JNCC Report
No. 706

Protocol for Sampling Marine Benthic Sediment for DNA-based Analysis

NatureMetrics

May 2022

ISSN 0963 8091

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This report was produced for JNCC & NE under an external contract, by NatureMetrics.

This report should be cited as:
NatureMetrics. 2022. Protocol for Sampling Marine Benthic Sediment for DNA-based Analysis. *JNCC Report No. 706*, JNCC, Peterborough, ISSN 0963-8091.

[*https://hub.jncc.gov.uk/assets/121b1d65-866a-47c9-ab71-7088c706770c*]

Acknowledgments:
This review and associated protocol were undertaken by NatureMetrics. We are very grateful to the following reviewers for providing critical evaluation, comments and insight to the initial draft of this document and the associated protocol:

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Contents

1 Introduction

The purpose of this document is to provide a standard protocol for collection and preservation of samples from the subtidal benthic environment for DNA analysis. This is with a particular focus on statutory monitoring of benthic species and habitats of conservation importance. These features are currently monitored in the UK by the Statutory Nature Conservation Bodies (SNCBs). This will enable monitoring bodies to have confidence in the use of these techniques for the long-term monitoring of the benthic environment, both within and beyond the UK's Marine Protected Area (MPA) network. This protocol has been compiled following a thorough review of scientific publications from a range of sources to determine best practice and to standardize sample collection methodology to suit a range of different projects. For further information relating to the findings of the literature review please refer to associated review (Wort *et al.* 2022).

DNA-based methods for species detection and identification have transformed our abilities to monitor biodiversity in both aquatic and terrestrial systems. DNA metabarcoding is used for community assessments by characterising the overall biodiversity within a selected taxonomic group. This can be used for rapid baselining and monitoring to gain an understanding of which species are present within the survey area, including any that may carry significant mitigation requirements. DNA metabarcoding can be applied to marine sediment samples for monitoring of benthic taxa. Compared to traditional methods using morphology-based identification of benthic taxa, DNA metabarcoding offers a scalable method which is time and cost efficient, to infer the environmental quality of, and assess the biodiversity present within sediments. It can also be used to generate biotic index scores to track how species, communities, and ecosystems change in response to impacts and management activities.

2 Sampling Considerations

2.1 Sampling Strategy

The sampling strategy is highly dependent on the aims of the study. Where a sampling strategy has been established for conventional benthic macrofauna and physico-chemical data, DNA sampling can be incorporated into the existing design. Sampling design needs to ensure that there are sufficient stations (sampling locations) and replicates (sediment samples within a station) to compare communities with a high degree of statistical power. A sufficiently large sample size is required to account for the levels of variation expected. Three to five replicate samples are typically included for each station.

Where investigating biological communities in areas such as MPAs, the following approaches are most common (not specific to DNA surveys):

- When reliable habitat maps are available and defined habitat types are present, sampling stations should represent the range of habitat types.
- If the aim of the study is to investigate anthropogenic stressors and the pressure gradient within the area is known, sampling stations are placed at regular intervals along the pressure gradient.
- When the benthos is not mapped or there is low confidence in the mapping data, systemic grid sampling is conducted to ensure full coverage of the sample area.

Where investigating point sources of impact, the following approaches are most common:

- To include a reference station of the same habitat type within a reasonably short distance (for example between 200 m and 2 km) from the point source.

- In areas with a strong prevailing current, to align stations with the current with short intervals for coastal projects (such as 25 m) and longer intervals for offshore projects (such as 250 m). These intervals should approximately scale with depth and mean current velocity, with larger intervals for deeper, faster flowing waters. This should also be at a scale to show the impact gradient appropriate to the geographic scale of the expected impact, for example as specified with regards to the Allowable Zone of Effects and Mixed Zone Limits for Scottish aquaculture.
- In areas where there is a weak prevailing current, use a cruciform station design with four stations in each cardinal direction.

2.2 When to sample

Where the study focus is on long term interannual monitoring of the benthic communities, sampling should be carried out at a similar time of year as the community is likely to undergo seasonal variation. To align with recommendations from broader Environment Agency sampling, sampling should take place within +/- 2 weeks of the original date for interannual sampling where feasible (Environment Agency 2016). For studies at seasonal or other temporal scales, sampling should be adopted to ensure a statistically robust repeated measures design, following the methods recommended in Noble-James *et al.* (2018).

2.3 Contamination

When collecting sediment samples for DNA analysis, it is important to consider potential sources of DNA contamination. Taking steps to minimise contamination is essential to ensure that samples are representative of the DNA at each location. Disposable gloves should always be worn during sampling and these should be changed between samples. The grab/core device should be rinsed down by hose with seawater between deployments to remove residual sediment. Any syringe mini-corers used to collect subsamples should not be reused between stations. Any other reusable equipment should be decontaminated between samples (with a DNA decontaminant such as 10% bleach solution).

2.4 Record keeping

An adapted version of current JNCC metadata recording form and the MEDIN grab/core forms are available in Appendix A. This ensures that the sample collection and records follow the MEDIN guidelines for metadata records. This information should be added to a spreadsheet on the vessel, using the field log sheet where necessary. At the end of the project, the completed forms and photographs of all field log sheets should be copied either onto a secure cloud storage system or hard drive before being transferred to the final secure data storage location.

2.5 Chemical use

Some of the sampling protocols in this document involve the use of chemicals such as ethanol, bleach, or other DNA contaminant solutions. The relevant Safety Data Sheets should be consulted before chemical use, and a COSHH risk assessment carried out if required. Appropriate gloves and safety glasses should always be worn when handling chemicals and steps should be taken to prevent escape of chemicals into the environment.

3 Sediment Collection

Sediment sampling devices should minimize mixing of the sediment profile and retain an undisturbed surface sediment layer for at least the top 6 cm. Considering depth and sediment substrate, acceptable equipment includes:

- Van Veen and Day grabs (soft sediments and gravels).
- Box cores (preferred method where possible for soft sediments).
- Multicorers.

Small Hamon grabs are commonly used for collection of conventional macrofauna samples in coarse or gravelly sediments (Cefas 2006). As it penetrates up to 15 cm into the seabed and does not maintain the sediment profile during collection, it is not the preferred option if sampling for bacteria and/or meiofauna analysis as these studies typically target surface sediments. However, separate instructions have been provided for when Hamon grab sampling is the only option (Section 5).

The following items will be needed for data recording:

- field data log sheets for each sample (printed on waterproof paper) or field recording app;
- pencil for filling in field data log sheet or device for recording sampling information;
- GPS device;
- Camera.

Sediment collection steps:

1. Deploy the sediment sampling device from the vessel. This is to be carried out by a trained operator following existing standard operating procedures in strict accordance with any relevant health and safety requirements contained therein. For the final few metres before contact with the seabed, deployment speed should be limited to 0.3 m/s to prevent bow wave dispersing the surface layer of sediment.
2. When the sampling device hits the seabed, record the sampling station information (eg. ID, date, time, coordinates, depth) on the field sample log sheet (Appendix A).
3. Retrieve the sampling device onto the deck and visually inspect the sediment from above (through the observation door if applicable).
4. If the sample does not meet the specified criteria, the entire sample should be discarded and the sampling device redeployed. At least three attempts should be made at each sampling station before abandoning. If a sample is discarded, this should be noted on the log sheet.
    - Criterion 1: sample must be of an acceptable volume, usually > 5 L with a 0.1 m2 grab (or 2.5 L in hard packed sand)
    - Criterion 2: there must be no obvious malfunction of the grab resulting in significant loss of sample material.
5. Complete the field sample log sheet with all required information (e.g. substrate type). Take a photo of the sediment sample surface before collecting subsample cores. It is suggested to include a written sample ID and timestamp within the shot to later help relate the photo to the sediment sample, or to use an app recording the sample ID and timestamp with the photo. If any of the following are observed, they should be noted on the log sheet:
    - Incomplete closure
    - Obvious uneven bite
    - Spillage during transfer of samples
    - Washout or disturbed surface layer
    - Samples clearly deviate from the other samples taken in the same area, for example, there is an observed change from clean sand samples to Mytilus bank samples. Nevertheless, the samples should be kept, to record faunal patchiness, but another sample should be taken, to replace it in calculating the mean for the station. Synoptic samplings could be useful, to reveal extent and nature of patchiness.

6. The sampling device should be thoroughly rinsed with a water hose between deployments to remove any residual sediment.
7. Sediments samples for DNA analysis should be collected in accordance with the relevant sampling protocol as determined by the workflow diagram (Figure 1).

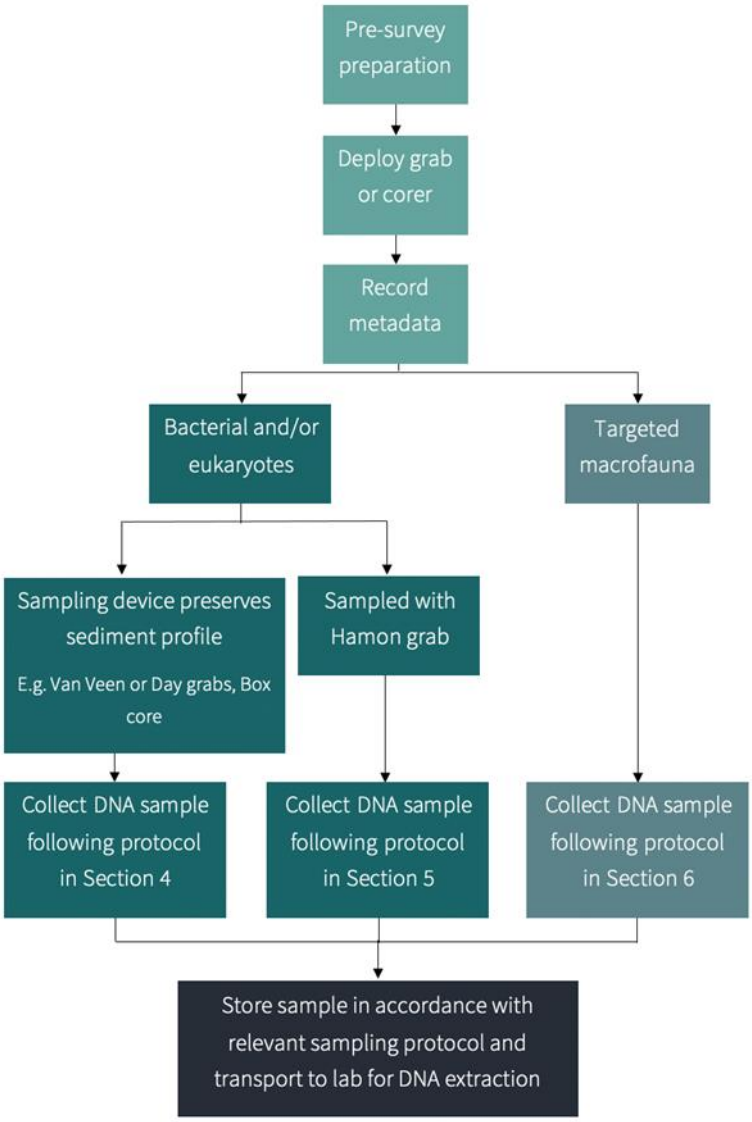

Figure 1. Sampling workflow diagram.

4 Sediment sampling methodology suitable for Bacteria and Eukaryotes only

This protocol is suitable for surveying bacteria and eukaryote communities in marine sediments with a sampling device that preserves the sediment profile, such as a Day grab or box core. As the sediment is not sieved, it will detect more meiofauna than conventional sampling methods. However, the number of macrofaunal taxa detected will be fewer than for conventional sampling due to a lower volume of sediment being processed. If sampling sediment with a Hamon grab, proceed to sampling instructions in Section 5. If sediment is only to be analysed for macrofauna, proceed directly to Section 6.

4.1 Preparation

For each sediment sample the following items are required:

1 x pair of sterile nitrile gloves	
1 x sterile mini-corer with plunger (50 mL syringe with end cut off) in a snap lock bag with write-on panel	
1 x additional snaplock bag with write-on panel	
Fine point permanent marker pens	

Mini-corers and sample kits should be prepared prior to deployment (see Appendix B for further details). It should be ascertained what volume marking on the mini-corers equates to 5 cm depth on your syringe after it has been cut. The items listed above should be combined into a single larger snaplock bag so that each bag contains the materials required for one sampling event. This further safeguards against contamination of equipment between samples.

4.2 Collect subsamples from grab

Samples for DNA analysis should be collected before any other sample type to prevent cross contamination from other sampling equipment. Cores should be collected from the top of the sampling device so that the sediment profile is as undisturbed as possible, via the observation doors if applicable.

1. Label both snaplock bags (those with and without a syringe) with a unique ID using a permanent marker on the write on panel.
2. Put on a fresh pair of nitrile gloves.
3. Remove the syringe from the snaplock bag. Do not dispose of the bag as you will put your sample into this.
4. Brush aside any debris (such as large shells, cobbles, rubbish, plant material) from the surface of where you intend to collect the core. This can be done with the end of the mini-corer or a sterile gloved hand (if it has not been in contact with the deck or other equipment).
5. Remove the plunger from the syringe and push the mini-core tube vertically down into the sediment with a twisting motion until the core is pushed 5 cm into the surface.
6. While the mini-corer is still in the sediment, insert the plunger into the very top of the mini-corer but do not push in further at this stage. In fine sediments this will help create a suction when pulling the core out of the substrate. In coarser sands and gravel, there will not be a suction effect within the tube and it will be necessary to tilt the core at an angle before pulling up out of the sediment to avoid the sample falling out the bottom of the core. If you fail to collect a complete sediment core on the first try, discard the remaining sample and try again while tweaking your technique.
7. Position the syringe with complete sediment core over one of the labelled snaplock bags and push the plunger all the way down to expel the entire core into the bag. Seal or fold over the top of the bag between subsamples to prevent contamination of the sample.
8. Using the same syringe, repeat steps 4-7 to collect a total of four subsamples, one from each quadrant of the grab (or from a random selection of cores if sampled with a multicorer), avoiding sediment that has been in contact with the sides or the bottom of the sampling device.
9. When all the subsamples have been collected in the bag, carefully pour off any excess water, close the seal, then mix the cores together within the bag to homogenise. This can be done by “massaging” the outside of the bag with your hands and some careful shaking. The composite sample should weigh at least 30 g, which is enough for at least two DNA samples using the largest commercially available kit.
10. Once homogenised, push/shake the sample to the bottom of the bag, slightly open the bag seal, roll the bag around the sample to expel excess air from the bag, and then reseal.
11. Place the sample bag into the second labelled snaplock bag, push out excess air, and seal. This is to minimise cross contamination in the event of water leakage or split sample bag.
12. Immediately place sample into a fridge or freezer. Alternatively, samples can be kept in a closed cool box with ice packs for a few hours before transferring to a fridge or freezer. Samples can be stored in a fridge for a couple of days. If storage time will be longer, samples should be transferred to a -20°C freezer as soon as possible after sampling. If samples are frozen, they must be kept frozen until arrival at the laboratory because repeat freeze/thaw samples will affect the results. For transportation to the laboratory, samples should be packed in a cool box with ice packs leaving minimal headspace. For long term storage (several months), it may be

advisable to store samples at -80°C, although this is unnecessary for short term storage (several weeks).

5 Sediment sampling methodology for Hamon grab

When sampling sediments with a Hamon grab, the sediment profile is not maintained as there will be some mixing of the sediment. It is therefore not possible to collect 0-5 cm depth cores and a different approach will be required if sediment is to be analysed for bacteria and/or eukaryotes. As the sediment profile will already be partially mixed, the entire grab should be homogenised to ensure better comparability amongst samples. As mixing will reduce the density of the sediment, it is recommended that subsamples are collected from the homogenised grab using a scoop instead of a syringe mini-core. To ensure a similar amount of sediment is collected for each sample, sediment should be collected to a specified weight (~30 grams).

If sediment is only to be analysed for macrofauna, proceed directly to Section 6. If sample is to be analysed for macrofauna and bacteria/meiofauna, follow this protocol first, and then with the remaining sediment carry onto the sieving protocol in Section 6.

5.1 Preparation

For each sediment sample the following items are required:

1 x pair of sterile nitrile gloves

2 x snaplock bags with write-on panels

The following equipment is also needed for sampling:

- Waterproof digital kitchen scale
- Fine point permanent marker pens
- Plastic tub large enough to hold entire grab sample
- 10% bleach solution or bleach wipes
- Water supply and hose
- Large plastic scoop

NOTE: the plastic tub and scoop must be decontaminated between samples. First rinse off all remaining sediment with seawater, then clean with a DNA decontaminant such as 10% bleach solution or bleach wipes.

5.2 Collect subsamples

1. Empty the grab sample into a sufficiently large plastic tub.
2. Label both snaplock bags with a unique ID using a permanent marker on the write on panel.
3. Put on a fresh pair of nitrile gloves.
4. Homogenise the grab sample in the plastic tub by thoroughly mixing and turning using a sturdy plastic scoop.
5. Open one of the labelled snaplock bags, hold it over the scales, and use the scoop to collect sediment from several different locations around the homogenised sediment until approximately 30 grams has been collected into the bag.
6. Carefully pour off any excess water that was scooped into the bag, close the seal, then mix the sediment within the bag. This can be done by “massaging” the outside of the bag with your hands and some careful shaking.

7. Once homogenised, push/shake the sample to the bottom of the bag, slightly open the bag seal, roll the bag around the sample to expel excess air from the bag, and then reseal.
8. Place the sample bag into the second labelled snaplock bag, push out excess air, and seal. This is to minimise cross contamination in the event of water leakage or split sample bag.
9. Immediately place sample into a fridge or freezer. Alternatively, samples can be kept in a closed cool box with ice packs for a few hours before transferring to a fridge or freezer. Samples can be stored in a fridge for a couple of days. If storage time will be longer, samples should be transferred to a -20°C freezer as soon as possible after sampling. If samples are frozen, they must be kept frozen until arrival at the laboratory because repeat freeze/thaw samples will affect the results. For transportation to the laboratory, samples should be packed in a cool box with ice packs leaving minimal headspace. For long term storage (several months), it may be advisable to store samples at -80°C, although this is unnecessary for short term storage (several weeks).

6 Bulk macrofauna sampling

If DNA surveys need to align with conventional macrofauna surveys, then the whole grab/core sample will have to be processed for more comparable results. However, the volume and size of retained macrofauna collected can present a problem when considering preservation for DNA analysis. It is therefore recommended to sieve the whole grab/core sample, removing megabenthos (>10 mm), mixing the remaining macrofauna, transferring a subsample of the macrofauna to 96% ethanol, then cool storage until DNA extraction. Any megabenthos should be collected separately, for identification either by DNA barcoding or morphological analysis. If desired, photos can be taken of macrofauna that are retained on the sieves for later reference.

If bacterial and meiofauna sampling is required in addition to macrofauna, a separate sample will first have to be collected before sediment is transferred to the sieve (see sampling instructions provided in Sections 4 and 5).

6.1 Preparation

For each macrofauna sample the following items are required:

- 1 x pair of sterile nitrile gloves
- 1 x labelled 500 mL plastic sample pot with screw lid
- 96% ethanol

The following equipment is also needed for sampling:

- Large diameter 1 mm and 10 mm sieves
- A large plastic scoop
- A small plastic scoop or spoon
- 10% bleach solution
- 2 x large plastic crates (one for washing sieves and one for receiving grab/core sample)
- Water supply and hose
- 2 x plastic buckets
- Scrubbing brush

Between samples, all reusable equipment (including crates, buckets, scoops, and sieves) must be thoroughly rinsed with water to remove remnant particles, decontaminated using a 10% bleach solution, then rinsed again with water. Plastic items should be left to soak in bleach solution for at least five minutes then rinsed with water. As bleach corrodes stainless steel and copper, metal sieves should just be scrubbed in bleach solution with a scrubbing brush, then rinsed with water immediately instead of soaking.

Sample pots should be labelled with chemical resistant labels to minimise the chance of unique IDs being rubbed off by residual ethanol.

6.2 Sample processing and collection

1. Transfer sediment from sampling device to a large crate.
2. Ensure sampling pot is labelled with a unique ID.
3. Put on a fresh pair of nitrile gloves.
4. Use the large plastic scoop to transfer sediment to the two sieves that have been stacked in descending size order.
5. Sieve the sediment using a wet-wash method by agitating the sample with the aid of running water. A containment device should be used to collect the sieved sediment and drain the water (eg. spill bin and overspill pipe).
6. Transfer anything retained on the 10 mm sieve to one bucket, and anything retained on the 1 mm sieve to a separate bucket.
7. Repeat steps 2-4 until all sediment has been sieved.
8. Stir/mix contents of the bucket containing 1-10 mm sized macrofauna with the large plastic scoop to homogenise.
9. Remove lid from sample pot and use the small plastic scoop/spoon to transfer 1-10 mm sized macrofauna from the bucket until the pot is half full.
10. Add 96% ethanol to mostly fill sample pot then firmly tighten lid onto pot.
11. For specimens that are over 10 mm, there are three options:
    - preserve in a formaldehyde solution for conventional morphological identification (refer to Cefas SOP 1380 or 1381 for collection of macrofaunal samples);
    - collect small subsamples of body tissue and add to the DNA macrofauna sample pot (if doing this, take photos of whole specimens and record details on the log sheet); or
    - preserve in a second, larger sampling container with 96% ethanol for DNA barcoding.
12. Sample pots for DNA analysis should be stored in a fridge. If samples will be stored for several weeks prior to analysis, it is advisable to store samples at -20°C. For transportation to the laboratory, samples should be packed into a cool box with ice packs and sufficient cushioning material to prevent movement and breakage of pots during transit. As ethanol is classed as dangerous goods, its transportation is subject to certain restrictions. Please confirm labelling and packaging requirements, as well as permitted quantities with your courier company prior to deployment.

References

Cefas. (2006) Standard Operating Procedure 1380: Collection of macrofaunal samples using a Hamon grab. Burnham Laboratory, Benthos Function. Issue number 3.

Environment Agency. (2016) Sampling and processing marine benthic invertebrates.

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Appendix 1 – Example field sample log sheet

Vessel		Project name	
Coordinate system		Sampling Device	
Country		Sample retained	Yes/No
Date	dd/mm/yyyy	Time (UTC)	hh:mm
Station ID		Deployment ID	
Photo ID		Person sampling	
Latitude		Longitude	
Easting		Northing	
Depth (m)		Sediment type	
Total subsamples		Depth subsampled (cm)	
Anoxic layer included in subsample	Yes/No	Vessel storage method	
Sample container		Sample sieved	No/1 mm/ 0.5 mm
Other comments			

Appendix 2 – Preparation of Mini-corers

Mini-corers should be prepared prior to deployment to minimise contamination while on-board the vessel.

You will need:

- Sterile 50 mL plastic syringes (without needles)
- Plastic pipe shears
- Snap-lock bags with write on panels
- Bleach wipes or a 10% blech solution
- Sterile disposable gloves
- Disposable face mask
- Clear bench area (with non-porous surface)
- Permanent marker pen
- A ruler

Instructions

1. Put on sterile gloves and a face mask to minimise contamination of syringes.
2. Wipe down bench area and pipe shears with bleach wipes or bleach solution and wipe dry with paper towels.

3. Remove syringe from wrapper and partially pull up the plunger so that it is away from the cutting zone.
4. Position syringe in the shears where you want to make the cut (as close to the bottom on the straight cylinder section as possible), apply light pressure and carefully rotate the syringe within the shears to score the line to be cut. This will help to give a cleaner cut.
5. Carefully use the pipe shears to cut off the closed end of the syringe along the scored line, ensuring that your fingers are well clear of the cutting zone before squeezing shears.

6. Use the ruler to measure 5 cm from the cut and mark with a permanent marker pen.

7. Put cut syringe directly into a snap lock bag and seal bag closed.

8. Repeat for as many syringes as needed, only opening one packaged syringe at a time to minimise exposure time to the air.

Notes

- For ease of sampling in the field and to minimise potential cross contamination, the bagged syringe should be combined with the other required sampling items listed in Section 4.1 into a single larger snaplock bag so that each bag contains the materials required for one sampling event.

- Not all pipe shears are the same. Some experimentation may be required to find the best cutting technique for your equipment.