Environmental DNA Expeditions in UNESCO World Heritage marine sites

Implementation plan for UNESCO World Heritage marine sites

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1. Introduction

In November 2021, UNESCO launched a global environmental DNA (eDNA) project in UNESCO World Heritage marine sites to study species richness at the sites and their vulnerability to climate change. The project has a duration of two years (1 January 2022 – 31 December 2023) and foresees the collection of eDNA in about 25 UNESCO World Heritage marine sites, through 500 water samples obtained through a syringe (21 samples available per UNESCO World Heritage marine site). The project will concentrate on fish species and other vertebrates that reflect the Outstanding Universal Value of marine sites on the UNESCO World Heritage List. All data will be made available free-of-charge to the site management and be processed and published on the UNESCO Ocean Biodiversity Information System (OBIS), the world's largest open-access data system on the distribution and diversity of marine species.

Environmental DNA is DNA that is collected from a variety of environmental samples such as soil, seawater, snow or air, rather than directly sampled from an individual organism. Environmental DNA sampling is user friendly, cost effective, and non-invasive, allowing the active engagement of citizens in sample collection. It has the potential to revolutionize the world's knowledge on ecosystems and species biodiversity.

The project will engage local citizens, guided by international experts, to collect water samples at UNESCO World Heritage marine sites, filter them and preserve the eDNA, which will then be processed and sequenced by a specialized university laboratory that is contracted by UNESCO.

The project is guided by an international advisory board that brings together some of the world's leading science and expertise in molecular ecology, eDNA, bioinformatics, fish metabarcoding, ocean science.

The project is a joint collaboration between UNESCO's World Heritage Centre and UNESCO's Intergovernmental Oceanographic Commission (IOC) and aims to achieve the following results:

- Measure species diversity through eDNA sampling and estimate the vulnerability of fish species to climate change across about 25 UNESCO World Heritage marine sites;
- Inspire and educate the next generation of ocean scientists through the participation of local youth in the collection of eDNA, thereby increasing awareness of the importance of the protection of the UNESCO World Heritage marine sites;
- Showcase how eDNA can revolutionize marine biodiversity monitoring and facilitate the work of local managers, by using case studies from UNESCO World Heritage marine sites;
- Promote the standardization of eDNA sampling and analysis protocols and the principle of open science. Environmental DNA sampling is already being used at about 20 UNESCO World Heritage marine sites but differing techniques make it difficult to combine and compare datasets. Importantly, much of the data is not openly accessible.

More information of the project:
https://www.unesco.org/en/edna-expeditions

2. Choosing the sampling locations

UNESCO World Heritage marine sites will be responsible for choosing the final sampling locations to ensure that the obtained data is as useful as possible for conservation and management. The following guidance for the choice of sampling locations apply:

- For each UNESCO World Heritage marine site, the aim should be to choose 5 locations with different habitats and take 4 replicate samples at each location (replicate samples are samples taken with the same methodology at the same time and in close proximity). It is recommended to take replicate samples within 5-10 meters from each other, but this can vary depending on the size of the habitat being sampled.

- Since the samples will be taken in surface water, the total water depth of the sampling locations should be shallower than 15 m (to ensure that the collected eDNA is representative for the sample location).

- Each sampling will provide a snapshot of marine biodiversity. The objective is to take all samples during one day across the UNESCO World Heritage marine site. If this is not possible, there is flexibility to choose two different dates for sampling. *Note: the objective of this pilot campaign is <u>not</u> to sample time-series (repeated sampling at the same location).*

It is recommended that UNESCO World Heritage marine sites choose the sampling locations considering the below points.

2.1. Known target species distribution

The project's main target species are fish (bony fishes, sharks and rays) and other marine vertebrates which are part of sites' Outstanding Universal Value, in particular those present on the IUCN Red List of Threatened Species. During sample processing, the analysis will focus on fish, other marine vertebrates as well as other metazoans. It is recommended to sample in locations where the target species is most abundant (and thus where there is more genetic material available), or where eDNA is likely to survive the longest (locations with less currents, less wave action, less turbidity, less coastal runoff).

The description of Outstanding Universal Value for each UNESCO World Heritage marine site is available here: https://whc.unesco.org/en/list/?search=&themes=7&order=country

More information about the IUCN Red List of Threatened Species is available here: https://www.iucnredlist.org/

2.2. Habitat

The objective of the project is to get a snapshot of sites' marine biodiversity. Therefore ideally multiple habitats should be included in the sampling, to ensure as much biodiversity as possible is caught during the sampling campaign. If possible, 5 different habitats should be chosen and at each habitat 4 replicate samples should be taken. The replicate samples should not be further than 5-10 m from each other. Depending on the location, examples of important habitat types include: seagrass beds, coral reefs, kelp forests, maerl beds, sand, mud, rock, polychaete reefs, mussel beds, mangrove forests, bare sediment, etc. The habitat type should be recorded on the sample information sheet (see section 6).

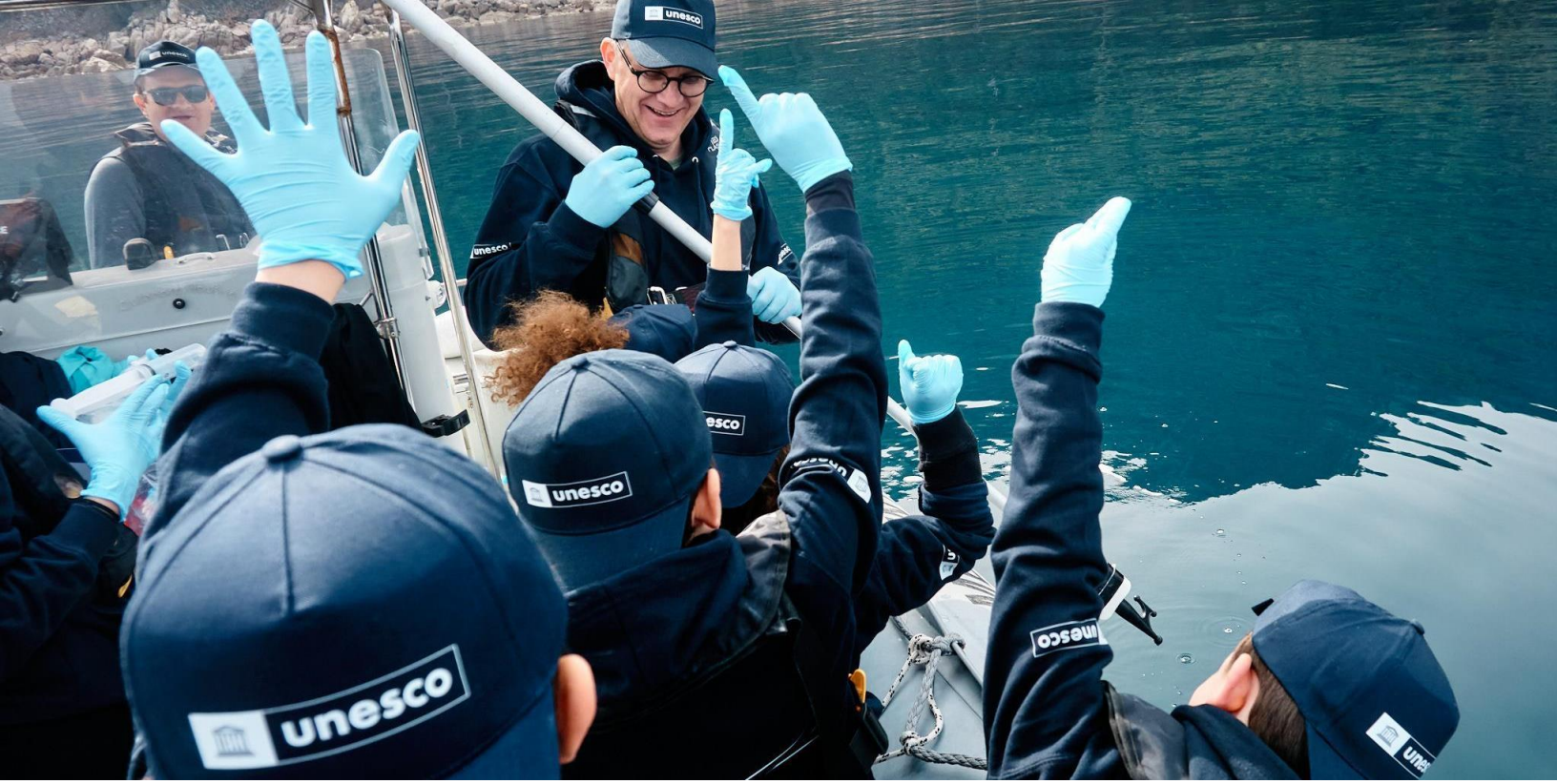
2.3. Season/sampling time

Timing of the sampling should take into consideration the seasonality of marine biodiversity. In addition, the time of day for sampling, and the tide, should also be taken into account. Ideally sampling is done through the peak time or mating/spawning season of the target species, when there is most genetic material in the water. Note that many fish species move to shallow waters for mating and move to deeper waters in the winter, while some species prefer cold deep water in the summer season. There might also be other considerations which are important for choosing the best sampling time, such as logistics, weather, etc. and **above all, safety comes first**.

2.4. Sources of contamination

The choice of sampling locations should take into account any sources of contamination that could interfere with the biodiversity signal. The following areas should be strictly avoided: sewage runoff (either from underwater pipes or from rivers), bilge pumps of vessels used for sampling as well as other vessels, and other places of human activities. Special attention should be given to avoid sampling in or near fish cleaning stations if local communities in your UNESCO World Heritage marine site are known to use fish cleaning stations to discard garbage or unwanted catches caught far away. Otherwise a sample taken at a fish cleaning station could find deep water fish in a coral reef, for example.

It is highly recommended to avoid taking samples inside or close to a port or river plume, because contamination might have a huge impact (because of sewage pipes, more invasive species, contamination from boats, people, etc.). If it is completely unavoidable to take samples near a port, it is recommended to take samples on the outer side of the port, not inside the port.



3. Choosing your sampling community

As an initial step after the required permits are in place and the time of the sampling has been chosen, the UNESCO World Heritage marine site should identify an appropriate group of local citizens to undertake the sampling. It is encouraged to hold information sessions before the sampling campaign on the importance of protecting the UNESCO World Heritage marine site, and on what is eDNA. UNESCO can assist in organizing such information sessions.

At least two or three people are required for the collection of each sample, but each pair can collect multiple samples during the sampling day if so required.

The sampling campaigns will constitute a crucial element of the global communication campaign. UNESCO World Heritage marine sites are invited to contact UNESCO to prepare the communication well in advance (photography, video, potential participation of journalists, etc.)

4. Required agreements

4.1. Permits for sampling

Each UNESCO World Heritage marine site will ensure, with support from their national focal points and, where needed, UNESCO, that all required sampling permits are in place. These might include local permits (e.g. a permit for scientific research in your site), and/or permits in the context of the Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization (or “The Nagoya Protocol”). Samples which have been collected without the required permits can not be taken into account by the project.

The Nagoya Protocol is a supplementary agreement to the Convention on Biological Diversity (CBD). Almost 140 countries are currently a party to the Nagoya protocol.

The Nagoya Protocol aims to avoid, for example, that an entity from country X collects a DNA sample in country Y, and then earns benefits from the knowledge gained (for example through a patent, or by using it in a medicine), without sharing those benefits with the local communities from country Y.

The UNESCO eDNA project does not have a purpose linked to monetary benefits. Environmental DNA will be used only as a tool for the taxonomic identification of species through the analysis of short DNA sequences. No specific genetic and/or biochemical properties of the function of genes will be analyzed, therefore it is considered that the project does not constitute utilization of genetic resources and therefore falls outside the scope of the Nagoya Protocol.

However, the Project Team advises that the UNESCO World Heritage marine site consults with the national focal point and confirm agreement with the interpretation of the Project Team, (i.e. that metabarcoding of genetic biomarkers does not fall under the Nagoya Protocol), or, if your country is not a signatory to the Nagoya Protocol, if there is any specific legislation under your national law regarding marine genetic resources. It is also recommended to collect advice from scientists who have already collected genetic material in your country, and who will typically also have this information.

The data collected during the project will be used to build a snapshot of baseline information on marine biodiversity at the UNESCO marine World Heritage sites. Further benefits to the UNESCO World Heritage marine sites include:

1) increased knowledge about the marine biodiversity of the UNESCO World Heritage marine site which is useful for conservation and management,
2) increased knowledge on the climate change vulnerability of the UNESCO World Heritage marine site's marine biodiversity which is useful for conservation and management,
3) increased capacity of local site management to use eDNA for biodiversity monitoring,
4) outreach to citizen scientists, young people and World Heritage stakeholders on the importance of World Heritage conservation and ocean science,
5) education.

Country focal points for the Nagoya Protocol are available here: https://absch.cbd.int/en/countries

UNESCO will not use the eDNA samples for purposes other than this project. The sample processing will be undertaken by the KU Leuven in Belgium, which has been contracted by UNESCO for this work. The contract between the laboratory and UNESCO ensures that the samples cannot be used for other purposes than this project.

Before sampling begins, each UNESCO World Heritage marine site is requested to:

- Complete the project's Collaboration Consent Form with UNESCO (Annex 1) and agree on the open sharing of data resulting from the project as detailed in the project's data policy (Annex 2).

- If legally required by your country, also complete a Material Transfer Agreement. A Material Transfer Agreement is a legal contract between the entity sending the sample abroad, and the entity receiving the sample (in this case, the laboratory contracted by UNESCO). Please note that drawing up a Material Transfer Agreement might require a substantial amount of time and approvals. It should only be undertaken if strictly necessary by country regulations.

4.2. Biobanking of samples

To ensure that the samples are not used for any purposes other than this project, all remaining samples or genetic material will be automatically discarded by the UNESCO-contracted laboratory at the end of this project.

However, recognizing that samples provide a unique genetic snapshot which might be useful for future researchers, and acknowledging that a substantial amount of investment has been made to collect the samples, UNESCO World Heritage marine sites may also wish to provide the samples for permanent archiving at a biobank. As owners of the samples, this decision is up to the respective UNESCO World Heritage marine sites, and might come at additional costs.

Benefits of adding the samples to a biobank include:

- Preserving the 'genetic heritage' of a UNESCO World Heritage marine site for future generations,
- Future researchers can do more comprehensive analysis, or time series analyses,
- The future discovery of a new biomarker might reveal different species.

If a UNESCO World Heritage marine site is interested in preserving their sample (or one of the samples) in a biobank, it is recommended to contact the Project Team who can provide further information.

5. Materials for sampling

UNESCO will provide 21 sampling kits to each participating UNESCO World Heritage marine site. The kits will contain all material required for sampling. 20 of these kits will be for filtering marine water for the analysis of marine biodiversity. One kit will be provided to be used as a negative control. The kits will contain the individual filter, syringe, sample bag and sample information sheet which are required for clean sampling (see section 5.1 for details). Additionally, a sampling bottle, thermometers and safety glasses will be provided by UNESCO as part of the package and will need to be reused between the sampling locations and samples.

The following sections provide further details on sampling material.

5.1. Sampling kit contents

UNESCO will provide 21 sampling kits to each participating UNESCO World Heritage marine site with the following contents:

1. Two pairs of gloves for clean sampling, size M
2. 1 clean and sterile sample bag (2 l volume)
3. 1 falcon tube (15 ml) to collect a water sample for salinity
4. 1 sterile 60 ml syringe
5. 1 dual 0.8 um PES filter with a valve to allow continuous sampling

6. 2 caps to close the filter
7. Preservation liquid, composition:
    a. Longmire's buffer: 100 mM Tris, 100 mM EDTA, 10 mM NaCl, 0.5 % SDS
    b. A positive control: DNA from *Artemisia annua* at about 100 copies.
8. 3 waterproof labels with the unique sample ID, to be added to i) the filter, ii) salinity sample and iii) sample information sheet
9. Sample information sheet, and an infographic with the 9 key sampling steps

5.2. Equipment for sampling

In addition to the sampling kits, UNESCO will provide the following material for sampling:

- 4 simple aquarium thermometers for measuring temperature at the sampling locations
- A telescopic rod (95-280 cm) with an angular beaker of 1 liter volume
- 4 plastic containers in which the sampling bag can be placed during filtering (for support)
- 4 clipboards as a support for writing
- 21 pencils for writing down sampling information
- 4 safety glasses, for use when adding the preservation liquid
- 50 UNESCO-branded caps
- 5 copies of the Field Sampling Booklet with detailed written instructions

5.3. Materials to be acquired by the local sampling team

In addition, the local sampling team is kindly asked to bring:

- One bottle (1.5 liters) of drinking water required for the negative control,
- A cooler or box to store the collected samples with the filters and shield them from direct sunlight once they are completed (UV radiation will degrade the DNA). There is no need for cold storage, but in places with very high temperatures, some cooling of the samples can be beneficial,
- If easily available, extra gloves can be brought for clean sampling.

5.4. Available training information and instructions

UNESCO will provide several training materials to show how eDNA collection with the sampling kits is done.

UNESCO will provide the following training materials in English, French and Spanish:

1) Field Sampling Booklet with detailed instructions on how to use the sampling kits in the field (5 copies will be part of the package sent by UNESCO),
2) Sampling instruction video (will be available on the project website, and on the sample registration application),
3) Infographic with the 9 key sampling steps (each sampling kit will contain one copy).

Prior to undertaking the sampling, a video call with UNESCO can be organized to go over the sampling plan and the sampling kits in case any steps are unclear.

5.5. Shipping and return of samples

All materials (listed in sections 5.1 and 5.2) will be sent by UNESCO in one box to each of the participating UNESCO World Heritage marine sites after receipt of the Collaboration Consent Form. In addition to the above, the box will contain the shipping labels required for sending the completed eDNA samples back to UNESCO. After sampling, only the completed samples should be returned to UNESCO, no other materials of the sampling kits need to be returned.

The small package which will be shipped back to UNESCO should contain 21 bags (one for each sampling), each containing:

1. One collected eDNA filter (preserved and capped) in its own little ziplock bag
2. One collected salinity sample (15 ml of water)
3. One completed sample information sheet

The filter, salinity sample and sample information sheet should be labeled with the sticker containing the sample ID provided in the sampling kit bag. Each UNESCO World Heritage marine site is expected to return 20 filters with eDNA from marine samples, and 1 negative control filter. For more details on the sampling protocol see section 7.

It is not necessary to return other elements from the sampling kit (thermometers, rod, clipboards, safety glasses, UNESCO-branded caps, etc.). The return shipping label is prepaid and covers the costs of the shipment of the samples

The shipping labels will already include the shipping address to return the samples:

UNESCO / IOC Project Office for IODE
InnovOcean Campus
Jacobsenstraat 1
8400 Oostende
Belgium

6. Data collection during sampling

During sampling, data should be collected linked to each separate sample, and by each sampling team. The data should be registered two times, as follows:

1. In writing on the sample information sheet which will be provided with each sampling kit (a copy for information can be found in Annex 3)
2. In the sample registration application (see section 6.1. for how to download the application to your phone at: https://app.ednaexpeditions.org/).

The data will be linked to each separate sample, therefore the local sampling team should instruct the participants to download the sample registration application before the start of the sampling campaign.

The metadata (data which provides information about other data) collected during sampling is of high importance for the sample data analysis, and will allow to accurately report all of the obtained results (see list of data that should be registered in sections 6.2 and 6.3). Samples for which the required metadata (e.g. sample ID, location and time) was not registered, will not be processed. It is important to record the emails of the people involved with each unique sample so that at a later stage these citizen scientists can track what happened with the sample they collected, and which species were found.

6.1. Download sample registration application

Before starting the sampling campaign, make sure that everyone who will register the samples in the field has downloaded the sample registration application. The sample registration application will be used to collect information about the samples during the sampling campaign. The sample registration application can be downloaded onto any mobile phone. Each pair/group of people filtering samples should have the sample registration application available for use. To download and use the sample registration application correctly, follow these instructions:

1. Go to the address https://app.ednaexpeditions.org/ on your mobile phone
2. The page will open a prompt to ask to add to the homescreen. Allowing this will show the application as a normal application in the phone (no need to go to the website again).
3. Allow location services for the application. These are needed to register the exact location where samples were taken. All information on the application is strictly used in the context of this project.
4. Register with your email, and you are ready to go! Please note that data can only be shared with UNESCO once you are registered, and logged in.
5. To start sample registration, press Add sample.

Once downloaded, the sample registration application will also work without internet access. After recording the sample information, press save. The information will be automatically uploaded once the mobile phone has access to the internet again. To ensure that all information is uploaded after sampling, make sure to open the sample registration application at least once after your internet connection has been restored.

The sample registration application provides access to the sampling instructions in both written and video format.

Finally, the sample registration application makes it also possible to upload pictures and images of your sampling initiative. The Project Team highly encourages everyone to upload imagery of your sampling campaign, including "Sampling Selfies"!

The information which needs to be recorded on the sample registration application (https://app.ednaexpeditions.org/) and on the sample information sheet is detailed below.

6.2. Required information to be recorded

The below information should be recorded with each unique sampling kit, and has separate fields in both the sample registration application and the sample information sheet:

1. Sample identifier (the code located on three labels included in the kit, e.g. “EE0018”)
2. Name and contact details (email) of a contact person or the person(s) sampling
3. Date and time when the sample is taken
4. Amount of water filtered
5. Location coordinates as decimal degrees (can be extracted from the app)
6. Water temperature
7. Salinity sample (indicate in the sample information sheet and application when the sample is collected)

6.3. Other additional information

Local teams are invited to collect further general information during the sampling that will help with the sample analysis. Local teams are encouraged to include as much information as possible about the conditions of the sampling location. Any further remarks can all be added on the sample information sheet, or on the sample registration application (this information can be registered only once). For example:

8. Time used for filtering
9. Total water depth at sampling location
10. Biome (description of the habitat)
11. Visible algal bloom presence/absence
12. Weather conditions during sampling

6.4. Photos and “Sampling Selfies”

In addition to the collected sample metadata, it will be possible to upload photos with the sample registration application. Photos can be used to show the habitats/biomes at the location of sampling, but also to show the people sampling and the activities during the day. The Project Team encourages sharing your experience during the sampling through social media channels like Twitter, Instagram, TikTok or Facebook using the project hashtag #eDNAexpeditions. You could also use the hashtags #WorldHeritage and #OceanDecade. Photos will help to build the global community around the sampling campaigns.

UNESCO marine World Heritage sites are invited to upload photos here:
https://drive.google.com/drive/folders/1_8f3nkSzUsQa77izPc_VKUO39GAIw0q2?usp=sharing

If people take a “Sampling Selfie”, do not forget to ensure they are wearing a UNESCO cap.

7. Sampling protocol

The citizen science eDNA sampling will be based on filtering marine water samples collected from the sample locations. The goal is to collect 20 samples from each UNESCO World Heritage marine site, as well as filter one negative control sample (local bottled water) at the end of each sampling campaign. The objective will be to filter approximately 1.5 liters of water with each filter, and to preserve the DNA in the filters using the sampling kits. The Field Sampling Booklet provided to the UNESCO World Heritage marine sites will contain more elaborate instructions, with images for each step to help with the explanation.

Each sampling should be performed by at least two or three people, the same people can collect multiple samples. While one person is performing the sampling, the others should be writing down the collected information, holding the sampling bag during filtration and making sure that all sampling materials are collected in a clean way back into the sampling kit bag.

After 20 marine water samples are filtered, at the end of the sampling day, one team of people should filter the 1.5 liters of bottled water with the 21st sampling kit. This will be the negative control, and will be used to rule out common contaminants. Therefore, this water should also be filtered at the sampling location, to get an accurate signal of possible contaminants.

The sampling protocol with images of each step will be provided together with the sampling kits (5 paper copies of the Field Sampling Booklet for each UNESCO World Heritage marine site). The instructions will also be available through the sample registration application.

7.1. Step 1: Preparation

1. When you have arrived at the sampling location, open the sample registration application and take the sample information sheet.
    a. Write down the email of the person(s) sampling
    b. Record the **sample ID** (the code located on three labels inside the kit, e.g. “EE0018”)
    c. Record the **sampling time and location** by tapping on the application and write down the same information on the sample information sheet
    d. Write down also any notes on the sampling site: what is the biome, are there any visible blooms of algae, or any other organisms
    e. Remember you can also add photos of the site and of the sampling, to the application. This will be helpful in determining how the conditions were at the site, and in communication about the project
2. For salinity, collect 15 ml of water from the sampling location in the separately provided 15 ml tube, then return the tube to the sampling kit.
3. Record the surface water temperature at the sampling location with the use of the provided thermometer. Add to the application, and write down on the sample information sheet.

7.2. Step 2: eDNA sampling

Warning: Wear gloves at all times during sampling. Do not touch anything else than the sampling equipment to avoid contamination. Also, consider where to store the sampling equipment as it should be kept as clean as possible.

4. Rinse the sampling bottle with the rod three times with water from the sampling location.
    a. If you are close to the shore, aim to take samples as far away from land as possible with the rod, to avoid runoff from land

5. Collect the sample, make sure to immerse the bottle approximately 30 cm below the surface.
6. Prepare the sample bag. Rip open the top, and open the bag using the holders on the sides.
7. Place the sample bag inside an empty plastic box (for support).
8. Fill the sample bag 2x with water collected with the bottle, in total about 2 liters of water. One person should hold the sample bag, while the other does the filtering.
   Note: If you are in an area with a sandy bottom, let the sample water stand for a few minutes before filtering, to allow the sand to settle at the bottom of the bag.
9. Attach the filter to the syringe.
10. Be careful, proceed slowly, the next step will require some effort and patience. Take turns filtering if needed.
11. While the inlet of the filter is submerged in the sample water, filter the water from the bag by pulling in the plunger.
12. Discard the water outside of the bag by pushing out the piston. The water will come out of the other end of the valve.
13. Count the number of times the syringe is filled and/or the amount of water that you have filtered, in the sample registration application and on the sample information sheet.
14. When the filter is full, the filtering will be slower. To keep filtering, pull out the plunger of the syringe and allow the water to flow in slowly. Depending on the locality, there may or may not be color visible on the filter.
15. **Aim to repeat 20-30 times** (25 times = 1.5 liters) by continuously pumping the syringe and keeping the (bottom part of the) filter submerged. The final amount of water that can be filtered will depend on local conditions. Stop if the filtering is too slow, or is taking too long (for example one hour). The filter is then clogged.

    Warning: Avoid air bubbles in the filter, this will cause the filter to malfunction. To prevent air from being sucked in, let the plunger of the syringe loose before lifting the dual filter capsule from the water. If air bubbles appear in the filter while filtering, they can easily be removed. For example, if the filtering is hard already after <10 times, you may have an air bubble that is clogging the filter. See **Additional part: In case of an air bubble.**


7.3. Step 3: Preserve the sample

Perform these steps once you have filtered the volume of the syringe 25 times (1500 ml) or for more than one hour.

Warning: This step should be performed by an adult wearing gloves and safety glasses.

16. Empty the filter from water by sucking in air with the syringe.
17. Remove the filter from the syringe.
18. **Wear the safety glasses**, be very careful with the preservation liquid, do not swallow, avoid contact with skin and eyes, and do not discard in the environment.
19. Close the top end of the filter with the cap from the small syringe.
20. From the bottom end of the filter, add the preservation liquid to the filter from the provided small syringe.

Warning: Be careful, when adding liquid, pressure builds in the filter. Relieve the pressure by lifting the plunger before you remove the syringe (replacing the liquid with air from the filter in the small syringe).

21. Close the bottom end of the filter with the second cap provided in the sampling kit.
22. Attach the three labels provided in the sampling kit to **a)** the filter, **b)** the salinity sample and **c)** the sample information sheet. Make sure that the same sample ID is registered on the app. Make sure the surface of the filter is dry before applying the label.
23. Close the filter in its own ziplock bag. This will allow the filter to stay clean also when starting sample processing.
24. Close all materials including the filter (in its own bag), the salinity sample and the sample information sheet in the provided sample bag, and return back to the site management. Save the sample information collected in the app.

Warning: Keep the filters sheltered from the direct sunlight at all times, UV radiation will degrade the collected DNA

Note: When finished with the sampling kit, remember to save sample information also on the application by pressing save. Dispose of any other waste in a sustainable and clean manner.

7.4. Step 4: Filtering the negative control

25. Repeat the sampling protocol for 20 sampling kits across the predefined sampling locations. After all marine samples have been filtered, filter the negative control (1.5 liters of bottled water) while still in the field using the exact same protocol.
26. Start by recording the sample ID, and indicating on the sample information sheet and in the notes in the app, that this is the negative control. There is no need to collect environmental metadata.
27. Pour the bottled water in the sample bag, and proceed with filtering as before (follow steps 11-30).
28. As soon as all 21 samples are collected, the samples should be sent to UNESCO.

All samples should be collected together with the sampling team, kept in the dark, and at room temperature conditions, if possible (e.g. 20 °C). For example the organizers can bring one cooler to the sites, and once the filters are brought back to land, store them in an air-conditioned room. The most important thing is to keep the filters away from direct sunlight, the filters and DNA will be ok with some heat.

7.5. Additional step: In case of an air bubble

Warning: Only perform the steps 29 to 35 if you suspect you have an air bubble clogging the filter! Otherwise move on to step 16 when you have finished filtering.

Air bubbles block the filter membrane and have a negative effect on the filter capacity. These air bubbles are easy to remove from the capsule, by flushing a small amount of sample water through the outlet of the filter (reverse direction).

29. Remove the filter from the syringe, and the valve from the filter from the silicone tubing connected to the valve.
30. Fill the syringe with sample water.
31. Connect the syringe to the outlet of the eDNA dual filter capsule.
32. Turn the filter upwards, so that any air bubble moves up to the inlet.
33. Push in water slowly from the syringe to the filter.
34. Hold the capsule upwards and push the syringe plunger until all air bubbles have been expelled from the capsule. **Warning:** do not allow water to be flushed through the filter, this will result in the loss of already filtered material.
35. Reconnect the capsule and syringe to the valve connector and proceed with sampling.

8. Sample processing and analysis

UNESCO will forward the samples to the university eDNA laboratory contracted for the project: the university KU Leuven (Belgium). The eDNA laboratory will be responsible for the processing of the samples as well as the sequencing of the DNA for marine biodiversity information. A metabarcoding approach will be taken to describe the taxonomic diversity found in the collected DNA. It will be possible to check on the project website when samples have been received by UNESCO, when they have been dispatched to the lab, and when processing has been completed. The four stages of the sample processing workflow are detailed below.

8.1. DNA extraction

DNA is extracted from the collected eDNA filters in a clean laboratory. The DNA will be extracted ensuring maximum recovery of eDNA, and minimizing contamination during the process.

8.2. Amplification of target genes

Defined target genes chosen for their differentiation across species will be amplified using the polymerase chain reaction (PCR). Four different target genes will be amplified. The short target regions (e.g. < 600 base pairs long) are part of the mitochondrial genome, are very well known, and are not useful for other purposes than determining the taxonomy of the species in the sample. Each of the four target genes will target a different subset of animals. The project focuses on fish but the primers will be chosen largely to allow them to also capture other species that are part of sites' Outstanding Universal Value. Therefore, the target genes will be chosen to target the biodiversity of 1) fish, 2) sharks and rays, 3) vertebrates and 4) all animals, with the aim to arrive at a snapshot of marine biodiversity at the UNESCO World Heritage marine site, and therefore capturing the species that are part of sites' Outstanding Universal Value and part of the IUCN Red List of Threatened Species. However, whether a species is detected or not depends on many factors during sampling as well as processing, and therefore cannot be guaranteed.

8.3. Sequencing

Sequencing will be performed by the contracted eDNA laboratory. Sequencing of the amplified target genes will be done using an Illumina MiSeq platform. Approximately 100,000 reads will be generated for each sample.

8.4. Data analysis

The DNA sequences achieved from the sample processing will be processed by the UNESCO Project Team. The taxonomic assignment of the DNA sequences will be done with a bioinformatic pipeline that is publicly available, and based on publicly available genetic reference databases.

The bioinformatic analysis is done by cleaning and trimming DNA sequences based on quality, clustering all identical DNA sequences to amplicon sequence variants (ASVs) and taxonomic assignment of these DNA sequences by comparing to reference databases. The reference databases contain known, named DNA sequences of the target gene. The target gene regions (the sequenced regions) are chosen because of their power in taxonomic differentiation. Small differences in the short DNA sequences indicate different species. By comparing the DNA sequences to the reference databases the DNA sequences are named to the closest available known DNA sequence. The level of similarity will determine how precisely the taxonomy will be defined, e.g. if the DNA sequence is only 80% similar to a known (named) DNA sequence, it might be assigned a name at the Family or Class taxonomic rank.

Local scientists working on the project will be encouraged to check the taxonomic assignments, to identify any possible cases of mislabelled DNA sequences. Taxonomic assignments will be published on the UNESCO Ocean Biodiversity Information System (OBIS). Due to the incompleteness of genetic reference databases many DNA sequences are expected to remain unclassified, or classified only to a higher taxonomic category. However, these sequences will also be included in the datasets, and will give an idea of the remaining gaps in the reference databases.

8.5. Data sharing

A fundamental part of the project is the open sharing of project protocols and data. It is a prerequisite for participation to agree to open data sharing (Section 3.1). All protocols and methods will be published as a guide on the IOC Ocean Best Practices System (OBPS) and GitHub, allowing full transparency and reproducibility of the scientific results.

According to good scientific practice, raw (unanalysed) DNA sequence data will be submitted to public sequence databases that are part of the International Nucleotide Sequence Database Collaboration (INSDC) like NCBI or ENA. The INSDC is committed to the open sharing of DNA sequence data, and in so doing enabling scientific work, and has been one of the most celebrated global initiatives in the open sharing of data. Most scientific publications have required the open sharing of DNA sequences since the 1990s, a practice that has ensured the development of molecular sciences and was instrumental also in the rapid global response to the COVID-19 pandemic. The sharing of sequence data ensures that derived scientific results can be confirmed and checked by other scientists working in the field.

Species lists resulting from the analysis of the DNA sequence data will be shared through the UNESCO Ocean Biodiversity Information System portal using the Darwin Core Archive data structure and the DNA-derived data extension. Data will be summarized on the project website, where citizen scientists will be able to track their samples, receive notifications when results are available, and explore the data for their sample or site. Through the portal it will be possible to discover sampling sites on a map, and explore species lists, detections of charismatic megavertebrates, and biodiversity indicators for each UNESCO World Heritage marine site. A special emphasis will be put on showcasing the number of species categorized to global, local, endemic and threatened categories, as well as unknown species. The sampling, analysis and results will give priority attention to vulnerable and endangered species as specified through the IUCN Red List of Threatened Species.

9. Scientific analysis

The aim of the scientific analysis is to illustrate the baseline information that can be acquired about marine biodiversity by eDNA analysis for conservation, and with small-scale citizen science sampling. A UNESCO-authored flagship publication will be developed, which is expected to illustrate the central importance of UNESCO World Heritage marine sites as some of the world's last refuges for vulnerable and critically endangered species as defined in the IUCN Red List of Threatened Species.

The key outcome of the publication is expected to highlight the impact of climate change on species trends and distribution patterns and provide preliminary insights toward potential changes that might be needed to secure the effective conservation of the Outstanding Universal Value of UNESCO World Heritage marine sites over the next decades given the rapidly changing ocean environment as a result of climate change.

All participating UNESCO World Heritage marine sites involved in the sampling campaigns will be involved and credited in the final publication, alongside the members of the scientific advisory board that joined the effort. The Project Team will ensure that UNESCO World Heritage marine sites are invited to nominate co-authors and/or contributing authors of the publication, and indicate the agencies which should be mentioned in the acknowledgements.

The key outcome of the publication will be obtained through the comparison of existing species inventories in the UNESCO Ocean Biodiversity Information System (OBIS) combined with species occurrence data collected from the sites to the species occurrence data collected with eDNA sampling. Projections of future climate as well as known distributions and environmental ranges

of species will be used to estimate how species assemblages might be reacting to climate change at each UNESCO World Heritage marine site, and if the boundaries of the sites are expected to remain relevant for their conservation according to different IPCC climate change scenarios. Community Thermal Indices (CTIs) will be calculated to establish a baseline for measuring future shifts in community composition at the sites. The work will be coordinated with the help of leading eDNA scientists, ensuring that the analyses and conclusions of the publication are according to the best available scientific knowledge and understanding.

The key outcomes of the scientific publication include:

- Provide a one-off biodiversity snapshot, with focus on fish and megavertebrates of which several are on the IUCN Red List of Threatened Species
- Determine the richness of
    - Fish species
    - Reptiles (turtles, snakes, etc)
    - Cetaceans (whales, dolphins)
    - Other marine vertebrates
- Combined with future projections of ocean warming, an analysis will be made of how climate change is affecting the world's most exceptional marine biodiversity

Other possible outcomes of the scientific publication could include:

- Perform gap analysis, number of identified species vs number of dark taxa (for which no sequences are available in reference databases)
- Develop indicators
- Discover changes and trends in community species composition
- Discover the presence of rare, endemic or endangered species (IUCN Red List of Threatened Species)
- Discover food web structure and the ratio of large pelagic fish (high trophic level) vs small, cryptic fish (low trophic level)
- Discover the presence of charismatic megavertebrates (sharks, cetaceans, turtles, etc)
- Assess our knowledge by comparing the eDNA results against the data in the UNESCO Ocean Biodiversity Information System, completed with literature reviews and local knowledge available at the UNESCO World Heritage marine sites.

9.1. Existing research information on biodiversity already available at UNESCO World Heritage marine sites

In the second half of 2023, all eDNA data will be processed. In combination with ocean warming projected scenarios, an analysis will be made of how climate change is affecting the world's most exceptional marine biodiversity. If UNESCO World Heritage marine sites or partnering scientists / scientific institutions already have relevant datasets available, they are invited to share this information with UNESCO. Such datasets could include, for example:

- Results from earlier eDNA analysis in UNESCO World Heritage marine sites
- Earlier calculations of Community Thermal Indices (CTIs)
- Any existing data on biodiversity in UNESCO World Heritage marine sites. If available, it is recommended to include spatial and temporal information, as well as references to related reports, research papers, online datasets, etc.
- Maps or other spatial datasets on the distribution of habitat types within the UNESCO World Heritage marine site
- Reference datasets of local species, if available.

While species lists can be useful for the project, it is recommended to include the following fields when sharing biodiversity datasets:

- Highly recommended:
    - Species name or full taxonomy
    - Time
    - Location (including uncertainty if available)
- Nice to have:
    - Quantitative information (abundance, biomass)
    - Sampling methodology, protocol, SOPs
    - Taxonomic scope of the study or dataset
    - Links to voucher specimens
    - Links to papers or reports

Some examples of biodiversity papers, reports, and datasets

- Seabed biodiversity on the continental shelf of the Great Barrier Reef World Heritage Area
- Fish diversity patterns along coastal habitats of the southeastern Galapagos archipelago and their relationship with environmental variables
- Wadden Sea Quality Status Report
- Marine Habitats of Western Australia

10. Global communication

Communication and outreach play a major role in this project through a professionally run UNESCO global media campaign. Communication will be built around 1) the sampling campaigns in the UNESCO World Heritage marine sites (September 2022 - April 2023), and 2) the launch of a UNESCO flagship publication summarizing the results and outcomes of the project (end of 2023 / early 2024).

UNESCO World Heritage marine sites and citizen scientists will be encouraged to document sampling efforts through images and blog posts that will be shared through the website and social media, with the objective of inspiring and engaging effectively the next generation of ocean scientists and conservationists. For each of the participating sites, this is a unique opportunity to engage local communities in the protection and management of the World Heritage site, especially youth and indigenous communities.

All participating sites are encouraged to film, photograph and interview local citizens during their eDNA sampling activities in an effort to bring global attention to the project's ambitions and expected outcomes. Local teams conducting the eDNA sampling will be provided with UNESCO-branded caps and other branding material to facilitate communicating the core ambitions of the project, the critical importance of protecting UNESCO World Heritage marine sites for future generations, and more generally the mission of UNESCO in the field of science and ocean conservation.

UNESCO World Heritage marine sites are invited to help identify inspiring youth voices (young scientists, indigenous communities, young citizen scientists) whose interviews could be integrated in the global communication campaign.

11. Annexes

11.1. ANNEX 1: Collaboration Consent Form

Collaboration Consent Form - “Environmental DNA Expeditions in UNESCO World Heritage marine sites”

1. [name of Partner] hereafter called the “Partner” joins the Project “Environmental DNA Expeditions in UNESCO World Heritage marine sites” hereafter called the “The Project”, to be implemented in [name of the World Heritage Site] and agrees with the objectives, activities and terms listed below.

2. The Project aims to provide a snapshot of marine biodiversity, across about 25 UNESCO World Heritage marine sites, and to estimate the possible impacts of climate change on distribution patterns of marine organisms based on IPCC scenarios of global warming. The Project will focus on fish and other marine vertebrates that are part of the Outstanding Universal Value of UNESCO World Heritage marine sites, with special attention to vulnerable and endangered species as specified through the IUCN Red List of Threatened Species.

3. By joining this Project the Partner will benefit from:
    a. increased knowledge about marine biodiversity of the UNESCO World Heritage marine site,
    b. increased knowledge on the climate change vulnerability of the UNESCO World Heritage marine site’s marine biodiversity,
    c. increased capacity to use environmental DNA for biodiversity monitoring,
    d. increased awareness on the importance of World Heritage to local and indigenous communities, young people and World Heritage stakeholders
    e. training and education in ocean scientific innovation and best practices, and
    f. unique opportunity to engage youth in the protection of the UNESCO World Heritage marine site.

4. The Partner agrees to coordinate the collection of environmental DNA samples inside the UNESCO World Heritage marine site. UNESCO will ship 21 sampling kits to the Partner. Note that sampling kits will be sent once this present Collaboration Consent Form is signed and returned to UNESCO. The receipt by UNESCO of this consent form provides the confirmation to the Project that the Partner has obtained all necessary permissions for environmental DNA sampling and shipping of samples at the local and national levels.[1]

5. UNESCO will provide the necessary training material (video, Field Sampling Booklet) to undertake the sampling, in English, French and Spanish.

[1] Some countries apply a strict policy for the sampling and shipping of genetic resources. The Partner commits to handle, process and ship samples conforming to relevant legislation, and permits.

6. The Partner will choose the sampling locations within the UNESCO World Heritage marine site, taking into account the recommendations provided in the Project's Implementation Plan.

7. During the sampling, the Partner will ensure the collection of the necessary information via the sample information sheets and the sample registration application as indicated in the Field Sampling Booklet. The information includes the unique sample ID provided in each sampling kit, name and email addresses of the contact person or person(s) sampling, date and time of sampling, coordinates of the sampling location, water temperature, salinity, and total amount of water filtered, as well as further notes on the sampling location.

8. The Partner commits to return the samples to the address specified on the shipping label. The return shipping label is prepaid by UNESCO and covers the costs of the shipment of the samples. The Partner may keep the remaining sampling equipment. Maintenance of the material for other uses beyond the scope of this project is the responsibility of the Partner.

9. UNESCO has established a formal contract with the environmental DNA lab of the University KU Leuven (Belgium), hereafter called "the Lab". For each sample the Lab will extract DNA, amplify short target regions of DNA for the analysis of marine biodiversity, and sequence the amplified DNA regions. Samples will be analyzed for marine biodiversity targeting well-known short DNA sequences (genetic biomarkers) with four primer sets. The target regions will be chosen based on their differentiation across different species, including target groups such as Actinopterygii, Chondrichthyes, and vertebrates. The Lab has no rights for the further analysis of samples or use of data, including all communication and publishing of data and the analysis thereof, beyond the explicit scope of The Project.

10. The Project will use environmental DNA only as a tool for the taxonomic identification of species through the analysis of short well-known DNA sequences (genetic biomarkers). No specific functional and/or biochemical properties of the collected genetic material will be analyzed. Therefore, The Project considers that this does not constitute utilization of genetic resources. More details are specified in the Implementation Plan.

11. The Project complies with (i) the FAIR Guiding Principles (which means data, metadata and products should be findable, accessible, interoperable and reproducible) and (ii) the UNESCO Recommendation on Open Science[2].
    a. All steps undertaken during sample and data analysis will be documented and published in open access repositories such as UNESCO's IOC Ocean Best Practices system (OBPS) and the OBIS GitHub channel.
    b. The data and information resulting from The Project will only be used for scientific purposes, i.e., publishing in the UNESCO Ocean Biodiversity Information System (OBIS), the world's largest open-access data system on the distribution and diversity of marine species[3], publishing the raw DNA sequences in public repositories (e.g. NCBI or ENA), and compiling information in a UNESCO-authored flagship publication. UNESCO will ensure that the Partner is invited to nominate

[2] https://en.unesco.org/science-sustainable-future/open-science/recommendation
[3] https://obis.org/

co-authors and/or contributing authors of the publication, and indicate the agencies which should be mentioned in the acknowledgements. UNESCO will provide an electronic copy of any final publication product of the sampling effort in the UNESCO World Heritage marine site within 24 hours of official publication.

c. All data and information will be licensed with an open-access Creative Commons (CC BY 4.0) license, which allows anyone to copy, redistribute and make use of the data, while ensuring proper attribution to the data creators (the project and its partners).
d. If for legitimate reasons, such as for the protection of endangered species, the location information of species will be generalized, rather than providing the exact location. Such data will be shared free of charge to the local management of the respective UNESCO World Heritage marine site.
e. All data resulting from the sampling campaign will be published as a stand-alone dataset in the UNESCO Ocean Biodiversity Information System (OBIS) and will hold a unique and persistent Digital Object Identifier (DOI) and a dataset citation, which includes the names of the people responsible for the expedition including the local Partners. The dataset citation should provide proper credit to those that are involved in the Project.
f. UNESCO will discard the samples after the analysis for the Project is completed, unless sites explicitly request otherwise. Separate agreements and/or additional costs might apply for the Partner in the case of storage of the DNA material in a biobank.
g. The Partner commits to engage with indigenous peoples conform with the United Nations Declaration on the Rights of Indigenous Peoples (UNDRIP) and the UNESCO policy on engaging with indigenous peoples.[4]

By signing this form the Partner confirms that it has obtained all necessary national approvals or permits to join this Project with the terms stipulated above.

Signature and stamp:

Signed by:
(name of person and position)

Date:

[4] https://en.unesco.org/indigenous-peoples/policy

11.2. ANNEX 2: Project Data Policy

Project Data Policy

Preamble

The project partners underline the importance of timely, free and unrestricted international sharing of biodiversity data and metadata for the preservation of life, as well as for the advancement of scientific understanding that makes this possible.

The project partners agree with the FAIR Guiding Principles, which means data, metadata and products should be findable, accessible, interoperable and reproducible.

The project partners agree that the data and information resulting from the environmental DNA expeditions will be used for scientific purposes.

Purpose

The purpose of this data policy is to outline the requirements with respect to data sharing, access, preservation, and attribution to facilitate the broad use and reuse of data and information.

Conditions of use

To comply with the FAIR Guiding Principles the data and metadata will be:

- Quality controlled, standardized following Darwin Core, archived in and published via the UNESCO Ocean Biodiversity Information System (OBIS) before the end of the project (i.e. 31 December 2023).
- Licenced under the CC BY 4.0 license, ensures proper attribution and allows others to copy, distribute and make use of the data.
- Each dataset will hold a Digital Object Identifier (DOI) and a dataset citation.
- All data and metadata will be made available with minimal restrictions unless for legitimate reasons such as for the protection of endangered species. In that case the location information could be generalized, rather than providing the exact location.
- Raw DNA sequences will be submitted to public repositories (e.g. INSDC).

Definitions

Project partners: UNESCO, the environmental DNA lab and the UNESCO World Heritage marine sites or their partners which have signed the Collaboration Consent Form.

11.3. ANNEX 3: Example sample information sheet

Sample Information Sheet

Sample ID: _____
Name(s): _____
Email(s): _____

UNESCO Site: _____
Date: _____
Coordinates: _____

Environmental data

Temperature: _____
Salinity sample: Collected / Not collected

Sample filtration

Number of times the (60 ml) syringe is filled:

☐ 1	☐ 6	☐ 11	☐ 16	☐ 21	☐ 26
☐ 2	☐ 7	☐ 12	☐ 17	☐ 22	☐ 27
☐ 3	☐ 8	☐ 13	☐ 18	☐ 23	☐ 28
☐ 4	☐ 9	☐ 14	☐ 19	☐ 24	☐ 29
☐ 5	☐ 10	☐ 15	☐ 20	☐ 25	☐ 30

In total (ml): _____
Notes (e.g. information on the habitat, bottom depth):

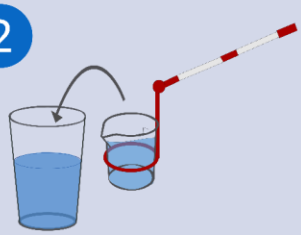
11.4. ANNEX 4. Example infographic

unesco

1

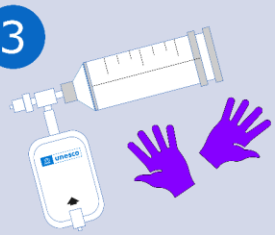
Open sample app, register sample-ID, time and coordinates. Collect salinity sample and measure temperature

2



Collect approximately 2 liters of water to the sample collection bag.

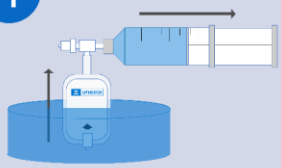
3



Wear gloves throughout the sampling. Attach the filter to the syringe.

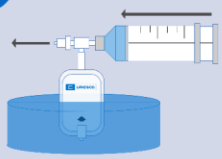
Repeat 20-30 times:

4



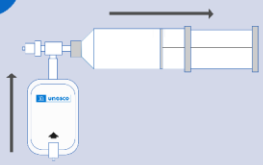
Filter 60 ml by filling the syringe from the bottom of the filter. Mark the amount filtered (60 ml). **Warning:** Do not remove from water.

5



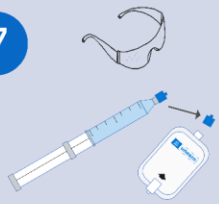
Discard the water by emptying the syringe. Repeat steps 4 and 5, 25 times or until clogged.

6



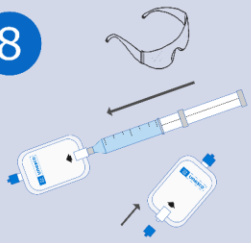
Empty the filter by sucking in air until no water is left in the filter

7



Warning: Wear safety glasses! Close the top of the filter with the cap from the small syringe.

8



Add the liquid from the small syringe carefully to the filter. Close the filter with the second cap.

9

Return the filter, salinity sample and sample information sheet in the sample bag