 4: Open filtration tripod with a filter membrane placed on it.

Figure 5: Open (left) and close (right) filtration tripods.

D. Seawater Collection

1. If a bucket is used to collect seawater, rinse it three times with seawater from the sampling site. The Niskin bottle is washed with seawater while on use.
2. Rinse the funnel, and the sample containers three times with seawater from the sampling site.
3. Sample seawater below the sea surface between 0-3 m. Make sure not to sample sea surface water. Collect the appropriate volume of seawater using the Niskin bottles. Seawater may also be collected by hand-pumping or by using a bucket.
4. The required seawater volume differs among observatories. As two sequential filtrations will take place, sample twice the required volume. For example, if an observatory has decided to sample and filter 10 l of seawater, then 20 l of seawater should be sampled in total and, in step 5, transferred in two 10 l containers.
5. Adjust a large piece of 200 µm mesh on a funnel and place it on the sample container mouth.
6. Transfer seawater in the sample containers through the mesh and the funnel. Each of the sample containers will be used for a separate filtration sequence. Therefore, each sample container should include the required volume for one filtration. For example, if an observatory has decided to sample and filter 10 l of seawater, then each of the two sample containers should contain 10 l of the 20 l of seawater that was collected (step 3). Wash the 200 µm mesh before filling up the second sample container to remove retained material.
7. Filtration should initiate as soon as possible after sampling. Sampled seawater may be transported to the laboratory if filtration in the field is not possible. Keep each of the sample containers in a dark and cool place until filtration.

E. Seawater Filtration and Sample Storage

1. Mix the sample containers gently by rotating at least 5 times.
2. Place the inlet tube of the first filtration tripod (3 µm filter membrane) in the first sample container and immerse it in the seawater.
3. Cover the mouth of the sampling container during filtration. The container does not need to be firmly closed, just enough to ensure that external material will not fall in. For example, cover the container using parafilm or shrink film.
4. Check the pumping speed and the flow direction and turn on the peristaltic pump. Flow rate should be adjusted to ensure a regular gentle continuous seawater flow.

5. Always initiate the filtration sequence with the filter holder vents open. Wait until all air bubbles get expulsed from the filter holders through the vents and once the water flows regularly through the vents, close them.
6. Seawater will be filtered sequentially through two filter membranes, first through the 3 µm and subsequently through the 0.2 µm pore size membrane.
7. Check the tubing system and the filtration tripod connections regularly for overpressure that may damage the cells and/or the filtration system. If pressure is too high, decrease the flow rate.
8. Check for the presence of bubbles and use the filter holder vents to expulse them.
9. Ensure membranes always remain moistened during filtration.
10. After filtration, it is important to allow the filters to dry. To do that:
    a. Keep the pump on and allow the system to pump air.
    b. Disconnect the tube after the second tripod (0.2 µm tripod).
    c. Empty the tube between the pump and the first tripod (3 µm tripod) by lifting it up.
    d. Slowly open the vent of the second tripod (0.2 µm tripod) and empty the tube between the two tripods by "waving it" a few times.
    e. Close the vent of the second tripod (0.2 µm tripod). All the seawater that remained on the second tripod filter will be pushed out by the increasing pressure.
    f. Gently shake each tripod to make sure there is no water trapped in the filter holder.
    g. Slowly open both vents while turning the peristaltic pump off.
11. Open the tripod filter holders. Avoid touching the inside of the filter holder area.
12. Use the forceps to handle the filter. Soak forceps in the HCl tube and then in the 0.2-prefiltered seawater tube before every use.
13. To avoid contaminations, cut rapidly the filter into 2 visually equal pieces by using a sterile scapel, after placing it on a clean, hard surface (e.g. in a Petri dish).
14. Immediately fold each piece twice and then fold again by rolling the filter in a cylinder. The two filter pieces will be the sample replicates.
15. Carefully place the filters into separate 5 ml cryotubes and gently push the filters to the bottom of the tube to minimize the space they occupy in the tube. Do not crumble the filter, just try to gently fold it once more while in the tube.
16. Add 4.5 ml of DNA/RNA Shield by using a pipette and make sure the filter is covered by the solution.
17. Label the tube appropriately using permanent marker (see Sample Labelling section). Each filtration produces two sample replicates; therefore after 2 filtrations, 4 replicated samples will be produced. The first filtration (for example, filtration A) will produce replicates 1 and 2 when filter A is cut in half.
18. Apply transparent tape firmly on top of the label to avoid bleaching.
19. Transfer tubes into liquid N_2 for flash freezing and subsequently store at -80°C.
20. Close the tripod and clean the filtration system by passing 2 l of purified water (Milli-Q or Nanopure). Place new filters in the filtration tripods as described in the setting up section and close the tripods.
21. Repeat steps 1-15 once more to filter all the collected seawater. Each filtration produces two sample replicates; therefore after 2 filtrations, 4 replicated samples will be produced. The first filtration (for example, filtration A) will produce replicates 1 and 2 when filter A is cut in half. The second filtration (for example, filtration B) will produce replicates 3 and 4 when filter B is cut in half.

22. Store the filter pieces for replicates 3 and 4 in 5 ml cryotubes without the addition of DNA/RNA Shield. Replicates 3 and 4 will be stored for biobanking.
23. Label the tube appropriately using permanent marker (see Sample Labelling section).
24. Apply transparent tape firmly on top of the label to avoid bleaching.
25. Transfer tubes into liquid N_2 for flash freezing and subsequently store at -80°C.

F. Negative Controls

As samples undergo different procedures at the operating stations and at the centralized sequencing centre laboratories, it is important to check for contaminations. For this reason, blank samples will be collected and processed together with the actual samples to serve as negative controls. The collection of the blank samples will take place after the seawater filtrations to account for the cleaning of the filtration apparatuses between different samples. After the seawater filtration follow the steps bellow to collect and store the negative control sample.

1. After the second filtration, close the tripod and clean the filtration system by passing 2 l of purified water (Milli-Q or Nanopure).
2. Place new filters in the filtration tripods as described in the setting up section above (Setting up steps 1-4) and close the tripods.
3. Filter purified water (Milli-Q or Nanopure). The volume of the purified water (Milli-Q or Nanopure) to be filtered should be equal to the volume of seawater filtered for one filtration. For example, if an observatory has decided to sample and filter 10 l of seawater in one filtration sequence, then 10 l of purified water (Milli-Q or Nanopure) should be filtered for the negative control as well.
4. Follow filtration procedure as described in the seawater filtration section (E. Seawater Filtration and Sample Storage, steps 6-10).
5. Open tripod filter holders. Avoid touching the inside of the filter holders.
6. Fold immediately each filter piece four times and then fold again by rolling the filter like a cigarette paper.
7. Carefully place the filters into 5 ml cryotubes and gently push the filters to minimize the space they occupy in the tube. Do not crumble the filter, just try to gently fold it once more while in the tube.
8. Add 4.5 ml of DNA/RNA Shield by using a pipette and make sure the filter is covered by the solution
9. Label the tube appropriately using permanent marker (see Sample Labelling section).
10. Apply transparent tape firmly on top of the label to avoid bleaching.
11. Transfer tubes into liquid N_2 for flash freezing and subsequently store at -80°C.

Water Column Standard Operating Procedures 2 – WaSOP 2 (optional)

Summary

This protocol aims at collecting plankton organisms sized >20 µm. EMO BON observatories have different characteristics and may already follow specific routines for sampling plankton >20 µm. Therefore, observatories will be allowed to deviate from the WaSOP 2 as long the procedures followed are described in detail and adhere to the data as metadata.

Here we suggest vertical tows by a 20 µm mesh size plankton net from 100 m depth to the surface of the water column or from bottom to the water column surface in case of a shallower site. The material collected in the cod end should be diluted and separated into 4 subsamples. Each subsample should be filtered through 10 µm polycarbonate membrane filters (47 mm in diameter) under vacuum pressure. After filtration, membranes should be left to dry, carefully folded, placed in individual tubes, preserved in DNA/RNA Shield, and stored in liquid nitrogen or at -80oC until further analyses.

Filtration may initiate in the field immediately after sampling or after the transportation of sampled seawater to the laboratory. Samples should remain in a cool dark place during transportation and until filtration. It is important to be consistent to when the filtration takes place among the different sampling events, therefore observatories are expected to either always filter at the field or always transport water and filter it in the lab.

Observatories may deviate from the protocol and opt for horizontal instead of vertical tow, or by introducing prefiltration at different mesh sizes. Nevertheless, it is important that all procedures are documented in detail and kept consistent during EMOBON. For a diagram representation of the procedures see Appendix 2.

Protocol

A. Materials, equipment, and supplies

Before starting, ensure all materials equipment, and supplies are available by checking the following table (Table 3).

Table 3: List of materials, equipment, and supplies for WaSOP 2.
10% HCl
70% ethanol
Distilled water
0.2 µm prefiltered seawater
Purified water (Milli-Q or Nanopure)
Gloves
50 ml screw cap tubes
Aluminium foil
Wash bottles
Forceps
Vacuum pump
Filtration system (47 mm in diameter) [1] and appropriate filter units
Appropriate tubes (adjustable to the vacuum pump and the filtration system)
Polycarbonate hydrophilic membranes, 10 µm pore size, 47 mm in diameter (see Appendix 1)

20 µm plankton net
2 ml cryotubes
Permanent marker
Transparent tape
2000 µm mesh
Nalgene-type bottle of 1 l (sample container)
250 ml graduated containers
Funnel
DNA/RNA Shield
Pipette (500-1000 µl) and suitable tips
Access to liquid N_2
Access to -80°C
[1] The filtration system may differ from a filtration ramp to Duran-type pressure resistant glass bottles.

B. Preparation and Cleaning

- Always wear gloves
- Prepare 10% HCl.
- Disinfect the workspace using 10% HCl.
- Filter seawater at 0.2 µm to prepare 0.2-prefiltered seawater. Ideally this should be seawater from the sampling site.
- Prepare a wash bottle filled with 0.2-prefiltered seawater.
- Prepare a 50 ml screw cap tube filled with 10% HCl and a 50 ml screw cap tube filled with 0.2-prefiltered seawater. Soak forceps and scalpel in the HCl tube and then in the 0.2-prefiltered seawater tube before every use. Store forceps in the 10% HCl tube after use.
- Clean the filtration system:
  - Clean the filtration ramp or the Duran-type pressure resistant glass bottles with tap water and distilled water.
  - Dissasemble the filtration units and rinse with tap water, distilled water, and EtOH 70%. Allow them to dry before reassembling.
- Cleaning of the filtration system may also take place at the end of every sampling event. Make sure it was cleaned and stored properly before using it.
- Rinse the 20 µm plankton net vigorously inside and outside using tap water and make sure material from previous samplings is washed away. Cleaning of the plankton net may also take place at the end of every sampling event. Make sure it was cleaned and stored properly before using it.
- Clean the sample containers (Nalgene-type bottles of 1 l) using 250 ml of 10% HCl and incubate overnight. The following day wash twice using distilled water and twice using purified water (Milli-Q or Nanopure). Rinse vigorously to ensure all interior surfaces are cleaned and acid is washed away. Cleaning and decontamination of the sample containers may also take place at the and of every sampling event. Make sure the containers were cleaned and stored properly before using them.

- Rinse funnels vigorously with tap water and purified water (Milli-Q or Nanopure). Cleaning and decontamination of funnels may also take place at the and of every sampling event. Make sure that materials were cleaned and stored properly before using them.
- Coordinate the appropriate adjustment of the plankton net to the winch cable and the proper towing with the RV sailing personel.

C. Setting up

Set up the filtration system as described below. Filtration may take place in parallel depending on the available equipment. The filtration system used may differ from a filtration ramp to Duran-type pressure resistant glass bottles. Filtration units of 47 mm in diameter are adjusted on the ramp or on the bottles. Figure 6 depicts a filtration ramp and the 4 adjusted filtration units.

1. Open the 47 mm filtration unit(s).
2. Humidify the filter holder area using 0.2-prefiltered seawater.
3. Soak forceps in the HCl tube and then in the 0.2-prefiltered seawater tube before every use. Handle the filter with the forceps not by hand.
4. Place a PC filter membrane (10 µm pore size; 47 mm diameter) on the filtration unit. If more than one filtration units are available, for parallel filtration place a filter on each of the filtration units. Each PC filter is separated by 2 blue inter-filters in the filter boxes. Make sure to remove the blue inter-filters or the water will not go through. Filters are non-polarized and both sides can be used.
5. Close the filtration units and cover them using a glove or aluminium foil to avoid contaminations from air particles.
6. Connect the system to a vacuum pump using appropriate tubing system.
7. If a filtration ramp is used, place the water outlet tube in a waste container or in the sink.

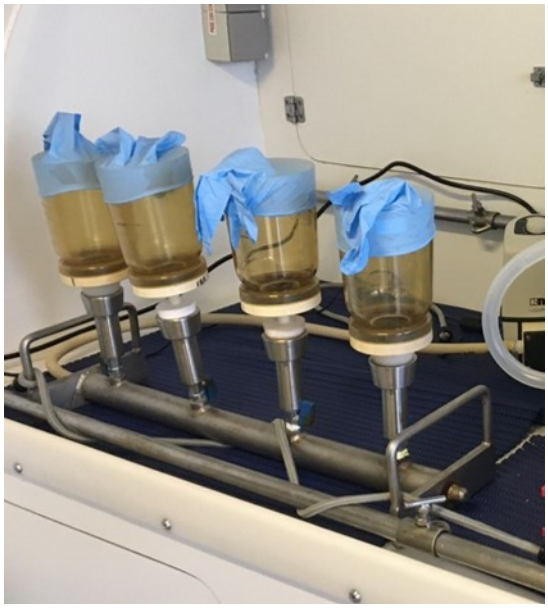

Figure 6: Set up of the WaSOP 2 and WaSOP 3 filtration equipment (filtration ramp and 47 mm filtration units) on the laboratory bench. The filtration system is connected to a vacuum pump and filtration takes place in parallel in the 4 filtration units.

D. Sample Collection

1. Perform a vertical tow from 100 m depth to the surface using a 20 µm plankton net. If the total water depth is less than 100 m, plankton net may be towed from the bottom to the surface of the water column (Figure 7).
2. Towing should be at a speed of ca. 0.5 m/s.
3. Once the plankton net is back on deck, rinse the net from the outside with seawater to concentrate collected plankton in the cod-end.
4. If the net is equipped with a flowmeter, record the sampled water volume.
5. Adjust a 2000 µm mesh on a funnel and place it on the Nalgene-type bottle mouth.

6. Transfer the plankton gently from the cod-end into a clean 1 l Nalgene-type bottle through the 2000 µm mesh. To ensure all material is collected, rinse vigorously using a wash bottles of 0.2 µm filtered seawater. Make sure the cod-end is rinsed well, especially the apertures on the sides (if any).
7. Gently fill the Nalgene-type bottle up to 1 l with 0.2-prefiltered seawater.
8. Mix the Nalgene-type bottle gently by rotating at least 5 times.
9. Separate the 1 l of seawater into 4 subsamples of 250 ml in separate graduated containers.

Filtration should initiate as soon as possible after sampling. Subsamples may be transported to the laboratory if filtration at the field is not possible. Keep each of the subsample containers in a dark and cool place until filtration.

E. Filtration and Sample Storage

1. Filter subsamples through 10 µm pore size PC filter membranes (47 mm in diameter).
2. Process each subsample in multiple filtration steps by adding 50 ml to the filtration tower in each step. This is because samples may be rich in plankton and clog the filter. Mix the Nalgene-type bottle gently for each of the 50 ml filtration steps. In case the filter clogs, stop the filtration and record the total volume filtered.
3. After filtration, allow filters to dry. Recover each membrane using forceps and fold membranes three times. Soak forceps in the HCl tube and then in the 0.2-prefiltered seawater tube before every use.
4. Place filter membrane into 2 ml cryotube.
5. Add 1.5 ml of DNA/RNA Shield by using a pipette and make sure the filter is covered by the solution.
6. Label the tube appropriately using permanent marker (see Sample Labelling section).
7. Apply transparent tape firmly on top of the label to avoid bleaching.
8. Transfer tubes into liquid N_2 for flash freezing and subsequently store into -80°C.
9. Repeat steps 1-8 for all 4 replicated subsamples. Vacuum filtrations of the replicates may also take place in parallel if appropriate filtration system is available.
10. For the third and fourth filter (replicates 3 and 4) skip step 5. Replicates 3 and 4 will be stored for biobanking and DNA/RNA Shield will not be used.

F. Negative Controls

As samples undergo different procedures at the operating stations and at the centralized sequencing centre laboratories, it is important to check for contaminations. For this reason, blank samples will be collected and processed together with the actual samples to serve as negative controls. The collection of the blank samples will take place after the seawater filtrations to account for the cleaning of the filtration apparatuses between different samples. After the seawater filtration follow the steps bellow to collect and store the negative control sample.

1. Filter 250 ml of purified water (Milli-Q or Nanopure) through a 10 µm pore size PC filter membrane (47 mm in diameter). Follow filtration procedure as described above.
2. After filtration, allow filter to dry. Recover the filter membrane using clean forceps and fold three times. Soak forceps in the HCl tube and then in the 0.2-prefiltered seawater tube before every use.
3. Place membrane into a 2 ml cryotube.

4. Add 1.5 ml of DNA/RNA Shield by using a pipette and make sure the filter is covered by the solution.
5. Label the tube appropriately using permanent marker (see Sample Labelling section).
6. Apply transparent tape firmly on top of the label to avoid bleaching.
7. Transfer tubes into liquid N_2 for flash freezing and subsequently store into -80°C.

Figure 7: Vertical tow onboard a RV. The WP2 net is clean and about to be

Water Column Standard Operating Procedures 3 – WaSOP 3 (optional)

Summary

This protocol aims at collecting plankton organisms sized >200 µm using WP2 plankton nets. EMO BON observatories have different characteristics and may already follow specific routines for sampling plankton >200 µm. Therefore, observatories will be allowed to deviate from the WaSOP 3 as long the procedures followed are described in detail and adhere to the data as metadata.

Here we suggest vertical tows by a 200 µm mesh WP2 plankton net from 100 m depth to the surface or from bottom to the water column surface in case of a shallower site. The material collected in the cod end should be diluted and separated into 4 subsamples. Each subsample should be filtered through 10 µm polycarbonate membrane filters (47 mm in diameter) under vacuum pressure. After filtration, membranes should be left to dry, carefully folded, placed in individual tubes, preserved in DNA/RNA Shield, and stored in liquid nitrogen or at -80°C until further analyses.

Filtration may initiate in the field immediately after sampling or following transportation of sampled seawater to the laboratory. Samples should remain in a cool dark place during transportation and until filtration. It is important to be consistent to when the filtration takes place among the different sampling events, therefore observatories are expected to either always filter at the field or always transport water and filter in the lab.

Observatories may deviate from the protocol and opt for horizontal instead of vertical tow, or by introducing prefiltration at different mesh sizes. Nevertheless, it is important that all procedures are documented in detail and kept consistent during EMOBON. For a diagram representation of the procedures see Appendix 2.

Protocol

A. Materials, equipment, and supplies

Before starting, ensure all materials equipment, and supplies are available by checking the following table (Table 4).

Table 4: List of materials, equipment, and supplies for WaSOP 3.
10% HCl
70% ethanol
Distilled water
0.2 µm prefiltered seawater
Purified water (Milli-Q or Nanopure)
Gloves
50 ml screw cap tubes
Aluminium foil
Wash bottles
Forceps
Vacuum pump
Filtration system (47 mm in diameter) and appropriate filter units [1]
Appropriate tubes (adjustable to the vacuum pump and the filtration system)
Polycarbonate hydrophilic membranes, 10 µm pore size, 47 mm in diameter (see Appendix 1)

200 µm WP2 plankton net
2 ml cryotubes
Permanent marker
Transparent tape
Nalgene-type bottle of 1 l (sample container)
250 ml graduated containers
Funnel
DNA/RNA Shield
Pipette (500-1000 µl) and suitable tips
Access to liquid N_2
Access to -80°C
[1] The filtration system may differ from a filtration ramp to Duran-type pressure resistant glass bottles.

B. Preparation and Cleaning

- Always wear gloves
- Prepare 10% HCl.
- Disinfect the workspace using 10% HCl.
- Filter seawater at 0.2 µm to prepare 0.2-prefiltered seawater. Ideally this should be seawater from the sampling site.
- Prepare a wash bottle filled with 0.2-prefiltered seawater.
- Prepare a 50 ml screw cap tube filled with 10% HCl and a 50 ml screw cap tube filled with 0.2-prefiltered seawater. Soak forceps and scalpel in the HCl tube and then in the 0.2-prefiltered seawater tube before every use. Store forceps in the 10% HCl tube after use.
- Clean the filtration system:
- Clean the filtration ramp or the Duran-type pressure resistant glass bottles with tap water and distilled water.
- Dissasemble the filtration units and rinse with tap water, distilled water, and EtOH 70%. Allow them to dry before reassembling.
- Cleaning of the filtration system may also take place at the end of every sampling event. Make sure it was cleaned and stored properly before using it.
- Rinse the 200 µm WP2 plankton net vigorously inside and outside using tap water and make sure material from previous samplings is washed away. Cleaning of the plankton net may also take place at the end of every sampling event. Make sure it was cleaned and stored properly before using it.
- Clean the sample containers (Nalgene-type bottles of 1 l) using 250 ml of 10% HCl and incubate overnight. The following day wash twice using distilled water and twice using purified water (Milli-Q or Nanopure). Rinse vigorously to ensure all interior surfaces are cleaned and acid is washed away. Cleaning and decontamination of the sample containers may also take place at the and of every sampling event. Make sure the containers were cleaned and stored properly before using them.
- Rinse funnels vigorously with tap water and purified water (Milli-Q or Nanopure). Cleaning and decontamination of funnels may also take place at the and of every sampling event. Make sure that materials were cleaned and stored properly before using them.

- Coordinate the appropriate adjustment of the plankton net to the winch cable and the proper towing with the RV sailing personnel.

C. Setting up

Set up the filtration system as described below. Filtration may take place in parallel depending on the available equipment. The filtration system used may differ from a filtration ramp to Duran-type pressure resistant glass bottles. Filtration units of 47 mm in diameter are adjusted on the ramp or on the bottles. Figure 6 depicts a filtration ramp and the 4 adjusted filtration units.

1. Open the 47 mm filtration unit(s).
2. Humidify the filter holder area using 0.2-prefiltered seawater.
3. Soak forceps in the HCl tube and then in the 0.2-prefiltered seawater tube before every use. Handle the filter with the forceps not by hand.
4. Place a PC filter membrane (10 µm pore size; 47 mm diameter) on the filtration unit. If more than one filtration units are available, for parallel filtration place a filter on each of the filtration units. Each PC filter is separated by 2 blue inter-filters in the filter boxes. Make sure to remove the blue inter-filters or the water will not go through. Filters are non-polarized and both sides can be used.
5. Close the filtration units and cover them using a glove or aluminium foil to avoid contaminations from air particles.
6. Connect the system to a vacuum pump using appropriate tubing system.
7. If a filtration ramp is used, place the water outlet tube in a waste container or in the sink.

D. Sample Collection

1. Perform a vertical tow from 100 m depth to surface using a WP2 200 µm plankton net. If the total water depth is less than 100 m, plankton net may be towed from the bottom to the surface of the water column (Figure 7).
2. Towing should be at a speed of ca. 0.5 m/s.
3. Once the plankton net is back on deck, rinse the net from the outside with seawater to concentrate collected plankton to the cod-end.
4. If the net is equipped with a flowmeter, record the sampled water volume.
5. Transfer the plankton gently from the cod-end into a clean 1 l Nalgene-type bottle through the 2000 µm mesh. To ensure all material is collected, rinse vigorously using a wash bottles of 0.2 µm filtered seawater. Make sure the cod-end is rinsed well, especially the apertures on the sides (if any).
6. Gently fill the Nalgene-type bottle up to 1 l with 0.2-prefiltered seawater.
7. Mix the Nalgene-type bottle gently by rotating at least 5 times.
8. Separate the 1 l of seawater into 4 subsamples of 250 ml in separate graduated containers.
9. Filtration should initiate as soon as possible after sampling. Subsamples may be transported to the laboratory if filtration at the field is not possible. Keep each of the subsample containers in a dark and cool place until filtration.

E. Filtration and Sample Storage

1. Filter subsamples through 10 µm pore size PC filter membranes (47 mm in diameter). Filtration should proceed fast.

2. After filtration, allow filters to dry. Recover each membrane using forceps and fold membranes three times. Soak forceps in the HCl tube and then in the 0.2-prefiltered seawater tube before every use.
3. Place membranes into 2 ml cryotubes.
4. Add 1.5 ml of DNA/RNA Shield by using a pipette and make sure the filter is covered by the solution.
5. Label the tube appropriately using permanent marker (see Sample Labelling section).
6. Apply transparent tape firmly on top of the label to avoid bleaching.
7. Transfer tubes into liquid N_2 for flash freezing and subsequently store into -80°C.
8. Repeat steps 1-7 for all 4 replicated subsamples. Vacuum filtrations of the replicates may also take place in parallel if appropriate filtration system is available.
9. For the third and fourth filter (replicates 3 and 4) skip step 5. Replicates 3 and 4 will be stored for biobanking and DNA/RNA Shield will not be used.

F. Negative Controls

As samples undergo different procedures at the operating stations and at the centralized sequencing centre laboratories, it is important to check for contaminations. For this reason, blank samples will be collected and processed together with the actual samples to serve as negative controls. The collection of the blank samples will take place after the seawater filtrations to account for the cleaning of the filtration apparatuses between different samples. After the seawater filtration follow the steps bellow to collect and store the negative control sample.

1. Filter 250 ml of purified water (Milli-Q or Nanopure) through a 10 μm pore size PC filter membrane (47 mm in diameter). Follow filtration procedure as described above.
2. After filtration, allow filter to dry. Recover the filter membrane using clean forceps and fold three times. Soak forceps in the HCl tube and then in the 0.2-prefiltered seawater tube before every use.
3. Place membrane into a 2 ml cryotube.
4. Add 1.5 ml of DNA/RNA Shield by using a pipette and make sure the filter is covered by the solution.
5. Label the tube appropriately using permanent marker (see Sample Labelling section).
6. Apply transparent tape firmly on top of the label to avoid bleaching.
7. Transfer tubes into liquid N_2 for flash freezing and subsequently store into -80°C.

Soft Substrate

Soft Substrate Standard Operating Procedures 1 – SoSOP 1 (intertidal sediments)

Summary

Operating stations that have access to intertidal sediments are encouraged to choose such habitats as soft substrate sampling sites. Intertidal soft substrates will be sampled during low tides using sediment corers to ensure the collection of undisturbed sediment. Sediment corers are recommended to be at least 10 cm in diameter so that adequate sediment is collected. The top 5 cm of sediment will be sliced and sampled for the collection of microorganisms and meiobenthos. For the collection of macrobenthos, all the sediment in the corers will be used. For each community sampled, 4 replicated samples should be collected. These replicates are designed to represent technical replicates and will be used for different purposes (more information on the Replication and BiobankingReplication and BiobankingReplication and BiobankingReplication and Biobanking section).

The finer the sampled sediment is, the more care should be given during handling. For example, silty sediment might leak when the corer is retrieved from the sediment. In that case, corers handling should be performed quickly. In case operating stations find it impossible to use sediment corers in their site of choice, it will also be acceptable to use other means for sediment collection. For example, a shovel might be used to dig out sediment from a defined area, or metal frames could define the area from where the sediment will be collected. However, all deviations from the present protocols should be explicitly described and follow the samples as metadata.

Sediment slicing or sieving may initiate in the field immediately after sampling or following the transportation of the sediment corers to the laboratory. Sediment corers should remain cool and in the dark during transportation and always be kept in a vertical position as retrieved from the field. It is important to be consistent to when the slicing or sieving takes place among the different sampling events, therefore observatories are expected to either always process samples in the field or always transport corers and process in the lab.

Before shipment for DNA extraction and sequencing, the meiobenthic organisms should be extracted from the sediment. After the meiobenthos extraction, the meiobenthic organisms will be resuspended in the preservative DNA/RNA shield for shipment.

Sediment collected for macrobenthos sampling will have to be sieved through a 1 mm sieve. Some marine areas, such as the Eastern Mediterranean, or polluted sediments are characterized by smaller macrobenthic organisms. Using a 1 mm sieve alone would result to missing a large part of the macrobenthos for those areas. In such cases, sequential sieving using both 1 mm and 0.5 mm sieves should take place. The organisms retained in the 1 mm and in the 0.5 mm sieves will be handled and stored separately as different fractions. After sieving all organisms will be crushed and homogenized using mortar and pestle to obtain a mixture of tissues. For a diagram representation of the procedures see Appendix 2.

Protocol

A. Materials, equipment, and supplies

Before starting, ensure all materials equipment, and supplies are available by checking the following table (Table 5).

Table 5: List of materials, equipment, and supplies for SoSOP 1 (intertidal sediments).
Bleach solution
Distilled water
Purified water (Milli-Q or Nanopure)
Gloves
Aluminium foil
Plexiglass or PVC sediment corers of at least 10 cm in diameter (microbial and meiobenthos community) [1]
Appropriate rubber stoppers for the 10 cm sediment corers (microbial and meiobenthos community)
Plexiglass or PVC sediment corers of at least 20 cm in diameter (macrobenthos community) [1]
Appropriate rubber stoppers for the 20 cm sediment corers (macrobenthos community)
Spatula and/or spoon
Plunger
Clean surface for mixing the sediment (e.g., large Petri dish or plastic bowl)
Pipette (1000-5000 µl) and pre-cut 5000 µl tips (optional for silty sediments)
1 mm sieve (macrobenthos community)
0.5 mm sieve (optional; macrobenthos community)
Mortar and pestle (macrobenthos community)
15 ml screw cap tubes resistant to liquid N_2 (microbial community)
Ziplock bags (meiobenthos community)
50 ml screw cap tubes (macrobenthos community)
Permanent marker
Transparent tape
Laboratory balance
DNA/RNA Shield (microbial and meiobenthos community)
DESS aqueous solution (macrobenthos community)
Pipette (500-1000 µl) and suitable tips
Cotton swabs
Access to liquid N_2 (microbial community)
Access to -80°C (microbial community)
Access to -20°C (meiobenthos community)
Access to 4°C (macrobenthos community)
[1] Alternatively, a multicorer may be used to collect several sediment corers simultaneously.

B. Preparation and Cleaning

- Always wear gloves.
- Prepare 10% bleach solution.
- Prepare DESS aquaous solution (see DESS recipe).
- Clean sediment corers and sieves using tap water. Rinse vigorously and make sure material from previous samplings is washed away.
- Desinfect plastic material (large Petri dish and/or plastic bowl) by incubating in 10% bleach solution overnight. Rinse with purified water (Milli-Q or Nanopure) to wash the solution away and cover in aluminium foil to store.
- Desinfect spatulas, spoons, and other metal tools by briefly soaking in 10% bleach solution. Rinse with purified water (Milli-Q or Nanopure) to wash the solution away and cover in aluminium foil to store.

C. Negative Control Samples

As samples undergo different procedures at the operating stations and at the centralized sequencing centre laboratories, it is important to check for contaminations. For this reason, blank samples will be collected and processed together with the sediment samples to serve as negative controls. Once the sediment is collected and before further processing, collect and store a negative control sample as described below:

Microbial community negative control

1. Use a cotton swab on the lab bench, the spatulas, and the spoons.
2. Cut the cotton part of the swab and store it in a 15 ml screw cap tube.
3. Add 10 ml of DNA/RNA Shield by using a pipette.
4. Label the tube appropriately using permanent marker (see Sample Labelling section).
5. Apply transparent tape firmly on top of the label to avoid bleaching.
6. Transfer tube into liquid N_2 for flash freezing and subsequently store at -80°C.

Meiobenthos community negative control

1. Use a cotton swab on the lab bench, the spatulas, and the spoons.
2. Cut the cotton part of the swab and store it in a Ziplock bag.
3. Label the tube appropriately using permanent marker (see Sample Labelling section).
4. Apply transparent tape firmly on top of the label to avoid bleaching.
5. Store the bag at -20°C.
6. Process the negative control sample together with the sediment samples during the meiobenthos extraction from sediment.

Macrobenthos community negative control

1. Use a cotton swab on the spatulas, the spoons, the sieves and the mortar and pestle.
2. Cut the cotton part of the swab and store it in a 50 ml screw cap tube.
3. Add 20 ml of DESS (see DESS recipe).
4. Label the tube appropriately using permanent marker (see Sample Labelling section).

5. Apply transparent tape firmly on top of the label to avoid bleaching.
6. Store at 4°C.

D. Microbial Community Sampling

1. Reach the sampling site during low tide.
2. Deploy 2 plexiglass or PVC sediment corers of at least 10 cm in diameter. Deploy the different sediment corers in proximity and ensure that the sediment surface is not visually different among the deployment points.
3. Use appropriate rubber stop to close the upper end of the corer while deployed in the sediment to create vacuum.
4. Gently bring the corer to surface and place a rubber stop to its lower end. If sampling silt or clay sediment, retrieving corers from the sediment will need extra care as sediment may leak. In that case, the bottom end rubber stop should be placed quickly or, if possible, while the corer is still deployed in the sediment. In case, it seems difficult to adjust the lower stop, loosen the upper stop slightly, adjust the lower stop, and then firmly close the upper stop again.
5. If available, a multicorer may be used to collect several sediment corers simultaneously (Figure 8). In that case, all the necessary corers for microorganisms, meiobenthos and macrobenthos may be collected during the multicorer deployment.
6. Ideally, the sediment core slicing should take place at the sampling site directly after collection. If this is not possible, corers should be kept in a cool and dark environment until transportation to the lab. Open the top rubber stop during transportation to prevent oxygen consumption which may alter the microbial community. In any case, handle sediment corers carefully and always keep them vertically (surface sediment on top) to avoid mixing surface to deep sediment (Figure 9).
7. Place a plunger in a sediment corer, push the corer to remove seawater and set the sediment at the surface of the corer.
8. Push the corer to 6 cm and slice sediment at the top 5 cm and using a spoon and spatula place the slice on a clean surface (e.g., Petri dish or plastic bowl).
9. Repeat steps 7-8 for both collected sediment corers and place the sediment slices on the same surface.
10. Gently homogenize sediment using a spoon or a spatula.
11. Pre-weigh a 15 ml screw cap tube and using a spoon and spatula transfer 10 g of sediment in the tube. An alternative, though less accurate, method would be to fill the 15 ml graduated screw cap tubes to approximately 10 ml tube indication. In the case of silty sediment, pre-cut 5000 µl tips might be used to transfer the sediment in the screw cap tubes by careful pipetting. Make sure that the 15 ml screw cap tubes used are resistant to liquid N_2.
12. Fill 4 screw cap tubes with the homogenized sediment.
13. Add 10 ml of DNA/RNA Shield by using a pipette and make sure the sediment is covered by the solution. For the third and fourth tube of replicate samples skip this step. Replicates 3 and 4 will be stored for biobanking and DNA/RNA Shield will not be used.
14. Label the tube appropriately using permanent marker (see Sample Labelling section).
15. Apply transparent tape firmly on top of the label to avoid bleaching.

E. Meiobenthos Community Sampling

1. Reach the sampling site during low tide.
2. Deploy 4 plexiglass or PVC sediment corers of at least 10 cm in diameter. Deploy the different sediment corers in proximity and ensure that the sediment surface is visually similar among the deployment points.
3. Follow steps 3-10 above (Microbial Community Sampling), for deploying and retrieving the corers.
4. Distribute the homogenised sediment into 4 plastic Ziplock bags and weigh the bags. Flatten the bags to allow stacking of samples.
5. Label the Ziplock bags appropriately using permanent marker (see Sample Labelling section) and apply transparent tape firmly on top of the label to avoid bleaching.
6. Store bags at -20°C. Sediment can be stored at -20°C safely for 2 years until meiobenthos extraction from sediment.

F. Meiobenthos Extraction from Sediment

Extract meiobenthos from the sampled sediment into purified water (Milli-Q or Nanopure) by density-gradient centrifugation with the colloidal silica polymer Ludox HS-40 as a flotation medium, or equivalent (specific density 1.18 g cm−1). The meiobenthos extraction from sediment may take place any time after sampling and within 2 years. Before starting, ensure all materials equipment, and supplies are available by checking the following table (Table 6).

Table 6: List of materials, equipment, and supplies for meiobenthos extraction from sediment.

Purified water (Milli-Q or Nanopure)
Gloves
50 ml centrifuge tubes
Colloidal silica polymer Ludox HS-40
32 µm sieve
Funnel
Spoon and/or spatula
Wash bottle
Centrifuge
Isothermal box with ice
Pipette (10 ml) and suitable tips
Pipette (100-1000 µl) and suitable tips
1.5 ml centrifuge tube
Permanent fine-tip marker
Transparent tape
DNA/RNA Shield
Access to -20°C

1. Bring sediment Ziplock bags at room temperature.
2. Remove sediment from Ziplock bag and transfer onto 32 µm sieve.
3. Gently flush with water to thaw and reduce sediment volume.
4. Using a funnel and a wash bottle containing the Ludox (or equivalent) transfer the sample from the sieve into the large centrifuge tube and homogenize using a spoon or spatula.
5. Initiate first round of centrifugation (12 min, 1905 rcf, slow brake and acceleration). During centrifugation sediment will reside at the bottom of the large centrifuge tube while meiobenthos will move into the Ludox solution.
6. Once centrifugation is completed, pour supernatant over 32 µm sieve; using a funnel and a wash bottle containing purified water (Milli-Q or Nanopure) transfer the sample into the 50 ml centrifuge tube trying to minimise the volume of water used. Retain this tube on ice in an isothermal box for the duration of the meiobenthos extraction.
7. Re-fill the large centrifuge tube with Ludox and homogenize with a spoon or spatula.
8. Repeat for two more centrifugation rounds.
9. At the last round, pour the contents of the 50 ml centrifuge tube and that of the large centrifuge tube over the 32 µm sieve and subsequently transfer the entire sample back into the 50 ml centrifuge tube using a funnel and wash bottle containing purified water (Milli-Q or Nanopure). This tube now contains the meiobenthos.
10. Centrifuge this tube for 10 min at 1905 rcf (slow brake and acceleration), and carefully remove the supernatant using 10 ml pipette.
11. Resuspend the pellet containing the meiobenthos with 500 µl of DNA/RNA Shield and transfer the sample to a 1.5 ml centrifuge tube.
12. For replicates 3 and 4 resuspend the pellet containing the meiobenthos with 500 µl of purified water (Milli-Q or Nanopure) and transfer the sample to a 1.5 ml centrifuge tube. Replicates 3 and 4 will be stored for biobanking and DNA/RNA Shield will not be used.
13. Label the tubes appropriately using permanent marker (see Sample Labelling section).
14. Apply transparent tape firmly on top of the label to avoid bleaching.
15. Samples are now ready for DNA extraction. Store samples at -20°C until shipment.

G. Macrobenthos Sampling

1. Reach the sampling site during low tide.
2. Deploy 5 plexiglass or PVC sediment corers of at least 20 cm in diameter. Alternatively, deploy 10 plexiglass or PVC sediment corers of at least 10 cm in diameter. Deploy the different sediment corers in proximity and ensure that the sediment surface is not visually different among the deployment points.
3. Follow steps 3-5 above (Microbial Community Sampling), for deploying and retrieving the corers.
4. Place the total sediment in a 1 mm sieve and sieve it (see step 4 for sequential sieving). Make sure that the amount of remaining sediment in the sieve is minimized as much as possible.
5. Optional: Perform sequential sieving. This is recommended for stations that usually use smaller sieve size for macrobenthos collection. Place a 1 mm sieve on top of a 0.5 mm sieve and sieve sediment sequentially through both sieves. This way the material that flows through the 1 mm sieve will be

sequentially sieved through the 0.5 mm sieve. Make sure that the amount of remaining sediment in both sieves is minimized as much as possible.

6. Transfer macrobenthic organisms retained in the sieve(s) in a mortar for homogenization. The remaining sediment that may be transferred with the organisms will be removed during the DNA extraction. Sediment homogenization may take place in the field directly after sieving. If this is not possible, macrobenthic organisms may be collected in tubes and kept in a cool and dark environment until transportation to the lab.
7. Use mortar and pestle to crush and homogenize organisms until a tissue mixture is obtained.
8. Transfer 10 g of the tissue mixture into a screw cup tube of 50 ml. Repeat this to obtain 4 screw cup tubes of 50 ml each containing 10 g of the tissue mixture.
9. Fill tubes to 50 ml with DESS (see DESS recipe).
10. Label the tubes appropriately using permanent marker (see Sample Labelling section).
11. Apply transparent tape firmly on top of the label to avoid bleaching.
12. Store tubes at 4°C.

Figure 8: Multicorer deployment in an intertidal area.

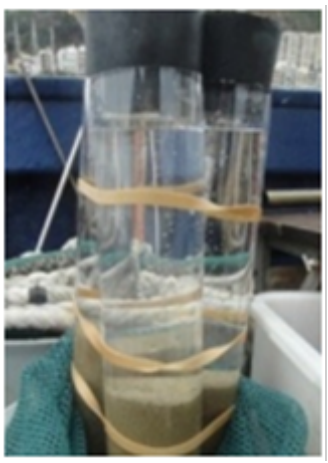

Figure 9: Example of corers that contain soft sediment and overlaying seawater.

Soft substrate Standard Operating Procedures 2 – SoSOP 2 (coastal sediments by diving)

Summary

If there is the capacity, operating stations may choose to sample coastal soft sediment by diving. This will lead to the collection of undisturbed sediment and would increase the precision of the sediment layers collected. Soft substrate sampling by diving will be performed using sediment corers as described above for the intertidal sediments [**SoSOP 1 (intertidal sediments)**].

Slicing and further handling of the sediment will take place upon retrieval of the sediment corers to the surface and transfer to a laboratory either onshore or onboard a research vessel. Sediment corers should remain cool and in the dark during transportation and always kept in a vertical position as retrieved from the field. During transportation, the corers should be open at the top to prevent oxygen consumption which may alter the microbial community composition. It is important to be consistent to when the slicing or sieving takes place among the different sampling events, therefore observatories are expected to either

always process samples in the field or always transport corers and process in the laboratory. For a diagram representation of the procedures see Appendix 2.

Protocol

Upon divers reach the sampling site, follow the Standard Operating Procedures described in SoSOP 1 (intertidal sediments). It is recommended to use plexiglass or PVC sediment corers of at least 10 cm in diameter for the macrobenthos as they are easier handled by the diver. Sediment core slicing and sediment sieving should be carried out after diving either directly in the field or after transportation to the lab. For a diagram representation of the procedures see Appendix 2.

Soft substrate Standard Operating Procedures 3 – SoSOP 3 (coastal sediments by research vessel)

Summary

Observatories may choose to sample coastal soft substrate onboard a research vessel. Soft substrate will be collected using a van Veen or a Smith-McIntyre grab (0.07–0.1 m^2) at the selected site. Sediment collection by grab is suitable for different types of sediment (clay to sandy sediments). The sediment should be brought onboard on a clean surface for subsampling. As the sediment collected in the grab is disturbed, subsampling should be performed carefully, and materials should not be immersed deep into the sediment to ensure the subsampling of surface sediment as much as possible. For each community sampled, 4 replicated samples should be collected. These replicates are designed to represent technical replicates and will be used for different purposes (more information on the Sample Labelling section). Sediment processing will take place onboard after collection.

In case other sediment sampling equipment is available on the research vessels, as for example a multicorer, a box corer or a multi-box corer, the observatories may use this equipment to collect sediment from the field. Once the sediment is brought onboard, proceed with handling the sediment according to SoSOP 1 (intertidal sediments) if a multicorer was used, or proceed with sampling the top sediment as described below (SoSOP 3).

Before shipment for DNA extraction and sequencing, the meiobenthic organisms should be extracted from the sediment. After the meiobenthos extraction, the meiobenthic organisms will be resuspended in the preservative DNA/RNA shield for shipment.

Sediment collected for macrobenthos sampling will have to be sieved through a 1 mm sieve. Some marine areas, such as the Eastern Mediterranean, or polluted sediments are characterized by smaller macrobenthic organisms. Using a 1 mm sieve alone would result to missing a large part of the macrobenthos for those areas. In such cases, sequential sieving using both 1 mm and 0.5 mm sieves will take place. The organisms retained in the 1 mm and in the 0.5 mm sieves will be handled and stored separately as different fractions. After sieving all organisms will be crushed and homogenized using mortar and pestle to obtain a mixture of tissues. For a diagram representation of the procedures see Appendix 2.

Protocol

A. **Materials, equipment, and supplies**

Before starting, ensure all materials equipment, and supplies are available by checking the following table (Table 7).

Table 7: List of materials, equipment, and supplies for SoSOP 3 (research vessel).
Bleach solution
Distilled water
Purified water (Milli-Q or Nanopure)
Gloves
Van Veen or Smith McIntyre grab (0.07–0.1 m^2) [1]
10 ml cut-off syringes
Spatula and/or spoon
Clean surface for mixing the sediment (e.g., large Petri dish or plastic bowl)
Pipette (1000-5000 µl) and pre-cut 5000 µl tips (optional for silty sediments)
1 mm sieve (macrobenthos community)
0.5 mm sieve (optional; macrobenthos community)
Mortar and pestle (macrobenthos community)
15 ml screw cap tubes resistant to liquid N_2 (microbial community)
Ziplock bags (meiobenthos community)
50 ml screw cap tubes (macrobenthos community)
Permanent marker
Transparent tape
Laboratory balance
DNA/RNA Shield (microbial and meiobenthos community)
DESS aqueous solution (macrobenthos community)
Pipette (500-1000 µl) and suitable tips
Cotton swabs
Access to liquid N_2 (microbial community)
Access to -80°C (microbial community)
Access to -20°C (meiobenthos community)
Access to 4°C (macrobenthos community)
[1] Alternatively, a multicorer, a box corer or a multi-box corer may be used to collect sediment and bring it onboard.

B. Preparation and Cleaning

- Always wear gloves when handling samples dedicated to microbial community analyses.
- Prepare 10% bleach solution.
- Prepare DESS aquaous solution (see DESS recipe).
- Prepare 10 ml cut-off syringes for subsampling. Use a scalpel to cut off the luer lock end of the syringe so as the suringe is open at this end.
- Clean sediment grab, shovel, bucket and sieves using tap water. Rinse vigorously and make sure material from previous samplings is washed away.

- Desinfect plastic materials (cut-off syringes, large Petri dish and/or plastic bowl) by incubating in 10% bleach solution overnight. Rinse with purified water (Milli-Q or Nanopure) to wash the solution away and cover in aluminium foil to store.
- Desinfect spatulas, spoons, and other metal tools by briefly soaking in 10% bleach solution. Rinse with purified water (Milli-Q or Nanopure) to wash the solution away and cover in aluminium foil to store.

C. Negative Control Samples

As samples undergo different procedures at the operating stations and at the centralized sequencing centre laboratories, it is important to check for contaminations. For this reason, blank samples will be collected and processed together with the sediment samples to serve as negative controls. Once the sediment is collected and before further processing, collect and store a negative control sample as described below:

Microbial community negative control

1. Use a cotton swab on the lab bench, the spatulas, and the spoons.
2. Cut the cotton part of the swab and store it in a 15 ml screw cap tube.
3. Add 10 ml of DNA/RNA Shield by using a pipette.
4. Label the tube appropriately using permanent marker (see Sample Labelling section).
5. Apply transparent tape firmly on top of the label to avoid bleaching.
6. Transfer tube into liquid N_2 for flash freezing and subsequently store at -80°C.

Meiobenthos community negative control

1. Use a cotton swab on the lab bench, the spatulas, and the spoons.
2. Cut the cotton part of the swab and store it in a Ziplock bag.
3. Label the tube appropriately using permanent marker (see Sample Labelling section).
4. Apply transparent tape firmly on top of the label to avoid bleaching.
5. Store the bag at -20°C.
6. Process the negative control sample together with the sediment samples during the meiobenthos extraction from sediment.

Macrobenthos community negative control

1. Use a cotton swab on the spatulas, the spoons, the sieves and the mortar and pestle.
2. Cut the cotton part of the swab and store it in a 50 ml screw cap tube.
3. Add 20 ml of DESS (see DESS recipe).
4. Label the tube appropriately using permanent marker (see Sample Labelling section).
5. Apply transparent tape firmly on top of the label to avoid bleaching.
6. Store at 4°C.

D. Microbial Community Sampling

1. Collect soft sediment using a van Veen or a Smith-McIntyre grab (0.07–0.1 m^2) equipped on the research vessel (Figure 10).

2. Bring sediment onboard on a clean surface for subsampling. In case a subsampling port is present at the top of the grab, perform subsampling from that port before the grab is opened.
3. Use 10 ml cut-off syringes to subsample for the sediment microbial community. Collect about 10 times a rather full 10 ml cut-off syringe from the same grab.
4. Transfer the sediment collected in the syringes on a clean surface (e.g., Petri dish or plastic bowl) and homogenize gently using a spoon or a spatula.
5. Pre-weigh a 15 ml screw cap tube and using a spoon and spatula transfer 10 g of sediment in the tube. An alternative, though less accurate, method would be to fill the 15 ml graduated screw cap tubes to approximately 10 ml tube indication. In the case of silty sediment, pre-cut 5000 µl tips might be used to transfer the sediment in the screw cap tubes by careful pipetting. Make sure that the 15 ml screw cap tubes used are resistant to liquid N_2.
6. In total, fill 4 screw cap tubes with the homogenized sediment.
7. Add 10 ml of DNA/RNA Shield by using a pipette and make sure the sediment is covered by the solution. For the third and fourth tube of replicate samples skip this step. Replicates 3 and 4 will be stored for biobanking and DNA/RNA Shield will not be used.
8. Label the tube appropriately using permanent marker (see Sample Labelling section).
9. Apply transparent tape firmly on top of the label to avoid bleaching.
10. Add 5 ml of Purified water (Milli-Q or Nanopure) in a 15 ml screw cap tube. Briefly dip a clean spatula in the tube (clean and disinfected as described in the Preparation and Cleaning
11. section above). Add 10 ml of DNA/RNA Shield by using a pipette. This tube will be used as negative control sample.
12. Label the tube appropriately using permanent marker (see Sample Labelling section).
13. Apply transparent tape firmly on top of the label to avoid bleaching.
14. Transfer tubes into liquid N_2 for flash freezing and subsequently store into -80°C.

E. Meiobenthos Community Sampling

1. Collect soft sediment using a van Veen or a Smith-McIntyre grab (0.07–0.1 m^2) equipped at the research vessel (Figure 10).
2. Bring sediment onboard on a clean surface for subsampling.
3. Use a shovel to transfer approximately half of the grabbed sediment in a clean bucket.
4. Gently homogenize the sediment in the bucket by stirring it with a large spoon or spatula.
5. Deploy a plexiglass or PVC sediment corer of 3.6 cm in diameter into the sediment.
6. Transfer the sediment into a plastic Ziplock bag and weigh it. Flatten the bag before placing it into the freezer to allow stacking of samples.
7. Repeat steps 5-6 to collect 4 subsamples in total.
8. Label the Ziplock bags appropriately (see Sample Labelling section).
9. Store the bags at -20°C. Sediment can be stored at -20°C safely for at least 2 years until meiobenthos extraction.

F. Meiobenthos Extraction from Sediment

Extract meiobenthos from the sampled sediment into purified water (Milli-Q or Nanopure) by density-gradient centrifugation with the colloidal silica polymer Ludox HS-40 as a flotation medium, or equivalent (specific density 1.18 g cm−1). The meiobenthos extraction from sediment may take place any time after sampling and within 2 years. Before starting, ensure all materials equipment, and supplies are available by checking the following table (Table 8).

Table 8: List of materials, equipment, and supplies for meiobenthos extraction from sediment.
Purified water (Milli-Q or Nanopure)
Gloves
50 ml centrifuge tubes
Colloidal silica polymer Ludox HS-40
32 µm sieve
Funnel
Spoon and/or spatula
Wash bottle
Centrifuge
Isothermal box and ice
Pipette (10 ml) and suitable tips
Pipette (100-1000 µl) and suitable tips
1.5 ml centrifuge tube
Permanent fine-tip marker
Transparent tape
DNA/RNA Shield
Access to -20°C

1. Bring sediment Ziplock bags at room temperature.
2. Remove sediment from Ziplock bag and transfer onto 32 µm sieve.
3. Gently flush with water to thaw and reduce sediment volume.
4. Using a funnel and a wash bottle containing the Ludox (or equivalent) transfer the sample from the sieve into the large centrifuge tube and homogenize using a spoon or spatula.
5. Initiate first round of centrifugation (12 min, 1905 rcf, slow brake and acceleration). During centrifugation sediment will reside at the bottom of the large centrifuge tube while meiobenthos will move into the Ludox solution.
6. Once centrifugation is completed, pour supernatant over 32 µm sieve; using a funnel and a wash bottle containing purified water (Milli-Q or Nanopure) transfer the sample into the 50 ml centrifuge tube trying to minimise the volume of water used. Retain this tube on ice in an isothermal box for the duration of the meiobenthos extraction.
7. Re-fill the large centrifuge tube with Ludox and homogenize with a spoon or spatula.
8. Repeat for two more centrifugation rounds.

9. At the last round, pour the contents of the 50 ml centrifuge tube and that of the large centrifuge tube over the 32 µm sieve and subsequently transfer the entire sample back into the 50 ml centrifuge tube using a funnel and squeezing bottle containing purified water (Milli-Q or Nanopure). This tube now contains the meiobenthos.
10. Centrifuge this tube for 10 min at 1905 rcf (slow brake and acceleration), and carefully remove the supernatant using 10 ml pipette.
11. Resuspend the pellet containing the meiobenthos with 500 µl of DNA/RNA Shield and transfer the sample to a 1.5 ml centrifuge tube.
12. For replicates 3 and 4 resuspend the pellet containing the meiobenthos with 500 µl of purified water (Milli-Q or Nanopure) and transfer the sample to a 1.5 ml centrifuge tube. Replicates 3 and 4 will be stored for biobanking and DNA/RNA Shield will not be used.
13. Label the tubes appropriately using permanent marker (see Sample Labelling section).
14. Apply transparent tape firmly on top of the label to avoid bleaching.
15. Samples are now ready for DNA extraction. Store samples at -20°C until shipment.

G. Macrobenthos Sampling

1. Collect soft sediment using a van Veen or a Smith-McIntyre grab (0.07–0.1 m^2) equipped at the research vessel (Figure 10).
2. Bring sediment onboard and place it in a 1 mm sieve (see step 4 for sequential sieving).
3. Sieve sediment. Make sure that the amount of remaining sediment in the sieve is minimized as much as possible.
4. Optional: Perform sequential sieving. This is recommended for stations that usually use smaller sieve size for macrobenthos collection. Place a 1 mm sieve on top of a 0.5 mm sieve and sieve sediment sequentially through both sieves. This way the material that flows through the 1 mm sieve will be sequentially sieved through the 0.5 mm sieve. Make sure that the amount of remaining sediment in both sieves is minimized as much as possible.
5. Transfer macrobenthic organisms retained in the sieve(s) in a mortar for homogenization. The remaining sediment that may be transferred with the organisms will be removed during the DNA extraction. Sediment homogenization may take place in the field site directly after sieving. If this is not possible, microbenthic organisms may be collected in tubes and kept in a cool and dark environment until transportation to the lab.
6. Use mortar and pestle to crush and homogenize organisms until a tissue mixture is obtained.
7. Transfer 10 g of the tissue mixture into a screw cup tube of 50 ml. Repeat this to obtain 4 screw cup tubes of 50 ml each containing 10 g of the tissue mixture.
8. Fill tubes to 50 ml with DESS (see DESS recipe).
9. Label the tubes appropriately using permanent marker (see Sample Labelling section).
10. Apply transparent tape firmly on top of the label to avoid bleaching.
11. Store tubes at 4°C.

Figure 10: Grab deployment from a RV.

Hard Substrate

Hard Substrate Standard Operating Procedures – HaSOP

Summary

The passive sampling of hard substrate communities will take place using ARMS units. More details on purchasing ARMS units, deployment and sample collection are available from the ARMS-MBON (http://www.arms-mbon.eu/).
Different sampling strategies may be followed while setting up an ARMS observatory. Here are some strategies that have been implemented in past:

1. Short-term surveys that aim at seasonal investigations and monitoring of alien species. ARMS are deployed for a period of 2-3 months usually during spring to summer season.
2. Long-term surveys that aim at continuous biological monitoring and ecological research. ARMS are deployed for a period of 12 months and at the time of retrieval the next ARMS unit is deployed.
3. Survey on community succession of macro-organisms. In this case 3 or more ARMS units are deployed on the same date and retrieved sequentially (i.e., 1 ARMS unit retrieved every 4 months) to investigate the succession of settled organisms over time.

Participating stations may opt for one of the sampling strategies or for a different sampling design depending on their scientific interests. However, if an observatory station has not performed ARMS deployment in the past, it is recommended to contact one of the current ARMS-MBON observatory stations to discuss possible sampling strategies. The sampling strategies usually reflect the internal research needs of the observatory stations.
Deployment and retrieval of ARMS in EMO BON will follow the standards and protocols of the Smithsonian Institution (https://www.oceanarms.org/) and the additional guidelines of ARMS-MBON (http://www.arms-mbon.eu/). Selecting the location of the ARMS observatory is of great importance both for short-term and for long-term surveys. For example, for non-indigenous species (NIS) monitoring, observatory sites should be placed in close vicinity to introduction hotspots, such as aquaculture facilities and ports or marinas, and they should be easy to reach to take samples. EMO BON recommends, if possible, that ARMS sampling sites are located near the water column and/or soft substrate sampling sites, as well as near long term monitoring sites. More information on the ARMS locations can be found in the ARMS-MBON Handbook (http://www.arms-mbon.eu/). Deploying more than one ARMS unit, will give a better representation of the biodiversity in the area. It is recommended that three ARMS units per sampling site are deployed in close proximity (10 metres maximum).
Sample processing after retrieval will follow the standards and protocols of the Smithsonian Institution (https://www.oceanarms.org/) and the additional guidelines of ARMS-MBON (http://www.arms-mbon.eu/); here only a brief description of the procedures may be found. After ARMS retrieval, units should be transferred in the laboratory where the disassembly and photography of ARMS plates will take place. Using scalpel and tweezers, sessile organisms are

collected from each ARMS plate and placed in a collection tube. Motile fractions should be recovered from the ARMS bin using a 2 mm sieve on top of a 0.5 mm on top of a 0.1 mm. Seawater and sediment in the ARMS bin should gently pass sequentially through the three sieves (2 mm, 0.5 mm, and 0.1 mm) and the bin should be rinsed thoroughly to ensure the collection of all organisms. The material collected on the 0.5 mm sieve may be concentrated using a 40 µm nytex mesh. The material collected in the sieves should be photographed prior to storage. All sievings should be carefully performed to avoid overflow.
In addition to the sessile fraction retrieved by plate scraping, each sieve will collect a different fraction of the motile organisms: a) motile fraction sieved with 2 mm (organisms >2 mm), b) motile fraction sieved with 0.5 mm (2 mm > organisms > 0.5 mm), and c) motile fraction sieved with 0.1 mm (0.5 mm > organisms > 0.1 mm). The material in motile fraction a (organisms >2 mm) will be processed only for optical identification by photographing the individual specimens. The sessile together with the motile fractions b (2 mm > organisms > 0.5 mm) and c (0.5 mm > organisms > 0.1 mm) will be homogenized and processed for DNA extraction and sequencing.
All tissues and material to be processed for sequencing (sessile fraction, b and c motile fractions) should be homogenized. The sessile fraction homogenization is a straightforward procedure performed using a blender. The motile fraction homogenization requires washing steps, and the grinding is performed using mortar and pestle. The procedures are described in detail by the Smithsonian Institution (https://www.oceanarms.org/) and in the ARMS-MBON Handbook. After thorough homogenization, the mixtures are stored in DESS at -20°C until shipping.
At least 4 technical replicates should be collected from each sampling site, therefore material from the sessile fraction and the motile fractions should be stored in 4 individual tubes. In case the material is not enough to collect 4 technical replicates, collect as many as possible; make sure to indicate the number of collected replicates in the metadata. In many cases, biomass of a few species may dominate the community in ARMS. This may cause biases during DNA amplification and sequencing and to decrease this bias it is recommended to reduce the amount of biomass included during tissue homogenization (http://www.arms-mbon.eu/).

Protocol

All procedures regarding ARMS assembly, deployment, retrieval, disassembly, and sample processing are described in detail by the Smithsonian Institution (https://www.oceanarms.org/). Before starting, ensure all materials equipment, and supplies are available by checking the following table (Table 1).

Table 1: List of materials, equipment, and supplies for HaSOP.
Bleach solution
90% ethanol
0.2-prefiltered seawater
Gloves
Elbow long gloves
ARMS unit
Crate to cover up the ARMS unit during retrieval
Recovery bin for retrieving the AMRS unit
Tie wraps of different sizes

Buckets
Wrenches
Forceps
Spatulas and/or spoons
Scalpels
Kimwipes
Wash bottle with 0.2-prefiltered seawater
Wash bottle with 90% ethanol
2 mm sieve
0.5 mm sieve
0.1 mm sieve
Mortar and pestle or Blender
15 ml screw cap tubes
Laboratory balance
Permanent marker
Pencil
Transparent tape
Parafilm
DESS aqueous solution
Access to -20°C
Camera with housing and photo tray
Sorting trays
Plate tags
Cotton swab
Access to 4°C

Negative control sample

As samples undergo different procedures at the operating stations and at the centralized sequencing centre laboratories, it is important to check for contaminations. For this reason, blank samples will be collected and processed together with the ARMS samples to serve as negative controls. Once the ARMS unit is brought in the laboratory and before samples processing, collect and store a negative control sample as described below:

1. Use a cotton swab on the lab bench, the spatulas, the spoons, and the sieves.
2. Cut the cotton part of the swab and store it in a 15 ml screw cap tube.
3. Add 10 ml of DESS.
4. Label the tube appropriately using permanent marker (see

5. Sample section).
6. Apply transparent tape firmly on top of the label to avoid bleaching.
7. Store at -20°C.

Sample Labelling

Correct labelling is of extreme importance when collecting samples and especially for a network of more than ten operational stations running in the long-term. Proper labelling will ensure that samples collected are organized and processed appropriately, that data and metadata produced in EMO BON will be properly catalogued and analysed in the future, and that the data produced can be correctly assigned to the sampling sites.
Participating institutions should decide on a name and a short ID for their observatory that will be maintained for the duration of EMO BON and will characterize all samples collected at this observatory. The name and ID of the observatory should be unique whether an observatory station samples for all three habitats (Wa: Water column, So: Soft substrate, Ha: Hard substrate) or for just one. For example, if "SMN99" is sampling water column 1 mile offshore and hard substrates in a marina, the observatory ID should be "SMN99" for both water column and hard substrate samplings. The different sampling sites of each observatory will be distinguished by the suffix "Wa" for water column, "So" for soft substrates and "Ha" for hard substrates. Therefore, if observatory "SMN99" is sampling all three habitats, it will include three different sites: "SMN99 Wa" (water column sampling site), "SMN99 So" (soft substrate sampling site) and "SMN99 Ha" (hard bottom sampling site).
Five terms will constitute the label of each sample:

1. Project ("EMO BON")
2. Sampling Site ID; that is the Observatory ID (for example "SMN99") supplemented with the sampling site indicator ("Wa" for water column, "So" for soft substrates and "Ha" for hard substrates)
3. Sampling Campaign Date formated as YYMMDD.For example "220315" would be the campaign on 15 March 2022. Especially for Hard substrates, both the deployment and retrieval dates have to be recorded formated as YYMMDD-YYMMDD.
4. Size fraction (Wa: "3 µm" / "0.2 µm" / "20 µm" / "200 µm") or organisms collected (So: "micro" for microorganisms, "meio" for meiobenthos and "macro" for macrobenthos) or ARMS fraction (Ha: "SF" for sessile fraction, "MF05" for motile fraction sieved through 0.5 mm, "MF01" for motile fraction sieved through 0.1 mm)
5. Replicate (number 1-4). For labelling the negative control, this term will be replaced by the notation "blank" in parenthesis.

A few examples of sample labelling may be found in Table 2. It is recommended to avoid hyphens, commas, underscores and to separate easily confused digits (for example 1 and 7, 0 and O). The combination of these terms will be unique for each individual sample within EMO BON. This unique Sample ID created from the combination of those terms will follow the sample and the generated data and metadata during all procedures and analyses.

Permanent markers should be used for labelling the tubes and Ziplock bags and transparent tape should be applied firmly on top of the label to avoid bleaching. Samples that are not properly labelled will be discarded prior to DNA extraction.

Table 2: Sample labeling examples

Sample Description	Sample ID
Replicate 1 from a water column sampling at the SMN99 observatory collected on 15 March 2022 on a 3 µm filter	EMO BON SMN99 Wa 220315 3 µm (1)
Replicate 3 from a soft substrate meiobenthos sample at the JIJO41 observatory collected on 28 August 2021	EMO BON JIJO41 So 210828 meio (3)
Replicate 2 from a hard substrate sample at the RFF055 observatory that was collected from an ARMS unit deployed on 2 October 2022 and retrieved on 2 December 2022, and sieved at 0.5 mm	EMO BON RFF055 Ha 221002-221202 MF05 (2)
Negative control sample from a water column sampling at the JIJO41 observatory collected on 15 March 2022 on a 3 µm filter	EMO BON JIJO41 Wa 220315 3 µm (blank)

Collection and Documentation of Metadata

The collection of rich and detailed metadata is of great importance for the longstanding life cycle of the data collected in EMOBON. Metadata are necessary in all the principles of FAIR data management. To assure the correct data FAIRification, metadata should be considered even before the collection of the biodiversity data. EMO BON will adhere to the FAIR data principles for all data generated within the network; therefore, the collection of metadata during sampling and laboratory procedure is essential and mandatory. More details on the data and metadata life cycle will be provided in the EMO BON Data Management Plan (DMP). EMO BON DMP will describe in detail the handling of the data and their processing towards FAIRification.
Observatories will collect and document all information related to the data from sampling to sequencing and quality control. There will be 5 categories of metadata that will adhere to the data:

1. Observatory Metadata
Here, all information regarding the observatory station, the sampling sites, their characteristics, and the contact people will be included. This information will be provided with the registration form that will confirm participation to EMO BON (see also **Erreur ! Source du renvoi introuvable.** section and the Registration Form document). Observatory Metadata refer to the observatory and will not change among sampling events. Observatory Metadata will be digitalized and submitted as described in the DMP.

2. Sampling Metadata
All information related to each sampling event will be documented as Sampling Metadata. This will include date and time of sampling, sample handling details, deviation from protocols and other characteristics of the sampling events. These metadata will be recorded in log sheet provided by EMO BON during each sampling event and will be digitalized and submitted as described in the DMP.

3. Measured Metadata
Observatories will have to measure additional variables that will make the sequencing dataset relevant to its ecological context. Some variables are characterized as mandatory and are to be measured during every sampling event. Observatories may choose to additionally measure any of the recommended variables. The more environmental variables connect to the dataset, the more relevant and reusable the dataset will be. Information regarding the sampling and laboratory methodologies of the measured variables will be documented here. EMO BON will not give specific instructions for the methodologies of the environmental variables measured, as observatory stations are already equipped with specific instruments and follow specific procedures for their sampling sites. However, it is important that the same procedure is followed for each variable for all EMO BON samplings and the procedure is adequately described.

Environmental variables are listed in Table 3 for each habitat (Water column, Soft substrate, Hard substrate). The DMP will describe all procedures to handle Measured Metadata after documentation.

Table 3: List of Measured Metadata for EMO BON sampling. Different variables are listed for each of the three habitats. Mandatory Environmental Variables are to be measured and recorded for every sampling event. Observatories may choose to additionally measure any of the Recommended Environmental Variables. Star symbol (*) indicates which variables have been characterized as Essential Ocean Variables by The Global Ocean Observing System (GOOS).

Habitat	Mandatory Environmental Variables	Recommended Environmental Variables
Water Column (Wa)	Sea Surface Temperature *	Dissolved Organic Carbon *
	Subsurface Temperature * (at the sampled depth)	Inorganic Carbon *
	Sea Surface Salinity *	Nutrients Concentration *: nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+), phosphate (PO_4^{3-}) and silicic acid ($Si(OH)_4$)
	Subsurface Salinity * (at the sampled depth)	Nitrous Oxide (N_2O) Concentration *
	Chlorophyll-a Concentration *	Oxygen Concentration *
		Particulate Matter *
		pH
		Bacterial Abundance and Biomass (*emerging)
		Other picoplankton Abundance and Biomass (*listed as phytoplankton by GOOS)
		Nanoplankton Abundance and Biomass (*listed as phytoplankton by GOOS)
		Microplankton Abundance and Biomass (*listed as phytoplankton by GOOS)
		Mesoplankton Abundance and Biomass (*listed as zooplankton by GOOS)
		Macroplankton Abundance and Biomass (*listed as zooplankton by GOOS)
		Primary Productivity *

Soft Substrates (So)	Sediment temperature at the top 0-5 cm	Sediment Granulometry (median grain size)
	Sea Surface Temperature *	Sediment Granulometry (percentage content of each fraction of clay, silt, sand, gravel, cobbles, and boulders)
Habitat	**Mandatory Environmental Variables**	**Recommended Environmental Variables**
Soft Substrates (So)	pH	Porosity
	Redox potential (Eh)	Wet Bulk Density
		Dry Weight
		Oxygen Concentration
		Sulphide Concentration (H_2S)
		Sulphate Concentration (SO_4^{2-})
		Organic Matter Content
		Chlorophyll-a Concentration
		Bacterial Abundance and Biomass
		Phytobenthos Abundance and Biomass
Hard Substrates (Ha)	Sea Surface Temperature *	Images of the specimens from the plates
	Subsurface Temperature * (at the sampled depth)	Images of the habitat and surrounding environment
	Sea Surface Salinity *	Images of the sampling event
	Subsurface Salinity * (at the sampled depth)	
	Images of the ARMS plates	

4. Molecular Work Metadata

This category will include information on all the procedures followed during the molecular work after the sample's shipment. The sequencing centre will provide information on the DNA extraction, cleaning, sequence library preparation and sequencing. This information will be submitted as described in the DMP.

5. Post-sequencing Metadata

All information regarding the procedures performed on the raw sequences dataset to obtain a quality controlled processed dataset. Post-sequencing Metadata will be submitted as described in the DMP and will apply only to the processed dataset.

EMO BON will provide e-log-sheets to document the metadata. The e-log-sheets will specify all the metadata that need to be collected and documented. All the described metadata will be mandatory with the only exception being the Recommended Environmental Variables of Measured Metadata. EMBRC will closely assist observatories during the metadata collection, documentation, and submission. Samples for which metadata are incomplete or improperly documented will be rejected from the observatory network.

DNA extraction

DNA extraction will be performed at a centralized facility to minimize biases. It is important that all EMO BON samples are processed in the same laboratory, under the same conditions and using the same equipment to minimise operator bias in the study. As the laboratory analyses of samples will take place at different time intervals, it is essential that the procedures followed are as standardized as possible. DNA extraction will be performed using commercialized kits, as the standardized solutions of the kits will minimize extraction deviations among samples.

For water column samples

For DNA extraction of the water column filter samples, the same protocol as described by Alberti et al. (2017) will be used. The procedure consists of a first step of cell disruption by cryogenic grinding of membrane filters followed by chemical lysis and then nucleic acid purification using NucleoSpin RNA kits combined with the NucleoSpin RNA/DNA buffer set (Macherey-Nagel, Düren, Germany).

Protocol

1. Briefly each filter is placed in a grinding vial with 1-2 ml lysis buffer (Macherey-Nagel RA1 lysis buffer at 1% β-mercaptoethanol -Sigma, St Louis, MO-) and ground with Freezer/Mill instrument (6770 or 6870 Freezer/Mill; SPEX SamplePrep, Metuchen, NJ) using the following program:
    a. 2 min pre-cooling
    b. first grinding cycle at 10 knocks per second for 1 min
    c. 1 min cooling time
    d. final grinding cycle at 10 knocks per second for 1 min
2. The powder from cryo-grinding is then transferred to a NucleoSpin filter (Nucleospin Filters XL, ref 740605).
3. The remaining powder in the grinding flasks is recovered by adding 1 ml of lysis buffer and then transferred to the filter.
4. The solution is left on the filter for ~30 min to pass by gravity.
5. The eluate is transferred to a 15ml falcon tube and centrifuged for 20 min at 1,550 g at 20°C.
6. Add 500 µl of lysis buffer to the eluate.
7. Centrifuge for 3 min at 1550 g at 20°C.
8. A volume of 70% ethanol is then added to precipitate the nucleic acids.

9. The final mixture is placed on a NucleoSpin RNA Mini spin column and two successive washes are performed with 500 µl of DNA wash solution.
10. The columns are dried for 3 min at room temperature.
11. DNA is eluted by three successive elutions with a total of 100 µl DNA elution buffer, and stored with 1 mM EDTA in sterile 1.5 ml DNA LoBind Eppendorf tubes at -20°C.

For sediment samples

DNA extraction from sediment samples is performed using the commercially available Quick-DNA Fecal/soil kit (Zymoresearch) with slight modifications.

Protocol

1. After thawing, each sample tube is briefly vortexed and 500 µl of sample is transferred to a ZR BashingBead tube supplied in the kit.
2. Add 250 µl of BashingBead Buffer in the tube, and ground at room temperature using the FastPrep according to the following program: Speed: 5.5 m/s; Adapter: quick prep; Time: 30 sec; Cycle: 2; Pause time: 120 sec.
3. The tube is recovered and centrifuged for 1 min at 14,000 g at room temperature.
4. Transfer 400 µl of supernatant to Zymo-Spin III-F filter column, and centrifuge for 1 min at 8,000 g.
5. Retrieve the filter and disard the column.
6. Add 800 µl of Genomic Lysis Buffer solution and 400µl EtOH 95% to the filtrate and mixed well.
7. Transfer 800 µl to a Zymo-spin IICR column.
8. Centrifuge for 1 min at 10,000 g.
9. Discard the filtrate and keep the column repeating this centrifugation 3-4 times until the mixture has completely passed through the column.
10. Transfer 200 µl of DNA Pre-Wash Buffers solution on the Zymo-Spin IICR column and incubate for 5 min at room temperature.
11. Centrifuge the column for 1 min at 10,000 g.
12. Discard the filtrate, and place the column in a new collection tube.
13. Add 500 µl of g-DNA Wash Buffer solution and centrifuge for 1 min at 10,000 g.
14. Discard the filtrate and place the column in a 1.5 ml tube, with the cap left open to dry for 1 min on the bench.
15. Add 55 µl of DNA Elution Buffer and incubate on the bench while the Zymo-Spin III-HRC purification column is prepared.
16. Place the Zymo-Spin III-HRC column on the collection tube and add 600 µl of Prep Solution.
17. Centrifuge the column for 3 min at 8,000 g.

18. Place the Zymo-Spin III-HRC column on a 1.5 ml DNA low binding tube, waiting for the DNA eluate.
19. Centrifuge the Zymo-Spin IICR column for 30 sec at 10,000 g to elute the DNA.
20. The total volume of eluted DNA is collected and transferred to the previously prepared Zymo-Spin IICR column.
21. The purified DNA is finally recovered after centrifugation for 3 min at 14,000 g and then stored and preserved at -20°C.

For macrobenthos tissues (Soft Substrate) and the tissue mixtures from the Hard Substrates

Protocol

1. Samples are thawed and homogenized by vigorously inverting each tube.
2. 10 ml of each mixture is immediately transferred into a 50 ml tube. C
3. Centrifugate for 3 minutes at 3,200 g
4. Remove the buffer and carry out 2 successive washes as follows:
    a. Add 5 ml of PBS1X buffer .
    b. Centrifuged for 3 minutes at 3,200 g.
    c. Remove the buffer.
5. Resuspend the pellet with 5 ml of DNA/RNA shield 1X and grinding with Fastprep is carried out with the following program: Speed: 5.5 m/s; Adapter: teen prep; Time: 30 sec; Cycle: 2; Pause time: 120 sec.
6. Recover the tube and centrifugate for 2 minutes at 3,200 g.
7. 3 ml of the supernatant are transferred in a 5 ml tube and run through a Zymo spin III-F column in 800 µl volumes, centrifuging for 15 seconds each time (1 min for the final centrifugation) at 10,000 g.
8. The filtrates are progressively transferred to a 15 ml tube.
9. Add 1200 µl of Lysis Buffer and 600 µl of EtOH. 95% to the filtrate.
10. Vortex the mixture for 5 sec and pass the lysate through a Zymo spin II-CR column placed in a 2 ml collection tube by 800 µl volume, centrifuging for 15 seconds at 10,000 g.
11. At this step, the filtrate is discarded after each centrifugation.
12. Once the lysate is exhausted, 200 µl of prewash buffer are added to the column.
13. Incubate for 5 min at room temperature.
14. Centrifuge for 1 minute at 10,000 g.
15. Wash the column with 500 µl of wash buffer, centrifuge for 1 minute at 10,000 g and place in a 1.5 ml DNALoBind tube.

16. Add 110 µl of DNA elution buffer to the column and incubate at room temperature while the Spin spin III-HCR purification column is prepared.
17. The Spin spin III-HCR column is placed in a collection tube.
18. Add 600 µl of preparation solution and it is centrifuge for 3 min at 8,000 g.
19. The spin III-HCR column is collected and placed in a 1.5 ml DNA LoBind tube waiting for the DNA eluate.
20. The Zymo-Spin IICR column is then centrifuged for 30 sec at 10000 g to elute the DNA.
21. The total volume of eluted DNA is collected and transferred to the previously prepared Zymo-Spin IICR column.
22. The purified DNA is finally recovered after centrifugation for 3 min at 14,000 g and then stored and preserved at -20°C.

DNA is quantified by a dsDNA-specific fluorometric quantitation method using a Qubit 2.0 Fluorometer instrument. A first assay is performed to roughly evaluate the concentration using the Qubit dsDNA BR (Broad range) Assays kit (ThermoFisher Scientific, Waltham, MA). Based on this concentration a dilution of DNA is done to lower the concentration within the threshold range of the Qubit dsDNA HS (High sensitivity) kit and 2 HS assays are then carried out using this diluted DNA solution. The estimation of DNA concentration is calculated by the average of concentrations from these 2 assays.

Sequencing

Sequencing will also be performed at a centralized sequencing facility to reduce biases as much as possible. The Water Column WaSOP1 and the Soft sediment samples for microorganisms will be analysed using metagenomics. All other samples will be analysed using DNA metabarcoding methodologies. Samples and analyses details are summarized in Table 4.

Metagenome libraries are constructed according to the available DNA: 10 to 100 ng of genomic DNA are sonicated to obtain fragments of around 350 bp using the Covaris E220 instrument (Covaris, Woburn, MA, USA). Fragments are repaired, 3'-adenylated and NEXTflex PCR free barcodes adapters (Bioo Scientific, Austin, TX, USA) are added using the NEBNext® Ultra II DNA Library prep kit for Illumina (New England Biolabs, Ipswich, MA, USA). Ligation products are purified by AMPure XP beads 0;8 vol (Beckmann Coulter, Brea, CA, USA). DNA fragments (>200 bp) are amplified by PCR (2 PCR reactions, 14 cycles) using Illumina adapter-specific primers and NEBNext® Ultra II Q5 Master Mix (NEB). All libraries are subjected to size profile analysis conducted by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and to qPCR quantification using the KAPA Library Quantification Kit for Illumina Libraries (KapaBiosystems, Wilmington, MA, USA). All metagenomic libraries validated by the quality-control are sequenced using 151-bp pairwise read chemistry on an Illumina NovaSeq6000 sequencer using S4 Flowcells (Illumina, San Diego, CA, USA). A minimum of 40,000 million useful paired-end reads are obtained per sample.

DNA metabarcoding is carried out using the BID strategy. This strategy uses primers composed of the sequence for specific amplification of target organisms plus a small sequence of 8 nucleotides in 5', corresponding to a barcode identifier (BID). We used 12 BIDs, the same in Forward and Reverse, in order to allow for a correct reassignment of sequences to samples after demultiplexing. This strategy is described in detail in Belser et al. (2023).

COI is amplified using m1COIintF and jgHCO2198 primers. The PCR mixtures (20 µl final volume) contain 2.5 ng or less of total DNA template with 0.5 µM final concentration of each primer, 3% of DMSO, 0.175 mM final concentration of dNTPs Mix and 1X Advantage 2 Polymerase Mix. PCR is carried out in four replicates in order to smooth the intra-sample variance while obtaining sufficient amounts of amplicons for sequencing. PCR conditions included a 10 min denaturation step followed by 16 cycles of 95 °C for 10 sec, 30 sec at 62°C (–1°C per cycle), 68 °C for 60 sec, followed by 15 cycles of 95 °C for 10 sec, 30 sec at 46°C, 68 °C for 60 sec and a final extension of 68 °C for 7 min. PCR products from replicates are pooled and are then purified using 1x volumes AMPure XP beads.

18S is amplified using SSUF04 and SSURmod primers. The PCR mixtures (25 µl final volume) contain 2.5 ng or less of total DNA template with 0.4 µM final concentration of each primer, 3% of DMSO and 1X Phusion Master Mix. PCR conditions were the following: 98 °C for 30 sec; 30 cycles, 98 °C for 10 sec, 45 °C for 30 sec, 72 °C for 30 sec; and 72 °C for 10 min. PCR amplifications

are carried out in triplicate to smooth the intra-sample variance while obtaining sufficient amounts of amplicons for sequencing. PCR products from replicates are pooled and purified using 1x volumes AMPure XP beads (Beckmann Coulter Genomics).

Aliquots of purified amplicons are run on an Agilent Bioanalyzer using the DNA High Sensitivity LabChip kit to check their lengths and quantified with Qubit 2.0 Fluorometer using a Qubit RNA HS assay kit. Metabarcoding sequencing libraries are prepared from 100 ng (or less) of an equimolar pool of 8 to 12 purified PCR products. Sequencing libraries are made by high throughput automatized instruments. In case of a failed library with robot (based on QC results), sequencing libraries are remade by hand following the same process as used with the robot and as described below. 100 ng of purified amplicons from the pool (or less when the quantity available is insufficient to engage 100 ng) are directly end-repaired, A-tailed and ligated to Illumina adapters on a Biomek FX Laboratory Automation Workstation. Then, library amplification is performed (2 PCR reactions, 12 cycles) using Kapa Hifi HotStart NGS library Amplification and purified by AMPure XP purification (1 volume). All libraries are quantified first by Quant-it dsDNA HS using a Fluoroskan Ascent instrument (Thermo scientific) then by qPCR with the KAPA Library Quantification Kit for Illumina Libraries (Kapa Biosystems) on an MXPro instrument (Agilent Technologies). Library profiles are assessed using high throughput microfluidic capillary electrophoresis system (LabChip GX, Perkin Elmer, Waltham, MA). All metabarcoding libraries validated by the quality-control are sequenced using 250-bp pairwise read chemistry on an Illumina NovaSeq6000 sequencer using SP Flowcells (Illumina, San Diego, CA, USA). A minimum of 800,000 useful paired-end reads are obtained per sample.

Table 4: Sequencing details for samples.				
Protocol	Sample	Analyses	Details	Primer references
WaSOP 1	Microorganisms (3 µm fraction)	Metagenomes	-150 bp fragments -paired reads -50 M reads per sample	
	Microorganisms (0.2 µm fraction)			
	Microorganisms (3 µm fraction)	Metabarcodes	-*ITS* amplicon -paired reads -1 M reads per sample per amplicon	ITS1F (Bruns and Gardes, 1993) and ITS2 (White et al., 1990)
	Microorganisms (0.2 µm fraction)			
WaSOP 2	Plankton >20 µm	Metabarcodes	-*18S* v4 region -*COI* amplicon -paired reads -1 M reads per sample per amplicon	-*18S* v4 region: *to be decided* -*COI*: mlCOIintF and jgHCO2198 (Leray et al., 2013)
WaSOP 3	Plankton >200 µm			
SoSOP 1 SoSOP 2 SoSOP 3	Microorganisms	Metagenomes	-150 bp fragments -paired reads -1 M reads per sample	
	Meiobenthos	Metabarcodes	-*18S* v1-v2 region -*COI* amplicon -paired reads -50 M reads per sample per amplicon	-*18S* v1-v2 region: SSU_FO4 (Fonseca et al., 2010) and SSU_R22mod (Sinniger et al., 2016) -*COI*: mlCOIintF and jgHCO2198 (Leray et al., 2013)
	Macrobenthos			
HaSOP	Sessile fraction	Metabarcodes	-*18S* v1-v2 region -*COI* amplicon -paired reads -1 M reads per sample per amplicon	-*18S* v1-v2 region: SSU_FO4 (Fonseca et al., 2010) and SSU_R22mod (Sinniger et al., 2016) -*COI*: mlCOIintF and jgHCO2198 (Leray et al., 2013)
	Motile 0.5 mm fraction			
	Motile 0.1 mm fraction			

Replication and Biobanking

The replicates collected within EMO BON are technical replicates, not true statistical replicates. They represent identical samples and are to be used for different purposes. All 4 replicates per sample are collected following the exact same procedures as described in the **Erreur ! Source du renvoi introuvable.** section.

Two of the replicates will be processed for the extraction of DNA and subsequently sequencing. The replication of processed samples will allow the recognition of false or accidental laboratory practices and ensure the highest data quality. Additionally, 1 replicate per sample will be provided as a service from EMBRC to external users. The purpose of this service provision is to encourage the collaboration among marine institutions, to give the chance for biodiversity information comparisons across the globe and to allow samples to be analysed using different methodologies and under alternative foci.

EMO BON aims to be part of global research effort in marine biodiversity and to interact with other scientific entities and disciplines. It will contribute to the UN Decade of the Ocean not only by producing accurate and complete time-series data on marine biodiversity but also by preserving material samples for re-analyses at the end of the decade or beyond. Particularly, one replicate will be collected and biobanked for decadal storage. Decadal samples will be stored at -80°C organized in containers dedicated only to decadal preservation. All marine operating stations are required to keep detailed records of the preserved samples.

Calibration efforts

The cross-validation of the generated data and the calibration of methods is of high importance for EMO BON. Data produced within EMO BON will be a contribution to the United Nations Decade of Ocean Science for Sustainable Development and to the marine science community. As data will follow FAIR principles, will be reusable and will help reply to different scientific questions over the years. Data may be analysed several times during the coming decade as different scientists may access them. Therefore, it is essential that the quality of the data produced is high and that there is the capacity to validate the sequencing results in various ways.

For this reason, during all samplings two of the technical replicates collected will be processed. DNA extraction and sequencing of the replicates will take place under the same conditions and all followed procedures will be the same. Results of the two processed replicated will be integrated to produce final quality control datasets. In addition, a mock community comprised of eukaryotic microorganisms and a mock community comprised of bacteria will be created at EMBRC culture collections. The mock communities will be processed together with the samples and serve as positive controls. As the diversity of the mock communities will be known, the sequencing results will be validated by examining the sequencing reads of the mock communities

During sampling blank samples will be collected to serve as negative controls during DNA extraction and sequencing. This will help detect contamination during sampling or laboratory processing. In case a contamination is detected, it will be possible to go back to the sampling and processing of samples and revise the procedures. Furthermore, the sequencing reads of the blank samples will allow for corrections during the final dataset refining.

A sampling calibration exercise is planned to take place during EMO BON. The personnel responsible for sampling will be exchanged among observatories. Personnel will participate in a sampling event at one or more different observatories. The idea behind this is to standardize the methods between EMO BON operating stations and to exchange expertise. In combination to the training workshops planned, the sampling methods used in EMO BON will be harmonized ang the different observatories.

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Appendix 1: Materials and equipment

Specifications of filters and filter holders

- Polycarbonate hydrophilic membranes, 0.2 µm pore size, 142 mm (WaSOP 1)
  - For example the following items may be used:

    MERCK - GTTP14250

    Sterlitech - PCT0214220
- Polycarbonate hydrophilic membranes, 3 µm pore size, 142 mm (WaSOP 1)
  - For example the following items may be used:

    MERCK - TSTP14250

    Sterlitech - PCT3014220
- Polycarbonate hydrophilic membranes, 10.0 pore size, 47mm (WaSOP 2 and 3)
  - For example the following items may be used:

    MERCK TCTP04700

    Sterlitech - PCT10047100
- Stainless steel filter holder, 142 mm (WaSOP 1)
  - For example the following items may be used:

    MERCK - YY3014236

    Sterlitech - 301900

    Sterlitech - 302000

    Sterlitech - 302100

    Sterlitech - 302200

DESS recipe

The recommended composition of DESS aqueous solution is 20% dimethyl sulfoxide (DMSO), 0.25 M ethylenediaminetetraacetic acid (EDTA), and saturated sodium chloride (NaCl) (Seutin et al., 1991). The pH of the DESS solution should be adjusted to 8. It is recommended to saturate the DMSO with salt overnight.

To make 250 ml of DESS:

1. Weigh 23.265 g of disodium EDTA (FW 372.24). If you have EDTA with a different formula weight, you should adjust the desired weight. Add 50ml of deionized water to the EDTA and stir. Please, make sure to use disodium EDTA salt otherwise more NaOH will be needed to adjust the pH.

2. The EDTA should be around a pH of 3 or 4 to begin with. Add 1 M NaOH to adjust the pH of EDTA to about 8; roughly 50 ml of 1 M NaOH will be needed. The EDTA will then begin to dissolve slowly. Be patient and try heating to 30˚C to minimize the preparation time.

3. Once all the EDTA salt is dissolved, bring the volume up to 200 ml with deionized water. Then, add 50ml DMSO. Return to a beaker and stir for a few minutes.

4. Add NaCl until it no longer dissolves (heating will help dissolve the salt). Pour the solution into a bottle leaving most of the salt crystals in the beaker. The next day some salt may have settled or precipitated at the bottom of the bottle; it is normal for the salt to precipitate out of the solution once the liquid settles. If there is an excessive amount of crystals on the bottom of the bottle, it is best to move the solution into a new container leaving the crystals in the other bottle. Make sure that the container is sealed tightly to reduce the evaporation of the water in the solution.

Hydrochloric acid (HCl) 10% recipe

Hydrochloric acid aquatic solution is usually found at 37% w/w concentration (density 1.2 g/mL at 25˚C and molecular weight 36.5 g/mol). That corresponds to 12 M. To prepare 1 l of 10% HCl, add 270 ml of 37% HCl to 600 ml of distilled water. Stir gently to make sure the acid is evenly mixed. Add distilled water to the final volume of 1 l and stir again gently. It is important to add acid to water, never the opposite. Make sure to take all necessary precautions before working with acid (lab coat, fume hood, safety goggles). Read the specifications of the HCl aquatic solution before starting.

Bleach (sodium hypochlorite, NaClO) 10% recipe

Sodium hypochlorite aquatic solution is usually found at 5-8.25% concentration. It is recommended that to use a solution of at least 0.5% for disinfection. To prepare 10% bleach solution dilute the stock solution at a ratio 1:10, that is add 1 part of bleach solution to 9 parts of distilled water. For example, to prepare 1 l of 10% bleach, add 100 ml of bleach into 900 ml of distilled water. Make sure to take all necessary precautions before working with bleach (lab coat, fume hood, safety goggles). Read the specifications of the bleach solution before starting.

Appendix 2: Samples Summary

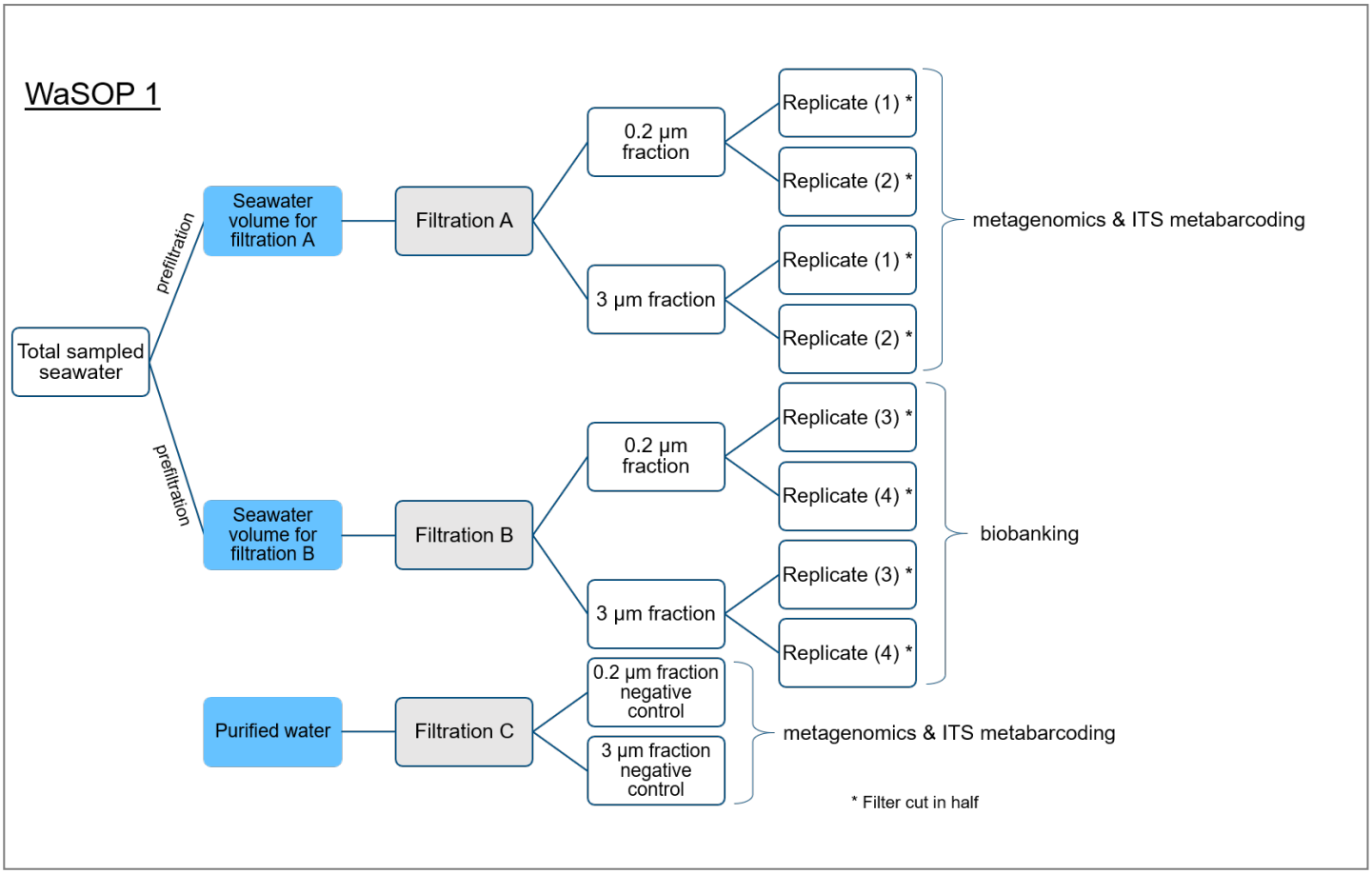

Figure 1: Diagram summarizing the WaSOP1 procedures, the samples collected, and the destination of the samples after collection.

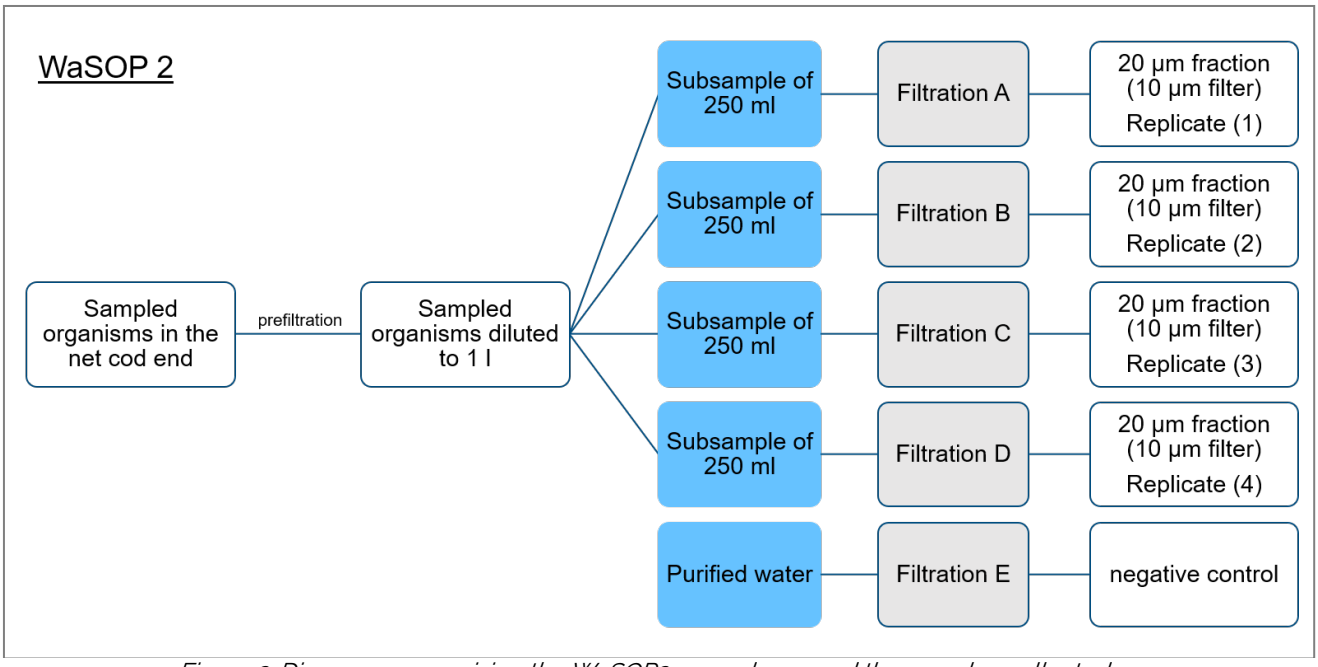

Figure 2: Diagram summarizing the WaSOP2 procedures, and the samples collected.

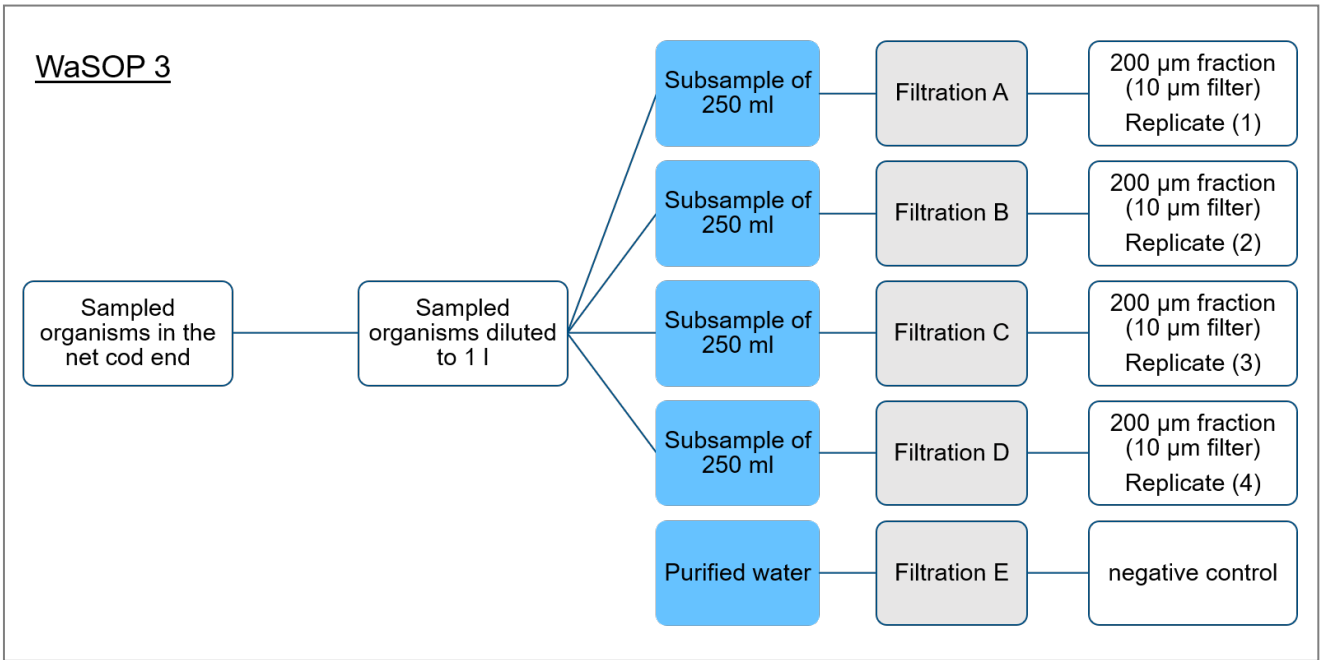

Figure 3: Diagram summarizing the WaSOP3 procedures, and the samples collected.

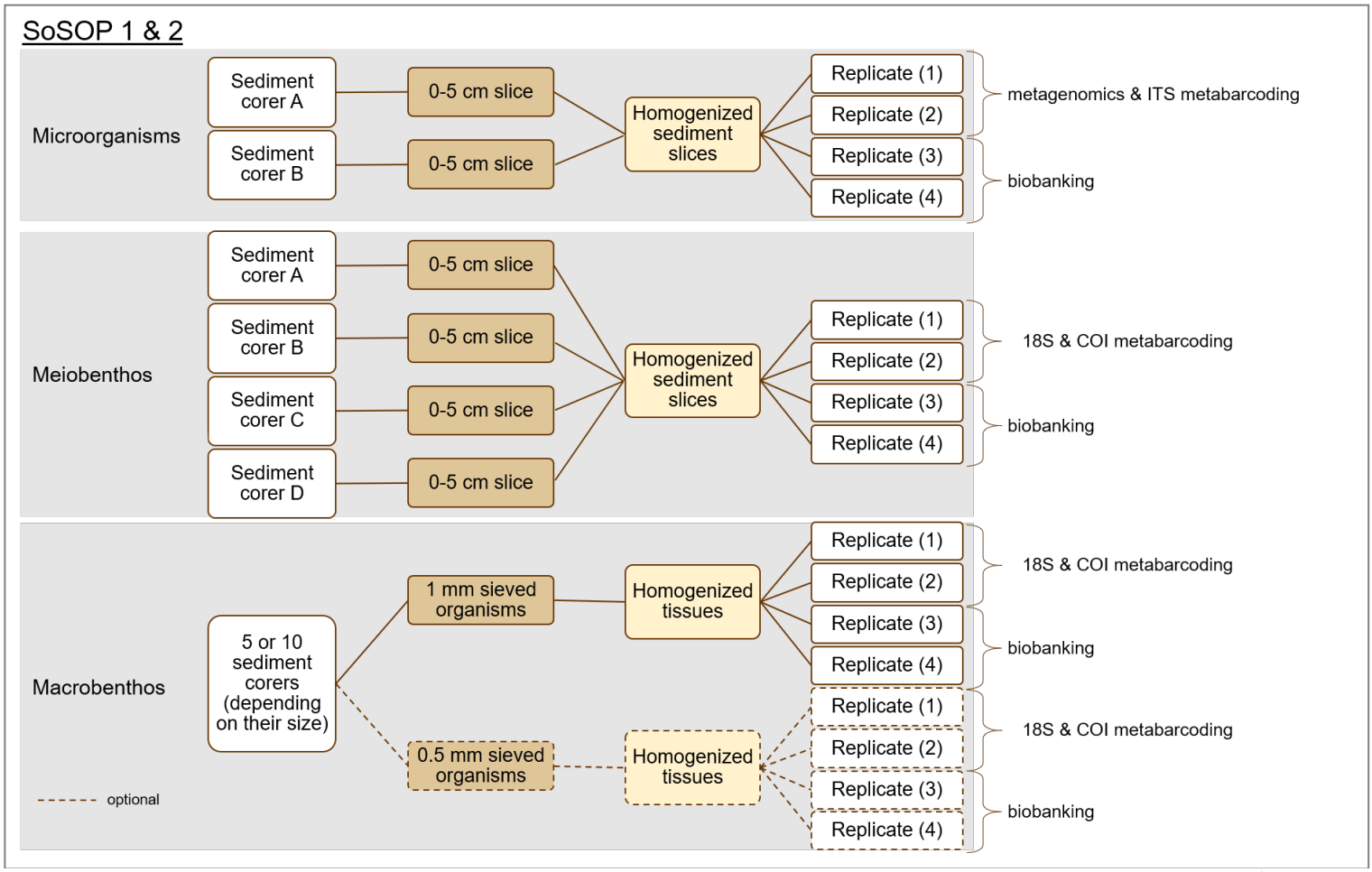

Figure 4: Diagram summarizing the SoSOP1 and SoSOP2 procedures, the samples collected, and the destination of the samples after collection. The dashed lines and boxes in the macrobenthos collection diagram indicate the sequential sieving performed optionally by the stations that usually use smaller sieve size for macrobenthos collection.

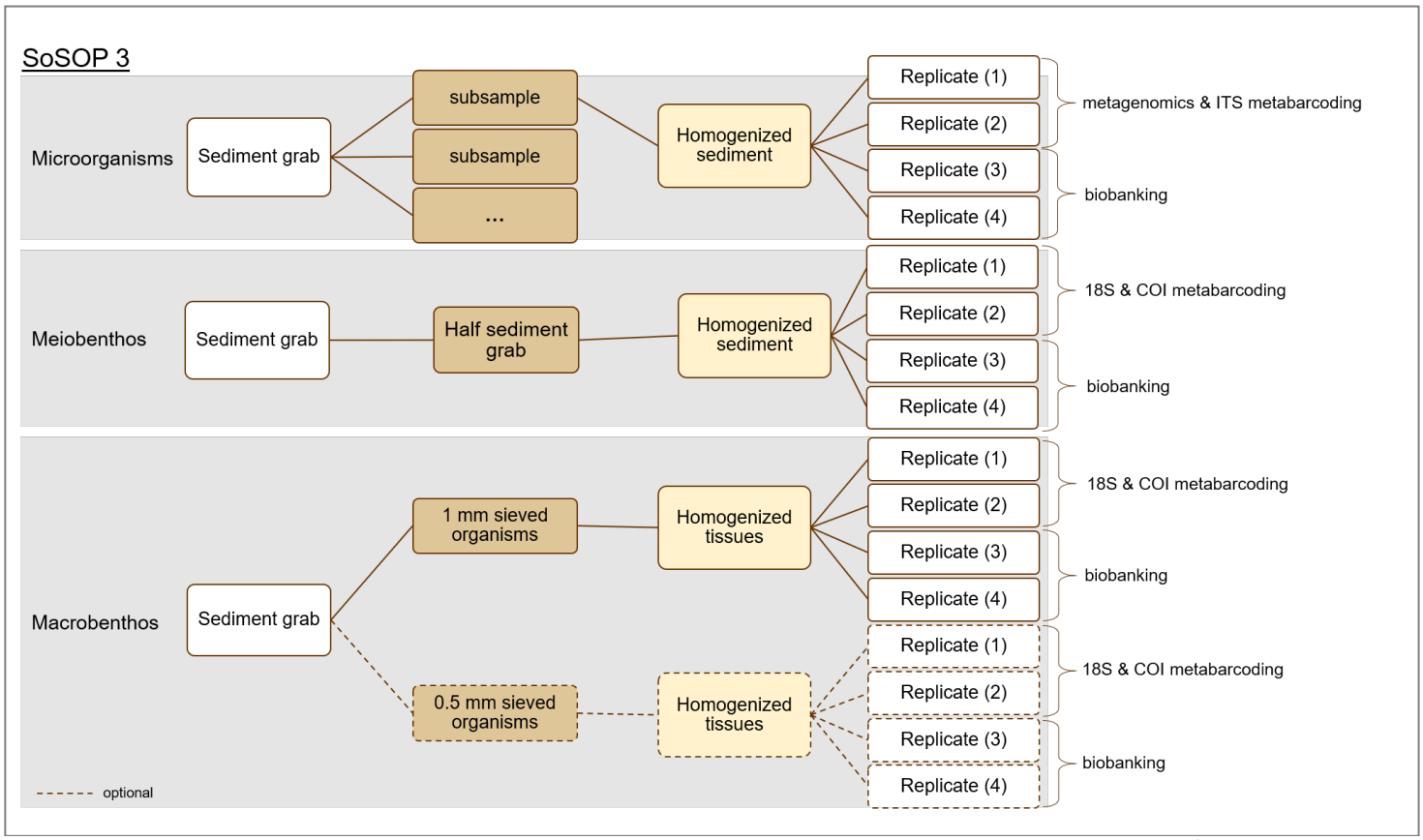

Figure 5: Diagram summarizing the SoSOP3 procedures, the samples collected, and the destination of the samples after collection. The dashed lines and boxes in the macrobenthos collection diagram indicate the sequential sieving performed optionally by the stations that usually use smaller sieve size for macrobenthos collection.