



Handbook

for

Marine Biodiversity Observation network for genetic monitoring of hard-bottom communities (ARMS-MBON)

Version 3.0

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1. Summary

This handbook provides a compilation of the standards required for setting up observatories as part of the Marine Biodiversity Observation Network for genetic monitoring of hard-bottom communities (ARMS-MBON). This document collects the guidelines, protocols, and recommendations for: the deployment and retrieval of ARMS observatories as established and further developed by the network; sample processing, including shipment and biobanking of samples; structuring the collected information (genetic data, image data, *in situ* measurements and recordings of biological and environmental variables) and the metadata and associated legal documents.

ARMS are highly standardised passive collectors for the assessment of epibenthic and hyperbenthic marine communities. In ARMS-MBON they are deployed at and for various periods depending on the scientific question being addressed, e.g. about 3 months for the monitoring of Non-Indigenous Species (NIS) in marine coastal environments, and 1-2 years for Long-term Ecological Research (LTER) sites to study the status and changes in hard-bottom species communities.

This is the Handbook for the ARMS-MBON project. Most of what is in here applies equally to those ARMS-MBON partners who are part of the

Changes since the last release

- Better instructions for creating the various sample IDs, in Sec 2.4
- Updates to the templates required for submitting data, and accompanying explanation in Sec. 2.4
- Simplified process for describing ARMS images and manual observations submitted to PlutoF, in Sec. 4.4 and 4.5
- New address for sending ARMS samples, in Sec. 5

2. Design your observatory

Before going ahead with ordering your ARMS unit(s), a certain amount of preparation is necessary.

2.1. Choosing observatory sites and deployment periods

Finding the area of interest. Currently, ARMS-MBON supports two kinds of investigations,

- i) Non-indigenous species (NIS) surveys and monitoring, based on short-term deployments during summer season in presumed introduction hotspots (see below)
- ii) Biological monitoring, based on long-term deployments for 1-2 years (i.e., Long-term ecological research; LTER)

For both investigations we encourage our partners to establish "observatory sites", which are localities where ARMS can be re-deployed on a regular basis, and hence build up a timeseries. Choosing a site that is accessible will allow for regular deployment and retrieval, and can support NIS or LTER research. Examples are marinas, ports, Marine Protected Areas (MPAs), LTER sites, or areas with high oceanographic connectivity. You should expect to deploy and retrieve ARMS at regular intervals depending upon the needs of your observatory.

Finding the right spot in the area of interest. Finding a good spot for long-term monitoring is not easy. Sometimes there are places where regular biological monitoring is already taking place, and these are often good candidate sites for an ARMS observatory when the purpose is LTER monitoring.

For 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. There

has to be a good chance that the ARMS will not be removed or disturbed by visitors and people working in the area. As well as asking for authorisation from the local authorities (e.g., MPA managers, port managers), according to your local or national rules, you should add contact labels to ropes and buoys (Fig. 1D) to minimise the risk of the ARMS being removed or disturbed. In marinas and ports, it may be a good practice to deploy the ARMS under the floating pontoons, where many of the NIS typically settle and contribute to the biofouling communities in these artificial habitats. In temperate waters, we recommend that you deploy ARMS for a short period, not exceeding 3 months, and preferably run a first trial to select the appropriate time frame. Depending on your region, spring to summer should be prefered. We also recommend you deploy your ARMS every year in the same place.

Definition of replicates. Replicates are used to assess the reliability of the experimental procedure. But before getting into the definition of different levels of replication, we should identify the variable of interest. What we ultimately want to assess is not so much the community on the ARMS but the benthic community at the deployment locality. The ARMS unit itself is therefore part of the experimental procedure and should be considered as a standardised measuring device for the diversity of the local benthic community. We need several replicates for a reliable diversity assessment. With this consideration in mind, we define the following hierarchical structure of replicates within the ARMS programme:

- 1. Independent samples, i.e. several ARMS units deployed over a broad study area: here we would have a measure of the different colonisation pressure from different benthic communities due to deployment of ARMS units at different sites and/or in different habitats. This provides an independent measurement of species diversity. Such independent replicates allow one to, for example, assess gamma diversity within the study area.
- 2. Technical or field replicate, i.e. separate ARMS units deployed very close to each other: here we have ARMS replicates in the sense of technical replicates, being deployed close to each other (ca. 3-10 m apart to avoid direct interaction) in a given locality and habitat. This should produce comparable results if there are no errors associated with their deployment, retrieval, and processing (scraping and homogenisation) in the laboratory. Such field replicates are important to estimate alpha diversity within, and beta diversity between, sites or habitats of interest. They are also crucial for replicability of the measurement, which is especially important for time-series in LTER. We recommend using three ARMS per locality.

Note that material sample replicates are separate to these ARMS (units) replicates, and are discussed in Sections 4.3 and 5.2.

Saving time and money. The main costs for maintaining an ARMS observatory are associated with deployment and retrieval. Some places require permits (e.g. ports, MPAs), which often take time to get. We recommend that, during your design phase, you contact a partner who already runs an observatory of the same type as you would like to establish. This way you can get help and advice, minimising the chance of unplanned obstacles.

Many partners deploy a new ARMS at the same time when they retrieve a submerged ARMS. This way you can save on expenses.

Diving is expensive, and you need to think about long-term costs for deployment/retrieval if you choose a site that needs scuba diving.

How many replicates should be deployed? We recommend deploying three ARMS per site, in close proximity to each other. This will help to get a representative capture from the area (replicate type 2). However, many partners start by deploying only one ARMS, to test the protocols, acquire practical experience, and explore the potential of a candidate site. For this, one ARMS unit would be sufficient during the first year, with more ARMS to be deployed in subsequent years.

Spatio-temporal distribution of replicates. For NIS monitoring, the three ARMS should be deployed as independent samples (replicate type 1), at different sites within the study area (e.g. the marina that is being monitored) which represent different microhabitats and thus maximising the number of observed NIS. For LTER the number of samples is less important, however for time-series the replicability is crucial: we recommend selecting one specific site within the area of interest, and deploying three ARMS in close vicinity within the same habitat, these would be field replicates (type 2 in the list above).

Practical details. Especially for NIS studies, ARMS can be attached to the jetties of marinas (e.g. underneath). In this case you have to make sure that: i) the connecting rope is clearly marked with a label (Fig. 1D), and ii) the ARMS and connecting rope are not in the way of propellers or currents made by vessels and boats.

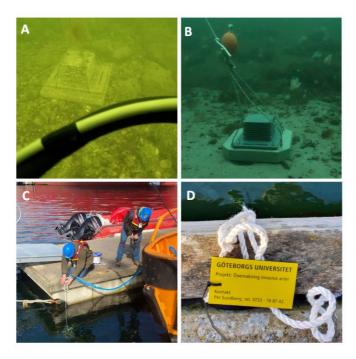


Fig 1. Examples of ARMS deployments at LTER sites with divers (A, B), in industrial ports (C), and marinas (D). In cases where ARMS are deployed with a rope and a buoy attached, please make sure you add a weather-proof contact label with your contact address and telephone number (D). Photograph credits: ARMS-MBON network.

2.2. Registration

We recommend that you start preparing for the deployment of the ARMS at your chosen observatory site(s) about 3-4 months ahead of time. You should register as a new partner of ARMS-MBON using the registration form available on the ARMS-MBON website (www.arms-mbon.eu). Once registered, you will be added to the ARMS-MBON email list and your observatory will be added to the ARMS-MBON database. As part of the registration process, you will be asked to read and comply with the Data Management Plan and supply some basic metadata for your observatory. We will then help you with the practical preparations, and discuss the specific design of your observatory with regard to purpose, location, number of ARMS replicates, and deployment periods.

2.3. Purchasing ARMS

ARMS should be purchased from the Smithsonian Institution (https://www.oceanarms.org/). They offer a good price for the units, and this also ensures that samples over the entire ARMS-MBON network will be comparable. Contact the ARMS-MBON network before ordering, because we may organise larger batch-orders during the winter months.

2.4. Sample terminology

Using the right naming for samples is essential, because this will allow all partners in the network to organise and understand the data collected from the samples. Bear in mind that we will have 100s of individual datasets to organise and link to each other, each year of the project!

We will use the following terms to describe and distinguish each sample (Fig. 2):

- 1. Observatory ID(s). An observatory is an area in which ARMS units are placed. Most partners have just one observatory, but if the units are placed in very distinct areas one may wish to define multiple observatories. Example: "Koster".
- 2. The IDs for the ARMS units placed in an observatory. Note that the ID is not for a physical unit, rather for the location it is placed. A new unit in an existing place adopts the existing ID. Example: "VH2". These are also chosen when you join or create new ARMS locations.

- 3. <u>Event IDs</u> are used to identify an event (unit in, unit out). Example: ARMS_Koster_VH2_20180415_20180906, with the date in followed by date out (YYYYMMDD)
- 4. <u>Material sample IDs</u> are made up of the eventID, the fraction (usually motile or sessile), filter size (in microns), and preservative (as of 2021 this is no longer necessary unless DMSO is not used). Example ARMS_Koster_VH2_20180415_20180906_SF40_DMSO. This ID is also to be written on the material sample labels (Secs 4.3 and 5.1).
- 5. For replicate material samples, add _A, B etc to the Material Sample ID

The observatory and ARMS IDs are chosen when you join the ARMS consortium or if you create new ARMS locations. The IDs should be short, simple, and not include spaces, hashes, etc. Event IDs are unique to each event (=a unit has been retrieved and successfully processed). Material Sample IDs are, fairly obviously, unique to each material sample. These IDs are to be used in PlutoF (the data management platform; Sec 7.) and in the ARMS data overview spreadsheet (Sec. 7).

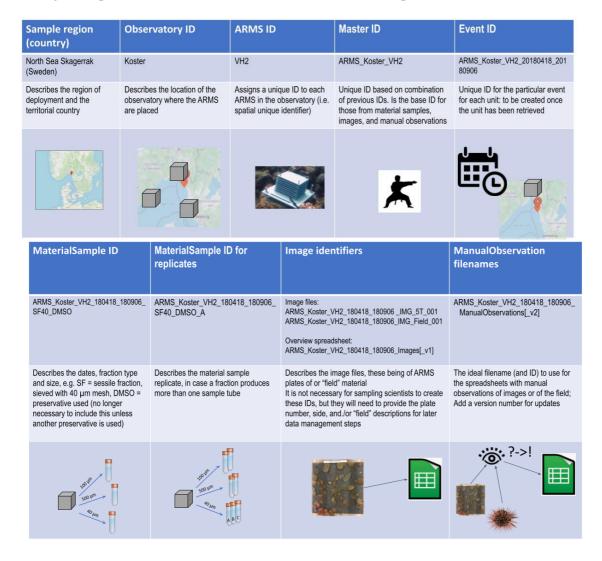


Fig 2. Sample terminology explaining how ARMS observatories and derived samples should be named.

3. Deployment and retrieval

3.1. General guidelines

For deployment and retrieval, ARMS-MBON follows the standards and protocols established by the Smithsonian Institution (https://www.oceanarms.org/), with some additional guidelines.

3.2. Additional guidelines

Base plate. In many cases it is difficult to attach the ARMS to the sea floor. We recommend using commercially-available base plates and drill holes into them (Fig. 3A-B).

Cover container. In order to capture all motile and epi/hyperbenthic fauna on the ARMS, we recommend putting a plastic container over the ARMS before retrieval. The container can be secured with rubber ropes to ensure it stays attached to the ARMS on the way to the surface (see Fig. 5). Openings can be drilled to the sides of the container and lined with 40 μm mesh to allow for partial drain of excess water above the surface to facilitate transportation to the lab, while preventing the smaller portion of epifauna from escaping. Please inform us if you use or do not use a crate cover for each unit you retrieve.







Fig 3. Photograph showing ARMS deployment mounted on a commercially-available base plate (A-B). An underwater buoy is attached to help relocate the ARMS (B). C. Photograph of the plastic container that is put over the ARMS on the seafloor before retrieval (C). Photograph credits A-B: ARMS-MBON network. C: https://www.oceanarms.org/.

4. Sample processing (physical and digital)

For all sample processing steps, ARMS-MBON follows the standards and protocols established by the Smithsonian Institution (https://www.oceanarms.org/). Please do read these carefully: here we outline only the amendments and specific additions to these protocols that customise the ARMS for hard-bottom monitoring in European coastal waters.

Note: the oceanarms.org website is often unavailable, we have therefore downloaded the protocols (as of Oct 2021) and provided them as a zipfile on the ARMS-MBON GitHub site.

4.1. Preservation

ARMS-MBON preserves all biological samples in DMSO (Dimethyl sulfoxide). The recommended recipe is DMSO salt-saturated buffer (20% DMSO, 0.25 M EDTA, pH 7.5, NaCl saturated), as described by Seutin et al. (1991) (https://cdnsciencepub.com/doi/10.1139/z91-013).

4.2. How to deal with large biomass or sediment

Overcrowding with a single or a few species can lead to bias in the amplification of the DNA. This may increase the chance of missing a rare species during the molecular genetic processing, and clearly we want to avoid this! One way to reduce the tissue bias is to include only a small proportion of the crowding species in your homogenisation, although it must be noted that this can similarly increase the chance of missing out a rare species in the actual sample processing. We recommend homogenisation of all tissue **ONLY** if you expect a NIS or a rare species among (or associated with) the crowding species. If that is

not the case, you should reduce the biomass of the crowding species that is homogenised and processed. Appropriate notes should be included with the metadata for each ARMS that approximates the biomass (g) of the removed dominant species.

Sometimes ARMS are heavily covered with sand and silt that can create large sample volumes. In these cases, you need to separate the sand/silt from the organic material before you separate the different fractions by shaking the sample and decanting the organic suspension right after the sand/slit has sedimented. Thereafter you can filter the different fractions (i.e. 100, 500 micrometer).



Fig 4. Overcrowding of the ARMS surface with one or a few dominating species. Photograph credits: ARMS-MBON network.

4.3. Material Samples

From each ARMS you should collect three size fractions (material samples). All material samples should be **preserved in DMSO**:

- Motile fraction sieved with 500 µm (MF500)
- Motile fraction sieved with 100 µm (MF100)
- Sessile fraction sieved with 40 µm (SF40)

In many cases you will produce several Falcon tubes from each material sample. You should label these as described in Secs 2.4 and 5.1, i.e. using the MaterialSampleID. We recommend that you only ship one replicate of each fraction (material sample) and keep the remaining tubes as "backup-replicate" in long-term storage at your institution (see Sec. 6). Please remember to label these backups in the same way as the primary tubes.

Note that while it has been decided to now use DMSO, in the past both DMSO and EtOH were used as a preservative, and this is why the preservative forms part of the MaterialSampleID (Sec. 2.4). From now on, you only need to specify the preservative it is *not* DMSO.

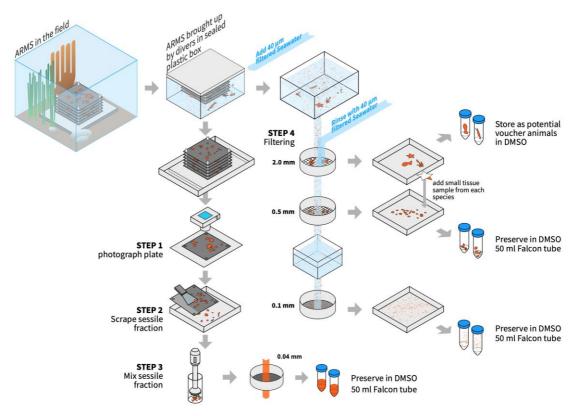


Fig 5. Schematic illustration of sample processing in ARMS-MBON. For details see the protocols established by the Smithsonian Institution (https://www.oceanarms.org/).

4.4. Images

From each ARMS you should take high-resolution images (see Fig. 6 for examples) of

- the plates (mandatory)
- specimens from the plates (optional)
- the habitat and surrounding environment (optional)
- the sampling event (optional)

These images will be uploaded to the data management platform, PlutoF ()

Image-IDs: We appreciate that it is boring to rename all the images that you offload from a camera. However, it is also necessary to be able to know certain things about each image based not only on a label that is photographed with the image, but with metadata provided for each image. Therefore, we request that for each set of images that are uploaded to PlutoF (Sec. 7), a spreadsheet that links each image to these metadata is provided. A template of this spreadsheet can be found on the <u>ARMS-MBON GitHub site</u>. You will be required to provide

- EventID (see Sec. 2.4)
- Plate location (number, counting from baseplate upwards, and face, being Top or Bottom)
- An iterator, if multiple images were taken of the same plate location
- Whether the image is of an ARMS plate, is of a specimen, or was taken in the field

These metadata will be used to construct image IDs, unique to each image you provide

ARMS_<Observatory-ID>_<ARMS-ID>_<DateIn>_<DateOut>_IMG_<Plate-ID>_<###>.tif[jpg|png
For example: ARMS_Koster_VH2_20180418_20180906_IMG_5B_3.jpg

Habitat/Specimen/Event Image-IDs: These images are also to be described in the previously-mentioned template, and the metadata will be used to construct similar IDs

ARMS_<Observatory-ID>_<ARMS-ID>_<DateIn>_<DateOut>_IMG_Field_<###>.tif|jpg|png For example: ARMS_Koster_VH2_20180418_20180906_IMG_Field_2.jpg

All IDs are as described in Sec. 2.4. Plate-ID is to indicate the plate number being photographed: [#][T|B] (for top or bottom), where plate 1 is the plate closest to the baseplate.



Fig 6. Examples of images of plates, close-ups, and isolated specimens, including labeling. Photograph credits: ARMS-MBON network.

4.5 Manual observations

There are two types of manual/visual observations that can be made: "plate or "field" observations, which are visual observations of the ARMS plates or surroundings, and "image" observations, which are an eyeball-based inspection of the images. *Please note that eyeball inspection of images is not the same as eyeball inspection of actual plates:* the difference is not irrelevant, as the source data in the first case are digital image files (which may later be analysed by someone else), while the source data in the second case is your eyeball--brain (and no-one will be able to redo that later).

4.5.1 "Plate" or "Field" observations

In many cases partners make manual observations of species, for example during the ARMS retrieval or the sample processing. These are valuable records that can be added to the images and the sequences. It is therefore important to link these observations to the environment where they were recorded.

If you want to describe your manual observations, you should use a template (see this GitHub folder) where metadata related to the: eventID, type of observation (plate, field, image...), and species information can be filled in. This file should then be uploaded to PlutoF (Sec. 7). It is not necessary to provide an ID for this uploaded file, however a suitable filename should be given from which an ID can be created by the data management team. This ID will be constructed as

If you could name your file this same way, that would be much appreciated.

4.5.2 Image observations

The main difference for manual observations made of images is that you will list which image the observation was made from. Otherwise, the rest is the same as for plate/field observations.

5. Shipment

5.1. Sample labelling and address for destined for ARMS-MBON

To ship samples for centralised processing and sequencing, please prepare following these instructions. Make sure all samples and all falcon tubes are properly labelled with the following information. Please use a printed label and NOT handwriting on the falcon tube (see Fig. 7), as that can be removed in time:

MaterialSample-ID: ARMS_Koster_VH2_20180418_20180906_SF40_A

Observatory-ID: Koster ARMS-ID: VH2

Date in: 2018-04-18 Date out: 2018-09-06 Name: Matthias Obst

Fraction/size: Sessile fraction/40 µm (SF40)

Replicate-ID: A Wet weight: 26 g Preserv: DMSO

Remember to include your ABS permits in your shipment, as specified in the ARMS <u>ABS HowTo</u>: your IRCC code, or copies of emails, and the signed Material Transfer Agreement (<u>MTA</u>).

Ship the sample to the following address:

Melanthia Stavroulaki

Institute of Marine Biology, Biotechnology and Aquaculture

Hellenic Centre for Marine Research (former US Base) Gournes Pediados

71500 Heraklion Crete

Greece (Hellas)

Telephone: +30 2810 33 77 41 Mobile phone: +30 6934 17 71 86

Email: Christina Pavloudi (cpavloud@hcmr.gr)

(Note that the name of the addressee has changed since the last version of the Handbook)

Important: Please make sure you write down correctly our <u>NAMES</u> and <u>TELEPHONE NUMBERS</u> and the full <u>SHIPPING ADDRESS</u>, when sending your parcels. This is very important, since parcels will get lost otherwise. Please also try to remember <u>NOT TO DECLARE ANY COMMERCIAL VALUE</u> for the contents of the parcel and to <u>SPECIFY</u>, with a note on the parcel, that they are <u>ARMS SAMPLES</u> (courier companies can easier recognise the parcel and deliver it to HCMR with no delays or customs issues).

Send an email to <u>Melanthia Stavroulaki (mstavroulaki@hcmr.gr)</u> and <u>Matthias Obst</u> (<u>matthias.obst@marine.gu.se</u>) with the shipping details and dates. When the samples arrive, you will be sent a short confirmation email with a photocopy of the bilaterally-signed MTA.



Fig 7. Samples ready for shipment. Photograph credits: ARMS-MBON network.

5.2. Checklist for the sample package

The following items need to be in the sample package:

- 1. At least three falcon tubes, i.e. at least one tube per fraction and with labels as explained above. We recommend that you keep the remaining, labelled, falcon tubes as "backup-replicates" for long-term storage in your institute.
- 2. Filled out, printed and signed Material Transfer Agreement (MTA)
- 3. ABS declaration of due diligence (see the ARMS <u>ABS HowTo</u> for an explanation of what this is)

Please keep copies of all documents together with the "backup-replicate" samples in your institution.

5.3. Sample labelling and address for destined for ARMS data for the EMO BON project

For those of you who are doing your ARMS work for EMO BON, rather than ARMS-MBON, the shipping address is different: please see your EMO BON Handbook for information on where to ship your samples. EMO BON will then handle all the subsequent stages of your ARMS samples, sequences, and any other digital data.

5.4. What happens next?

For those who have sent their samples to HCMR (i.e. ARMS-MBON project only). Once your samples arrive at HCMR, you will receive a confirmation email (if not, please send a reminder 2 weeks after shipping your samples). The samples will be processed as a batch approximately every 3 months. We sequence the following genetic markers: COI, 18S rRNA (V9 region), ITS1. Once the sequences are produced, they will be uploaded to European Nucleotide Archive (ENA) (under the submission account id Webin-55576: contact Matthias Obst or Christina Pavloudi for access details) and the run accession numbers will be added to the ARMS overview metadata googlesheet. You will have exclusive access to the sequences for a moratorium period of one year (meaning that you will need to log on to the ENA account to access those sequences). Thereafter these sequences will automatically be made public.

In addition, we will periodically run a sequence cleaning, trimming, and analysis for all raw sequences using the <u>PEMA pipeline</u> to generate a consistent data product from the raw sequence data. These data will likewise be made available to you through PlutoF and the ARMS-MBON GitHub pages.

Each year, all data in PlutoF will be linked to a metadata record in IMIS; for more detail see the <u>Data Management Plan</u>.

<u>For those who have sent their samples to EMO BON.</u> See the EMO-BON Handbook for more information on the sequencing steps and on how the sequences are added to ENA and the accession numbers provided to you.

6. Biobanking

We ask all partners to keep at least one "backup-sample" replicate from each of the three fractions of an ARMS sample event, together with a copy of the legal documents (ABS IRCC or due diligence, as explained in the <u>ABS HowTo</u>, and the <u>MTA</u>) as well as a digital copy of all original images from the sample event and from the processed plates. Please mark the samples as described in Sec. 5.1 and place them in a long-term storage freezer at -20 °C or colder in your institute. Similarly, HCMR will be archiving the extracted DNA for future use and cross-validation.

7. Data management

The data management is described in the ARMS-MBON Data Management Plan (DMP) which can be found on the ARMS-MBON <u>GitHub pages</u>.

All the data (metadata and any data files) from the ARMS events is managed by each observatory partner on PlutoF, a data management platform for which you will need to obtain an account. Use of PlutoF for ARMS-MBON is described in this <u>GitHub</u> folder. In PlutoF you will describe your observatories, ARMS units, events, material sample, and you will upload any data such as images and manual observations.

From PlutoF (and including any additional information the observatories send via email or with the samples), information is propagated to an ARMS googlesheet, which is on the ARMS google account but can also be viewed directly here. ENA accession numbers are also added here, once the sequences have been uploaded to ENA.

From PlutoF and from the googlesheets, periodic updates to the entire ARMS dataset (being all metadata and data that exist in these sources) is made in the ARMS-MBON GitHub site: https://github.com/arms-mbon or https://github.com/arms-mbon or https://github.com/arms-mbon.org/ NEED THAT SITE. Note that whether the data are destined for ARMS-MBON or EMO BON, they can be found in the ARMS-MBON GitHub site and the EMO-BON GitHub site (bearing in mind that these sites are still under development and the EMP-BON site is yet to be created).

8. Contacts

Matthias Obst, ARMS-MBON coordinator: matthias.obst@marine.gu.se Melanthia Stavroulaki sample shipping and sequencing: mstavroulaki@hcmr.gr

Christina Pavloudi, ENA submission: cpavloud@hcmr.gr Katrina Exter, Data management: katrina.exter@vliz.be

9. Links

The following documentation can be found on the ARMS-MBON GitHub documentation repository

- Data Management Plan
- PlutoF user guide
- Standard Operating Procedures taken from the Global ARMS Smithsonian site
- Our Molecular SOPs

- Access and Benefit Sharing guides
- This Handbook

The following templates can be found on the ARMS-MBON GitHub template repository

- Describing your photographs
- Creating manual observation spreadsheets
- How to create your various IDs
- Sample labels
- Material transfer agreement template
- Plate label template

Additional links

- The ARMS-MBON space on the data management platform PlutoF: https://plutof.ut.ee/#/study/view/81139
- The ARMS-MBON overview google spreadsheet: https://docs.google.com/spreadsheets/d/1j3yuY5lmoPMo91w6e3kkJ6pmp1X6FVGUt LealuKJ3wE
- The ARMS-MBON page on the ASSEMBLE Plus website: https://www.assembleplus.eu/research/ARMS-MBON

EMO BON website: https://www.embrc.eu/emo-bon

9. Checklist

Important things to remember when performing your ARMS-MBON activities

- Remember to create your Observatory and ARMS unit site pages in PlutoF as soon as they are known. Use the correct IDs please. (Sec. 2.4).
- Remember to create sampling events for each ARMS unit you manage in PlutoF after you have dropped your unit...and then to update it when you have retrieved your unit. Use the correct IDs please. (Sec. 2.4)
- Remember to create material sample pages in PlutoF when you have shipped your samples. Use the correct IDs please. (Sec. 2.4).
- Remember to create an image description spreadsheet and upload that to PlutoF along with your images, so that the plate number etc are documented for each image you provide. Use the provided templates (Sec. 4.4, 4.5)
- Remember that the shipping address is now different if you are working within ARMS-MBON or within EMO-BON. The post-shipping stages are also different, but for you this simply means that you go to a different address to get information about how the sequencing and archiving of sequences in ENA is proceeding. Sec. 5.