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# Review of DNA-based marine benthic monitoring protocols

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# Executive Summary

Natural England (NE) and the Joint Nature Conservation Committee (JNCC) commissioned NatureMetrics to produce a review and protocol for DNA-based marine benthic sampling. This was in the context of DNA metabarcoding and long-term monitoring of benthic species and habitats of conservation importance in UK Marine Protected Areas. For the same benthic DNA sampling method to be used across marine surveys with different spatial and temporal scales, the standardization of DNA sample collection and processing is essential. This will provide considerable benefits for the development of DNA methods for operational benthic monitoring, as the low variability in methods will enable valid interoperability of data for robust spatial and temporal analyses. The protocol is not intended to replace existing field subtidal sediment sampling methodologies for macrofaunal invertebrates but to identify the most suitable operational eDNA sampling protocols that can be applied alongside existing sediment sampling approaches conducted by JNCC and NE.

DNA-based biomonitoring has the potential to generate more biodiversity data for less effort compared with conventional monitoring methods. DNA-based monitoring usually gives much larger, more comprehensive and consistent datasets than traditional, morphological assessments. This is especially important for monitoring over long temporal scales. It also offers a pathway to obtaining data from robust biological indicators that can measure changes in response to environmental and anthropogenic impacts and mitigation measures.

This review outlines the current state of the field in DNA-based marine benthic biomonitoring to enable scientists and managers to make informed decisions and to maximise the benefits of this new technology. It is based on an extensive literature review, and on NatureMetrics' practical experience. DNA-based biomonitoring encompasses a suite of molecular analysis tools that can be applied to a wide range of different environmental sample types.

The scientific literature now abounds with proof-of-concept demonstrations, and DNA-based tools are being integrated into large-scale biomonitoring. There is little doubt about the potential for molecular methods to improve the power and extent of monitoring for environmental impact assessment in marine ecosystems. However, there is still significant room for improvement and there remain a number of areas for future research, particularly with regard to cross validation with existing methods.

Sampling methods for benthic taxa make use of well-established equipment and techniques to obtain sediment samples from the seabed. There is a range of different options available for sub-sampling and sample preservation, depending on project goals and logistical constraints. There are still open questions regarding the best sampling regimes and the optimal trade-off between resources and comprehensiveness of sampling. It is likely that these questions will be answered as DNA monitoring techniques are more widely adopted. This document aims to summarise the most common, practical and successful practices concerning the sample collection and preservation steps, whilst ensuring that sediment samples can be used for as wide a variety of subsequent DNA analyses as possible.

It is recommended that barcoding campaigns are conducted alongside DNA-based biomonitoring surveys to maximise the availability of high-quality reference data for the sampled population. The lack of reference barcodes is often a limiting factor in DNA metabarcoding studies. Expanding the number of reference sequences available in barcoding databases increases the likelihood of obtaining high-resolution taxonomic identifications. This builds strong foundations for the progression of DNA-based monitoring and increases compatibility with morphologically identified macrofauna datasets.

When commissioning DNA-based biomonitoring surveys, study goals will ideally be considered at the outset to determine the minimal sampling prerequisites. Here we consider

the different sampling strategies for broad environmental monitoring across a range of depths and areas, namely marine protected areas, offshore oil and gas infrastructure, and aquaculture. This review provides suggestions regarding the number and positioning of stations around sites, considering recommended JNCC and NE/EA sampling strategies, impact gradients and prevailing water currents to address research and biomonitoring goals. Three to five sampling replicates (grab or core deployments) per station are recommended, although it is understood that this may not always be possible due to resourcing constraints.

The sampling device being used has implications for downstream protocols. For finer sediments, Van Veen or Day grabs are commonly used for metabarcoding studies, although box corers may have more success in maintaining the sediment profile. For coarse sediments, a Shipek grab maintains the sediment profile while a Hamon grab does not. Neither of these grabs were used for DNA sampling in the studies reviewed, but recommendations have been made for their use. Alternative sampling methods may be suited to different environments, such as multicorers or remotely operated vehicles (ROVs) for the collection of deep-sea samples. For harder substrates, scraping the surface in quadrats is recommended for stations within SCUBA diving range, although a recently developed suction-based method may provide an alternative sampling method, particularly for deeper water.

When sampling for bacterial and meiofauna, collecting four mini-cores from each grab is recommended to subsample both the surface and sub-surface taxa. This is preferable to the alternative of surface scrapes which would only capture surface taxa, or scoops which are more difficult to regulate sampling depth. Mini-core subsampling will ensure good representation of DNA of the smaller biota such as bacteria and meiofauna. From the homogenised composite subsamples, a smaller subsample is then used for laboratory analyses. If using a Hamon grab, it is recommended that the full sample is thoroughly mixed prior to subsampling.

When sampling for macrofauna, it is recommended that the whole grab is sieved on board the vessel using a 1 mm mesh sieve (after any subsampling for bacteria and/or meiofauna), or that a minimum of 1000 $cm^3$ of unsieved material is collected as a subsample. This approach is broadly compatible with existing morphotaxonomy sampling approaches. It may be possible to use the sediment subsamples directly for DNA-based macrofaunal analysis. However, further research is required in this area and, as macrofauna are a key component in many biomonitoring programs, it is advisable to take separate macrofauna samples that can be subject to DNA-based analysis. Further research should include targeting macrofauna from both sediment directly and from sieved macrofauna portions of grab samples, to determine whether macrofauna diversity is adequately captured using sediment. The work should include investigation of the number of sediment samples to take from each grab. Ethanol preservation of sieved macrofauna is recommended, using a subsample where necessary. Megafauna (>10 mm) should be separated from the macrofauna bulk sample by including a 10 mm mesh sieve. These megafauna samples can then be processed by including small tissue samples in the DNA bulk sample, using morphological methods, or storage in a larger container of ethanol.

Cold storage is recommended for sample preservation targeting bacteria and meiofauna. If samples are to be delivered to the laboratory within two days of sampling, they can be stored in a fridge. 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 remain frozen until arrival at the laboratory as freeze/thaw cycles will affect the results. For transportation to the laboratory, samples should be packed in a cool box with ice packs leaving minimal headspace. Bulk macrofauna samples should be preserved in 96% ethanol and kept cold (6 °C) until analysis. Where cold storage is not feasible, DESS preservation is recommended.

This review has summarised information from over 80 publications and reports. The sampling methodology used in each study has been categorised and this is provided as a comprehensive appendix. This review has been used to generate an associated marine benthic sampling protocol, which has been reviewed by experts in this field. The protocol is intended to act as a best practice operational procedure that can be widely applied by marine monitoring agencies.

# Contents

# 1 Introduction

## 1.1 Scope

The scope of this work was to provide JNCC and NE with a review and protocol for benthic sampling in the context of eDNA metabarcoding and long-term monitoring in UK Marine Protected Areas. This was 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), who use traditional morphological identification as the primary tool for biodiversity assessment. DNA-based methods for species detection and identification have transformed our ability to monitor biodiversity in both aquatic and terrestrial systems. Introducing standardised marine benthic DNA sample collection and processing across SNCBs' marine surveys (with different spatial and temporal scales) could provide considerable benefits in terms of cost savings, sample turnaround times, and a massive increase in the amount of biodiversity data collected per sample. This has the potential to lead to more robust and holistic measures of Marine Habitat Classification and more extensive biodiversity inventories for Marine Protected Areas.

The scope is limited to the collection and preservation of benthic sediment samples, including macrofauna separated from sediment, in the context of eDNA metabarcoding. While an overview of considerations for ecological study design is provided, a thorough description of current monitoring programmes and statistical study design approaches is outside of the scope of this review. Generally, the broad ecological study design approaches for benthic marine sampling are similar whether using traditional morphological analysis or DNA-derived taxonomic data and these are detailed in other documents (Noble-James *et al.* 2018). It was also outside the scope of this work to review processes downstream of sample collection and preservation, such as DNA extraction, DNA amplification, bioinformatic processing, taxonomic assignment and reference databases. However, it is understood that sampling and preservation approaches can affect these processes and reference is made to these where appropriate.

## 1.2 Environmental DNA Overview

Environmental DNA (eDNA) in the strict sense refers to extra-organismal genetic material that has become detached from an organism and can be isolated from environmental samples such as water or sediment. This is in contrast to **organismal DNA**, which is isolated from an environmental sample containing the organism itself.

Environmental samples (i.e. water or sediment) typically contain a mixture of extra-organismal eDNA deposited by larger organisms, and organismal DNA of small (micro)organisms (Rodriguez-Ezpeleta *et al.* 2021). Water samples often target the extra-organismal eDNA component specifically, whereas sediment analyses typically target both organismal and extra-organismal DNA. Benthic sediment samples usually contain a high diversity of living organisms as well as eDNA and DNA from dead or dormant organisms and gametes. In aquatic systems this is also supplemented by eDNA settling into the sediment from the water column. Consequently, this makes sediment samples a rich source of data across the entire spectrum of biodiversity for operational MPA monitoring.

Molecular analysis of marine sediment can broadly be separated into three categories:

> **Targeted, single species, assays:** These are used to screen an environmental sample for the presence of the DNA from a particular species. These assays typically apply quantitative PCR (qPCR) or digital droplet PCR (ddPCR) to check for the presence of species that are of particular concern. This may be related to their

conservation significance, and associated legal protections, or their status as pathogens or invasive non-native species.

**DNA metabarcoding:** This is used for community assessment by characterising the overall biodiversity within a preselected taxonomic group. This can be used for biodiversity monitoring and baselining to gain an understanding of which species are present within the survey area, including those that may have implications for mitigation requirements.

**Functional analysis:** This can be DNA- or RNA- based, with RNA-based methods offering additional insights. For example, in one study bacterial eDNA was particularly useful in providing insights at petroleum impacted stations where an increased presence of hydrocarbonoclastic taxa was identified (Laroche *et al.* 2018). RNA-based analysis can characterise gene expression activity in environmental samples to map microbial activity, and hence the ecological processes taking place at the time of sampling. This provides further insights into ecosystem function, resilience and services. Although currently less well-developed than targeted assays or community assessments, functional analyses holds substantial potential for application in environmental impact assessment and for an improved understanding of ecosystem health. It should be noted that it is outside the scope of this review to consider methods for RNA sampling and preservation and any such studies would require additional considerations, particularly concerning sample preservation, as RNA is less stable than DNA.

## 1.3 Advantages of eDNA-Based Approaches

Compared to methods using morphology-based identification of benthic taxa, DNA metabarcoding offers a scalable, resource efficient method to assess biodiversity and infer the environmental quality of sediments. Aylagas *et al.* (2018) reported a cost saving of 55% and a time saving of 72% compared to traditional approaches when investigating the potential for metabarcoding to determine marine ecological status using the AZTI's Marine Biotic Index in the Basque estuarine and coastal monitoring network programme.

There is substantial evidence in the scientific literature of the power of metabarcoding in the context of marine benthic environmental impact assessment for tracking changes in sediment biological communities (Aylagas *et al.* 2014). Moreover, as eDNA approaches offer the potential to capture not only macrofauna data but microbial and meiofaunal data, it presents the opportunity to develop more advanced metrics based on a wider community of the benthic biome, potentially with greater sensitivity to detect changes in ecological status. This has been demonstrated through the development of machine learning approaches based on metabarcoding data from eukaryotes and prokaryotes (Cordier *et al.* 2017, 2018). When assigning habitat health scores, there is a strong relationship between this approach and traditional morphological analysis, and it is argued that these approaches could bypass morpho-taxonomic approaches in future biomonitoring.

A considerable body of research now demonstrates that DNA-based biomonitoring can significantly improve the capacity to assess, monitor, and manage both environmental risks and impacts. Accordingly, environmental practitioners and policy makers are now starting to integrate DNA-based methods into routine monitoring applications.

## 1.4 Important Considerations for eDNA-Based Approaches

All sampling methods have limitations and biases, and DNA-based approaches are no exception. Advantages and limitations of any method need to be understood to be able to make informed decisions about when and how to apply them. Here we provide a brief

overview of some key considerations for eDNA-based monitoring. Other aspects of benthic marine sampling and preservation in the context of eDNA metabarcoding are discussed in the relevant sections of this document.

### 1.4.1 Reference Databases

One of the key considerations is the availability of reference DNA sequences. There are large publicly available sequence databases (e.g. NCBI nt, BOLD, SILVA) but initiatives focused on specific taxa or geographical regions have also led to the development of separate reference collections, such as FishBase or the Fungal Barcoding Database. Presently, the data stored in NCBI databases exhibits a strong taxonomical bias towards animal taxa, with vertebrates being considerably better represented than invertebrates (Ferreira *et al.* 2018). There are known large taxonomic gaps in the databases which hampers taxonomic assignment, particularly to species level. This is further compounded by the fact that many sequences have been labelled with incorrect species assignments by the sequence contributors. For many taxonomic groups, assignment at the species, genus or even family level is not possible. Where monitoring programs require this resolution of data it is pertinent to: 1) check existing databasing for target taxa of interest; 2) check that the reference sequences are reliable; and/or 3) carry out barcoding to add these sequences to public or local databases.

### 1.4.2 The Barcoding Gap

Metabarcoding is based largely on taxa having particular DNA sequences that are unique within a specific and short (usually 70-400 base pairs) DNA region. Many researchers have sought a barcode region and associated assay (primer set) that can reliably detect taxa across wide taxonomic groups whilst also having sufficient variability to disambiguate taxa at the species level. In many cases this has been very successful but for large taxonomic groups there are many species that share identical barcodes within a barcode region. This makes taxonomic assignment difficult for those species and often analyses targeting different DNA regions need to be developed for certain taxonomic groups. In short, there is no single universal analysis that can reliably detect and disambiguate all taxa, meaning that multiple analyses are often required depending on the study goals. Furthermore, where taxonomically broad analyses are applied, data will tend to be dominated by microbes, which are present at high densities in the environment (Bakker *et al.* 2019).

### 1.4.3 Organism Viability and Life Stages

eDNA-based analysis cannot discern organism life stages (e.g. gametes, juveniles, adults) whereas this can be accomplished using morpho-taxonomy. Also, as eDNA can be detected from dead biota, its detection is not always strictly indicative that the organism was living in the sample at the time of sampling. In general, however, it can be assumed that the contribution of dead organisms to the eDNA component of sediments will be a far lower proportion of reads. These low signals should be interpreted with caution. Dependent on study goals these may pose a problem to some monitoring objectives. Targeting eRNA may offer ways to overcome this in the future but further work is required in this field before it can be incorporated into routine monitoring.

### 1.4.4 Abundance

DNA metabarcoding methods can provide an indication of relative abundance but there are numerous biases that play a role in this such as body type (e.g. soft-bodied versus heavily sclerotised) and primer bias (Martins *et al.* 2020). Abundance data is required for some common benthic indices (Steyaert *et al.* 2020) and further methodological development and optimization efforts are needed to reduce these biases. Research in this area could focus on

characterising the macrofauna community of a grab sample using morphotaxonomy, including measures of biomass and number of individuals, and comparing this with DNA sequencing read abundance data.

## 1.5 Aims

This document aims to provide a clear overview of marine benthic DNA-based methods and the different sampling and sample preservation options. It highlights their strengths and limitations to inform such decision making and enable scientists and managers to maximise the potential of this revolutionary new technology. In addition to the published scientific literature and our network in the academic community, we draw on the knowledge of NatureMetrics staff who have extensive experience in marine benthic sampling and analysis of invertebrates and microorganisms. Recommended field sampling strategies for benthic DNA samples are presented, as well as the different options for sample preservation and their respective merits. Remaining knowledge gaps and areas of potential research are considered.

This review is accompanied by a protocol for marine benthic sediment sampling for DNA-based analysis and the sampling methodologies used in each reviewed study has been categorised and is provided as a comprehensive appendix.

# 2 Methods

A literature search was carried out identifying published studies where metabarcoding had been applied to subtidal benthic samples. This was based on both previously obtained literature and additional searches using the terms “sediment metabarcoding”, “benthic monitoring”, “marine metabarcoding” and “sediment eDNA”.

A total of 65 scientific publications were found as well as 17 sources of information from the grey literature. From each of the scientific studies obtained, the following information was extracted:

- Referencing information
- Sampling location (e.g. North Sea)
- Water body (e.g. deep sea)
- Sediment substrate type
- Bottom depth
- Sampling instrument
- Grab deployment (details regarding the speed of instrument deployment)
- Number of study sites (locations of particular interest)
- Number of Sampling Station per Study Site
- Number of Replicate Grabs/Cores (per station)
- Number of Sub-samples (per grab/core)
- Total number of sediment samples for DNA analysis
- Sample Type (if particular layers were selected)
- Sub-sampling instrument
- Final sample/sub-sample depth (into the sediment)
- Preservation methods in the field and in the lab
- Contamination control protocols
- Target DNA marker and taxonomic group(s)
- Total number of taxa recorded
- Comparison of eDNA applications compared to other methods

Grey literature was also obtained concerning benthic sediment sampling without a DNA-based component, particularly focussing on current practice for the UK. This information is included in the review where relevant.

## 2.1 Sampling and Preservation Methods for DNA from Benthic Sediment Samples

### 2.1.1 Sampling Strategy

The sampling strategy is highly dependent on the aims and geographical scale of the study. Where a sampling strategy has been established for obtaining conventional benthic macrofauna samples and physico-chemical data, this can be expanded upon with DNA sampling (Lanzén *et al.* 2020). The main aims of marine benthic sampling can be described as follows:

- Characterising biological communities across coastal and offshore areas, including marine protected areas (MPAs).
- Monitoring for changes in the community or biological quality index.
- Tracking the presence of protected or invasive species, or other species of particular interest.
- Assigning areas according to habitat classifications or biotopes, for example the Marine Habitat Classification for Britain and Ireland.

The sampling design should be geared towards answering the main objectives with sufficient statistical robustness and maximising the evidence gathering potential. In the context of the aims given above, the main points to consider in relation to statistical analysis is the number of sampling stations and the number of samples and sample replicates within those stations. Where the goal is to compare sites or monitor for impacts, it is imperative that there are sufficient sampling stations and grab/core replicates included in the study to be able to compare communities with a high degree of statistical power. This will often involve a balance between minimising sampling costs and efforts while still collecting enough samples to be confident in the results. The statistical power of any dataset is determined by both sample size and the heterogeneity within the dataset. A sufficiently large sample size is required to account for the levels of variation expected between individual samples. As such, the chosen sampling design should be in line with the desired precision and accuracy of the results.

In the case of identifying habitats and biotopes the spatial scale of sampling is very important to ensure that the required granularity is achieved and not to miss important protected benthic habitats. This must be determined by topography and coastal processes of erosion and deposition and will be influenced by the likely level of habitat heterogeneity in the area.

For sediment sampling with the aim of general environmental biomonitoring in an estuarine or coastal environment, sampling stations and sites are commonly arranged along depth and salinity gradients (Lallias *et al.* 2015; Chariton *et al.* 2015; Fais *et al.* 2020; DiBattista *et al*. 2019). For benthic biomonitoring within Marine Protected Areas ideally multiple stations are sampled within each known habitat type (Noble-James *et al*. 2018). However, the sampling strategy will be dependent on the availability and resolution of the mapped habitat distribution. The same approaches as outlined in Noble-James *et al*. (2018) for marine benthic monitoring can be applied to DNA-based biomonitoring, namely:

- Probabilistic sampling design to allow inferences to be drawn about the wider population.

- Stratified random sampling where sediments are clearly stratified across the site and confidence in habitat maps is moderately high, as is frequently the case for MPAs.
- Systematic sampling where the seabed cannot be reliably stratified and full coverage of the survey area is required, particularly before the establishment of management or closure areas.
- Judgement sampling design (where the researcher subjectively selects sampling units without randomisation) should only be used where the researcher has a well-developed knowledge of the indicator and system, this is not initially recommended.

Several pilot studies using DNA-based biomonitoring have been carried out within MPAs, such as in the Stellwagen Bank National Marine Sanctuary in the USA (Polinski *et al.* 2019), and in the Cíes Islands and Cabrera Archipelago in Spain (Wangensteen *et al.* 2018; Wangensteen *et al.* 2018). However, both these studies were relatively limited in the number of sampling locations and samples. It is recommended that any sampling in MPAs should at least align with the pre-existing conventional sampling strategy (e.g. Downie *et al.* 2019).

One well-developed nearshore benthic DNA monitoring project was part of the Basque Monitoring Network (Lanzén *et al.* 2020; Aylagas *et al.* 2018). Two to five sampling stations were sampled within estuaries with a high number of replicates, whilst shallow (<50 m depth) offshore sampling stations were approximately 20 km apart (Lanzén *et al.* 2020). Two deeper (>100 m depth) stations represented the offshore environment (Lanzén *et al.* 2020). This type of sampling strategy may be particularly relevant for considering inshore and coastal areas. In the UK, a smaller scale study identified bacteria community differences that were associated with sediment particle size using metabarcoding (Cronin-O'Reilly *et al.* 2018). In a UK estuarine study, six stations were sampled for each section (inner and outer estuary; oligohaline, oligo-mesohaline and polyeuhaline) (Lallias *et al.* 2015), thus representing communities found over a range of salinities. Ideally, studies should encompass habitats at a reasonably high resolution of habitat classification (such as the EUNIS level 3 biotopes) when considering habitat variation such as sediment type and salinity within sites.

When considering the impact of estuarine barriers such as dams or sea dikes, samples have been collected from either side of these barriers. For example, Lee *et al.* (2020) sampled from one or two stations either side of estuarine dams at each of five sites, with annual sampling at each station over three years (Lee *et al*. 2020). This allowed interannual trends to be explored and revealed spatiotemporal changes linked to sediment pollution.

Marine sediment DNA sampling from waters deeper than the continental shelf seas can be used to target particular features such as submarine canyons (Guardiola *et al.* 2015, 2016; Atienza *et al.* 2020). Deep sea sediment sampling is often a component of broader research cruises (Sinniger *et al.* 2016; Kerrigan *et al.* 2019). In these cases, there is no easily transferable sampling strategy beyond ensuring good representation of varied depths and habitats (Fonseca *et al.* 2017). This should take into account the natural spatial variability and must be detailed enough to detect spatial differences in benthic communities (Environment Agency 2016).

Studies focussing on tracking sediment enrichment in relation to fish farms commonly arrange stations across an anticipated zone of impact. In a New Zealand study for example, sampling stations were distributed along an enrichment gradient up to 200 m from the site, with reference stations between 300 m and 4 km away from the farm (Keeley *et al.* 2018; Pochon *et al.* 2015). Similarly, in a study in Scotland, nine of ten stations were aligned in a transect following the prevailing water currents from the fish farm to a distance of 400 m (Lejzerowicz *et al.* 2015). Other Scottish studies (Stoeck *et al*. 2018) use set intervals along this transect, namely the allowable zone of effect (26 m), the intermediate zone of impact (60 m), and reference stations at 270 m and 400 m distance. The sampling distribution should

also account for the size of the fish farm, as discussed in monitoring for Scottish aquaculture (SEPA 2008). Other studies which also use this transect approach are found in Norway, and Canada and the Western Mediterranean (He *et al.* 2019, 2021; Stoeck *et al.* 2018; Aylagas *et al.* 2021).

For offshore oil and gas projects, the distribution of stations is typically in a cruciform around the oil or gas infrastructure site (Lanzén *et al.* 2016; Klunder, *et al*. 2020). The greatest number of stations per site identified in this review is 16 - four stations in each cardinal direction, with 150 or 250 m intervals for the first three stations (Lanzén *et al.* 2016; Klunder *et al.* 2020). Other studies have stations (six or nine) orientated along the major flow axis (Laroche *et al*. 2017, 2018). Sampling strategy should therefore account for the local prevailing currents as well as encompassing the wider area.

Where the study focus is on long term interannual monitoring of the benthic communities, sampling should be carried at a similar time of year (e.g. Keeley *et al.* 2018) as the community is likely to undergo seasonal variation (e.g. Guardiola *et al.* 2016). Timing and frequency should reflect the monitoring aims, for example at the end of algal growth season to capture the impact of coastal eutrophication on the sediment community (Salonen *et al.* 2018). The Environment Agency recommends that sampling should take place within +/- 2 weeks of the original date for interannual sampling where feasible (Environment Agency 2016).

### Recommendations

The sampling strategy will vary depending on the goals and sampling location, and where possible should be aligned with any pre-existing sampling strategies for macrofauna.

Where investigating a broader area using DNA sampling, such as coastal and estuarine monitoring, it is recommended that:

- sampling stations are placed at regular intervals along the salinity gradient, with an absolute minimum of two stations for smaller estuaries in a given coastal area
- a representative range of habitat types (different sediment composition) are sampled
- either stratified random sampling or systematic grid sampling should be used

Where investigating point sources of pollution, it is recommended that:

- a reference station on the same habitat type within a reasonably short distance (for example between 200 m and 2 km) from the point source is included
- in areas with a strong prevailing current, stations are aligned 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
- where there is weak prevailing current, a cruciform is used with four stations in each cardinal direction

Where sampling is being carried out for interannual comparison, it is recommended that repeat sampling is conducted within +/- 2 weeks of the original date for interannual sampling as is done for morphological macrofauna analysis (Environment Agency 2016).

### 2.1.2 Sample Replication at Stations

As each grab/core represents only a small spatial area of the seafloor, a major consideration in benthic survey design is ensuring a sufficient number of samples are collected to be representative of the feature or area being surveyed. Sample replication (repeated grabs/cores) at each sampling station can be used to help resolve the statistical power of such datasets where it is limited. This can therefore enhance the clarity of information on ecological trends over space and time. This must be balanced with the time and cost to deploy the sampler. Moreover, little is known about the spatial heterogeneity of benthic bacterial and meiofaunal communities or the extent to which a single grab/core can be considered representative of the wider area of the seafloor habitat for DNA analyses, although this is well explored for macrofauna (Holte & Buhl-Mortensen 2020; e.g. Álvarez *et al.* 2020).

The majority of the studies identified in this literature review used multiple replicates, whether sampling is part of broad environmental studies (e.g. Laroche *et al.* 2020b), those targeting oil and gas infrastructure impacts (Frontalini *et al*. 2020) or aquaculture (Dully *et al*. 2021). Three replicates per station is the most common number (Figure 1), and with the exception of one paper with only four sampling stations (Klunder *et al.* 2020), has been used as the upper number of replicates for oil and gas, and aquaculture impact sampling. For coastal sampling, a higher number of replicates is common, which is likely to be due to the lower sampling cost relative to offshore sampling. Although replicate cores generally show good clustering when comparing communities using DNA derived data, there can still be anomalous stations (Lallias *et al.* 2015). Furthermore, the use of spatial replicates for DNA extraction can prevent underestimation of alpha-diversity and overestimation of beta-diversity (Lanzén *et al.* 2017). For example, accumulation curves investigated by Fais *et al.* (2020) for multiple markers suggest that 5-6 sampling points per sampling station (with coordinates approximately 4-5 m apart) are the minimum required to represent the meiofaunal community using DNA. It is therefore recommended that multiple replicates are used initially to ensure that a representative beta diversity is generated, which may then be scaled back in the future subject to a power analysis. However, as discussed in Noble-James *et al.* (2018), a wider range of sampling locations should be prioritised over within-station replicates, although this will result in a lower understanding of localised variation. Obtaining sample replicates should therefore be conducted where possible, even if samples are not immediately analysed due to limited resources.

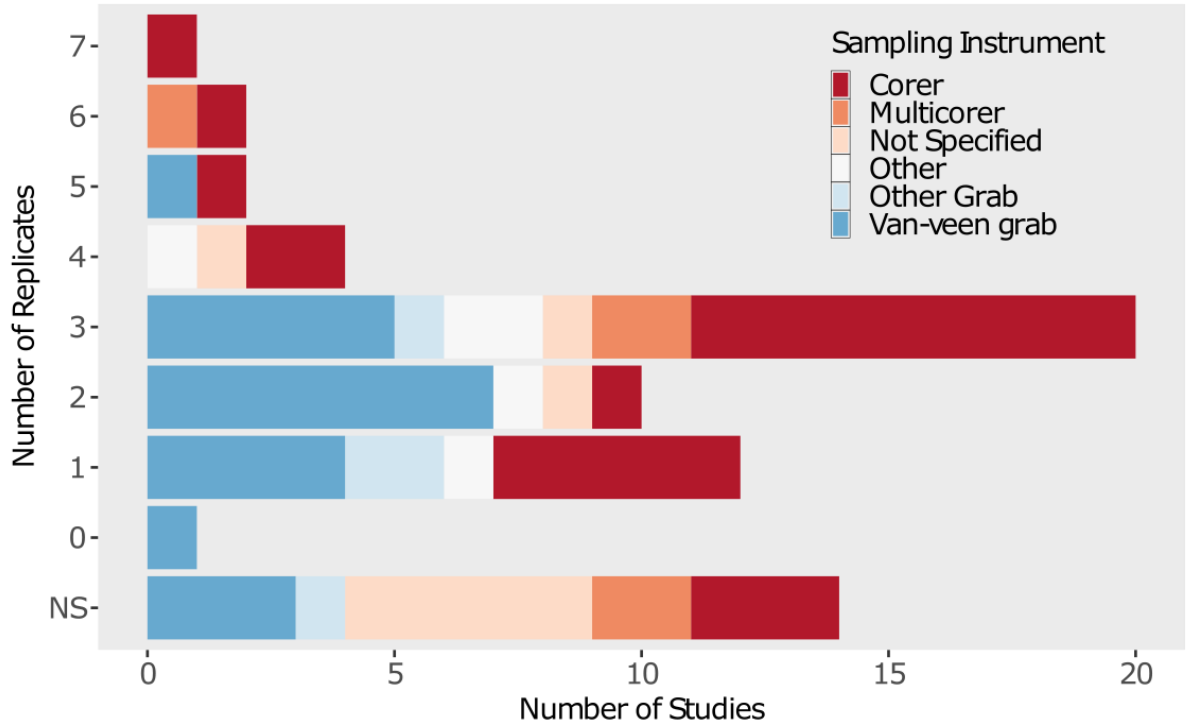


**Figure 1.** The number of replicates (repeated deployments of grabs/cores) at each sampling location for the reviewed DNA-based benthic sampling literature with different types of sampling equipment (NS = number of replicates not specified).

For conventional biomonitoring, it is usual to collect multiple sediment samples from each sampling station for benthic macrofauna assessment (OSPAR 2018; Environment Agency 2016), and physicochemical analysis (Kirby *et al.* 2018; Mcniven & Gilchrist 2017), although this is less common in JNCC monitoring (e.g. Downie *et al.* 2019). This is specified as 5 replicate samples per station for CSEMP monitoring (CEFAS 2012). This means that there is sufficient sediment available for molecular analysis without needing to deploy a dedicated grab/core (He *et al.* 2019). However, obtaining an independent sediment sample specifically for DNA analyses is more common (Cordier *et al.* 2019; Lallias *et al.* 2015) and prevents any confusion or compromising of sampling methods.

### Recommendations

Collecting three to five grab or core samples from each station is recommended where possible to ensure that data are statistically robust. Whilst this is currently above the number of sample replicates taken for some macrofauna and sediment monitoring programmes, it is advisable to obtain an excessive number of samples and perform a power analysis. Using this information, the number of replicates could be scaled back to a more cost-effective number.

## 2.1.3 Sampling Equipment

The choice of sediment sampling equipment used can be driven by the sediment type (Table 1). Marine sediment sampling from a survey vessel is most commonly carried out with a grab although alternative methods of sampling, such as box cores, are accepted (CEFAS 2012). For DNA analyses, the majority of sediment sampling studies have used a Van Veen grab (Figure 2). These have observation doors for sub-sampling purposes to avoid disturbing the sediment strata, as is commonly practiced when obtaining physico-chemical samples (Mcniven & Gilchrist 2017; CEFAS 2012). Van Veen grab sampling has been used on sediments up to depth of 367 m (Cordier 2020; Pawlowski *et al.* 2016; Stoeck *et al.* 2018).

Corers have been used in several examples as they can better preserve the profile for soft sediments, enabling greater precision in standardising samples (Cordier *et al.* 2019; Frontalini *et al.* 2020). For deep water samples (to a depth of 6326 m), multicorers are most commonly used (Scheckenbach *et al.* 2010; Pawlowski *et al.* 2011; Corinaldesi et al. 2005; Guardiola *et al.* 2016), in order to increase the number of samples obtained for each deployment. However, this may be considered pseudoreplication, as it is not the equivalent of conducting three separate deployments of a grab/corer.

Conventional sampling for macrofauna by the JNCC in UK offshore MPAs most commonly uses Hamon grabs (Noble-James et al. 2018). In the inshore waters, Natural England/ Environment Agency most commonly use mini-Hamon and Day grabs (Maija Marsh, personal communication). A Hamon grab was not used for DNA samples in any of the studies included in the review. In the one example where it has been used, it was applied only to collect conventional macrofauna for morphotaxonomy, while a corer was used for DNA sampling of bacteria (Massé *et al.* 2016). The apparent absence of using Hamon grabs in DNA-based studies is likely due to the fact that it mixes sediment layers during sampling, making it impossible to retain sediment stratification (Whomersley 2014). However, it is commonly used for coarser sediments (Cooper and Rees 2002) and this review includes considerations for using a Hamon grab in each section. Another option for coarse sediments that has not been identified in any of the review studies is the Shipek grab. This has the advantage of not disturbing the surface sediment layer, making it suited to sampling for contaminants historically (Whomersley 2014). This would also make it suited to subsampling specific layers for bacteria and meiofauna. Day grabs, which are also commonly used in MPA monitoring, are similar to Van Veen grabs and therefore considered suitable for DNA sampling.

We recommend using the similar grab rejection criteria as for conventional macrofaunal sampling:

Criterion 1: Less than 5 L of sample volume is obtained by a 0.1 m2 grab in soft sediments or less than 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

This covers the sample rejection criteria stated in Rumohr (2009) which should be noted namely:

1. Incomplete closure
2. Obvious uneven bite
3. Spillage during transfer of samples
4. 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.
5. Washout or disturbed surface layer

To reduce a bow wave displacing surface sediments, the grab deployment speed should be limited to 0.3 m/s (Laroche *et al.* 2017, 2018).

**Table 1.** Equipment, sample types and sediment penetration for benthic sampling *sensu* McNiven & Gilchrist (2017).

| Equipment | Applicability | |
|---|---|---|
| | Sample Type | Sediment penetration |
| Van Veen grab | Bulk samples of indurated or soft clay, mud, silt sand, gravel | Surface sediment (15-20 cm depth) |
| Box Corer | Undisturbed cores in soft fine-grained clay to coarse grained sand | Sub-surface sediment (~50 cm depth) |
| Gravity Corer | Short to medium length cores in soft fine-grained clay to medium-grained sand | Up to 1.8 m |
| Multicorer (e.g.MaxiCorer) | Deep sea soft sediment | Up to 15 cm depth |
| Uwitec Corer | Soft sediment (malfunctioned in Holman *et al.* 2019) | Up to 60 cm depth |
| HAPS Corer | Hard and soft sediments (KC Denmark Research Equipment, n.d.) | Up to 31.5 cm, 13.6 cm diameter |
| Gemax Twin Corer | Soft sediment (Winterhalter, n.d.) | Up to 60 cm depth |
| Day Grab | Not referenced in DNA studies, but generally similar to Van Veen grab | Surface sediment 15-20 cm depth |
| Hamon Grab (0.2 $m^2$) | Not referenced in DNA studies, does not preserve sediment profile. Used for coarse sediment sampling | Surface sediment 30 cm depth |
| Mini-Hamon Grab (0.1 $m^2$) | Not referenced in DNA studies, does not preserve sediment profile. Used for coarse sediment sampling in majority of MPA studies | Surface sediment 30 cm depth |
| Shipek Grab | Not referenced in DNA studies, is used for contaminant sampling as it preserves the sediment profile. Used for coarse sediments. | Surface sediment 10 cm depth |

Remotely operated vehicles (ROVs) have also been employed for deep water sampling (3133 m to 5240 m), to increase both accessibility and the number of samples that can be collected per deployment from the vessel (Laroche *et al.* 2020b). Use of ROVs may become more common as the costs decrease and deployment may become feasible from dockside or infrastructure. Automated sampling has already been piloted for water eDNA in Monterey Bay (Yamahara *et al.* 2019; Andruszkiewicz *et al.* 2017; Djurhuus *et al.* 2020), although vessel deployment has been necessary to date. JNCC has already developed benthic sediment sampling for other purposes using ROVs (JNCC 2018).

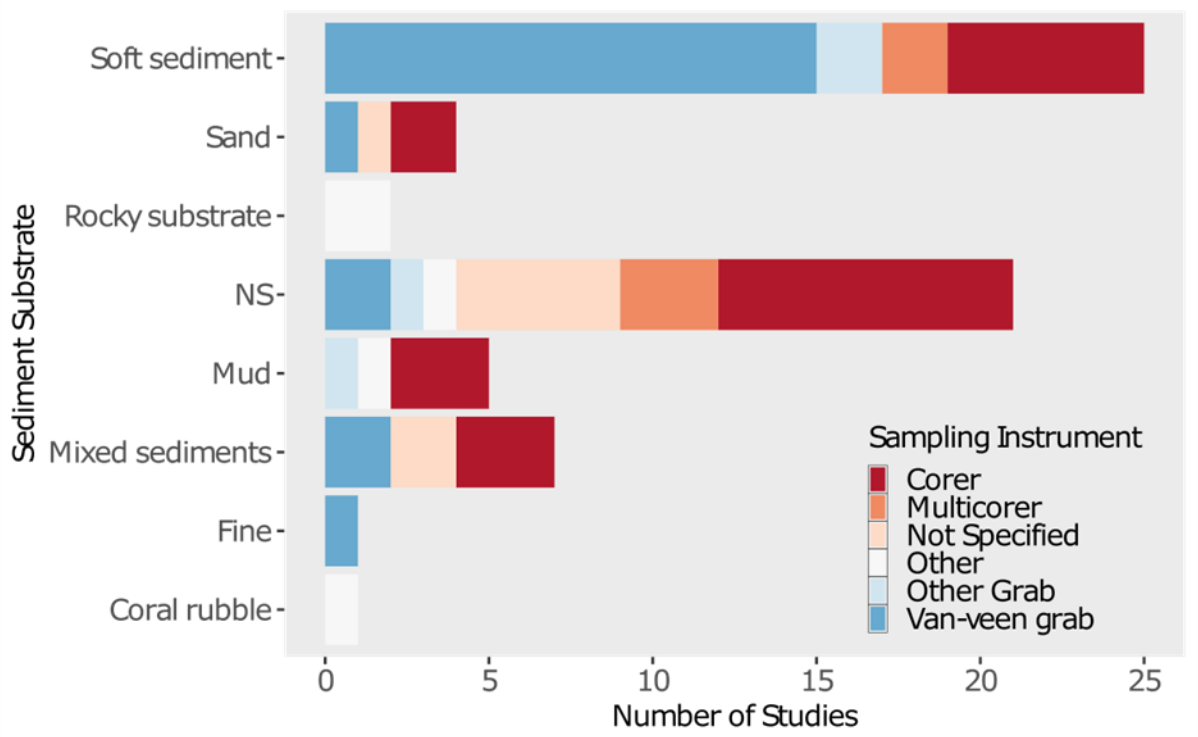


**Figure 2.** Sampling equipment used for different sediment types in reviewed DNA-based benthic sampling literature.

Sampling for taxa on hard-bottom communities, such as maerl reefs, is generally not possible using grabs and as such it has inherent challenges. One possibility is careful scraping of the surface of a 25 x 25 cm quadrat with a hammer and chisel (Turon *et al*. 2018; Wangensteen *et al.* 2018). For gravels and coral rubble, samples can be collected in falcon tubes by SCUBA divers (DiBattista *et al.* 2019, 2020). For shallow and intertidal sediment, hand-held corers are commonly used (Steyaert *et al.* 2020), which are usually plastic (Fais *et al.* 2020). For deeper hard-bottom substrates, one possibility is a suction-based method that removes the surface layer from rocky substrates. This sampling method has recently been developed (Keeley *et al.* 2021). These approaches are either limited to shallow areas or require additional specialised equipment.

Sampling the water that is recovered as part of sediment sample has been carried out (Scheckenbach *et al.* 2010) but is not a common component in benthic DNA monitoring. However, pelagic water sampling has been used in combination with benthic sediment sampling (Holman *et al.* 2019; Cordier *et al.* 2019; López-Escardó *et al.* 2018; Forster *et al.* 2016).

## Recommendations

It is recommended that a method be used that allows for recovery of the sample with negligible mixing. A box core can be used, except when grain size is greater than 1mm, or a van Veen or Day grab with inspection doors for subsampling for sandy sediments. For mixed and coarse sediments, a Shipek grab is recommended where possible or available. A Hamon grab can be used, although the sample will be mixed and therefore is subject to different subsampling and contamination considerations. For depths greater than 350 m, a multicorer is often used but ideally this should be deployed with the same number of replicate deployments as for other sampling approaches. ROVs are likely to become more widely used to maximise sampling efficiencies.

Where sampling is carried out on hard substrates, this will most frequently be in waters that can be reached through SCUBA diving. In these cases, it is recommended to sample carefully using a hammer and chisel in a 25 x 25 cm quadrat. Other devices such as the SIBS may become more widely used in future for sampling on hard substrates with a high proportion of gravels.

## 2.2 Bacteria and Meiofauna

Benthic sediment samples contain both extra-organismal eDNA shed by organisms passing through the sediment (particularly larger animals such as worms and molluscs), and organismal DNA from the small organisms living in the sediment (meiofauna such as nematodes and crustaceans, and microorganisms including single-celled eukaryotes and bacteria).

Sieving a sediment sample to remove extraneous material and keep fauna that are larger than the mesh size is a common approach for marine benthic macrofauna monitoring, with common mesh sizes being 0.5 – 1 mm (Environment Agency 2016). This approach is not suitable for DNA-based analysis targeting smaller fauna and/or microorganisms.

A disadvantage of non-sieved samples is that they may be more difficult to compare directly with the more familiar macrofauna data from conventional assessments. Steyaert *et al.* (2020) compared sieved and non-sieved samples using both metabarcoding and morphotaxonomy. They found that sieving reduced the number of invertebrate species identified regardless of the taxonomic approach used. They also found that while the metabarcoding approach and the morphotaxonomy approach yielded similar numbers of the taxa, many were exclusively found in only one approach. Being able to characterise faunal diversity without the necessity of sieving would greatly increase the utility of DNA metabarcoding in benthic marine sampling. However, as further research is needed in this area to ensure backward compatibility and calibration, we are considering both sieved and unsieved sampling approaches for this review.

In general, smaller taxa are likely to be more powerful in indicating ecological trends for two reasons (Steyaert *et al.* 2020):

1. The greater species diversity gives more statistical power
2. The smaller body sizes and greater abundance per volume of sediment means that a single sample is more representative of the overall community of small organisms than would be the case for the more widely dispersed macrofauna.

Indeed, published research supports the assumption of smaller taxa generating more useful results in the context of impact assessment in offshore oil and gas projects (Laroche *et al.* 2018) and fish farms (Stoeck *et al.* 2018; Cordier 2020).

### 2.2.1 Subsampling and Depth of Subsampling

The priority when collecting a sample for molecular analysis from the grab/core is to ensure the sample is as representative as possible of the whole area sampled by the grab or core. Obtaining representative samples is particularly important when sieving of the whole sample is not being carried out, as is done for conventional macrofauna. Composite samples, consisting of multiple subsamples mixed together, are the best way to ensure that a sample is as representative of the grab/core as possible, while minimising analysis costs. The equipment typically used to collect sediment subsamples for DNA analysis can be broadly divided into two groups:

- Syringes, mini-corers, glass and Perspex tubes used to sample to a depth that can range from 2 to 10 cm (Li *et al.* 2016; Laroche *et al.* 2020b; Guardiola *et al.* 2015; 2016; Klunder *et al.* 2020; He *et al.* 2019; He *et al*. 2020)
- Spoons and spatulas used to scrape the surface sediment up to 2 cm depth (Holman *et al.* 2019; Cordier *et al.* 2019; Stoeck *et al.* 2018; Cordier 2020)

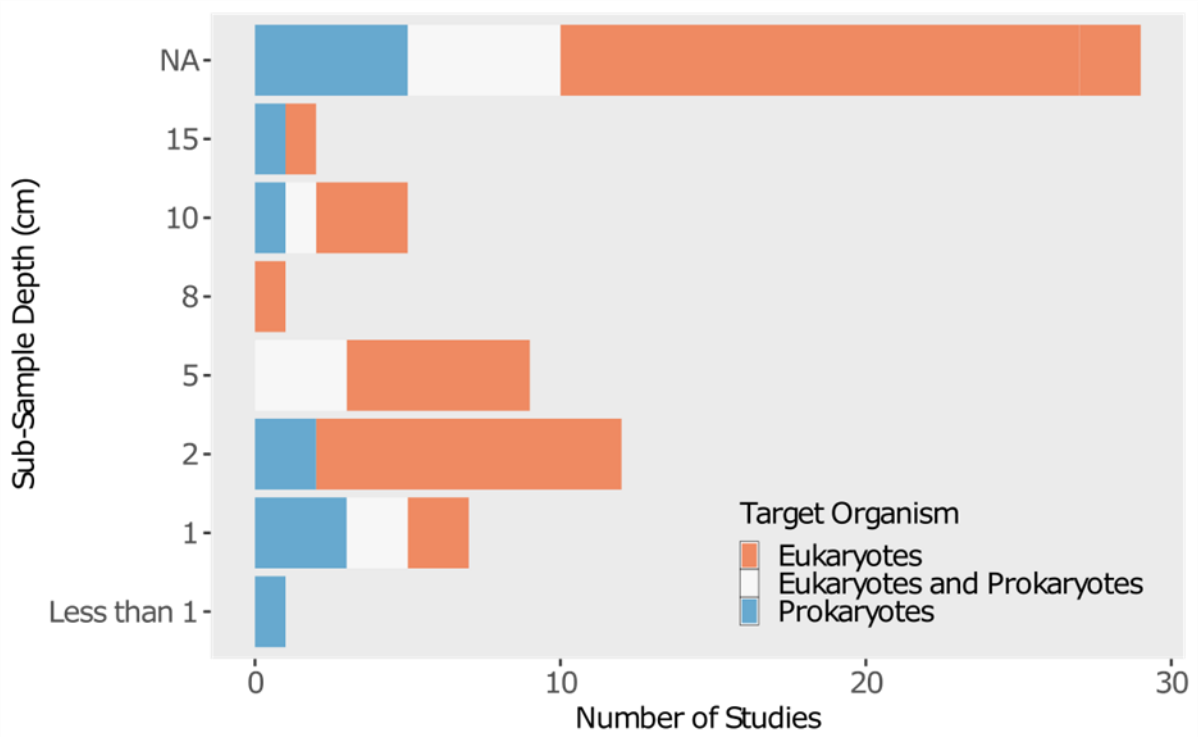


**Figure 3.** Depth of sub-samples in reviewed DNA-based benthic sampling literature for different target taxa (NA = Not Applicable).

Studies that target metazoa using markers targeting the 18S rRNA gene commonly use the mini-corer approach (Laroche *et al.* 2020a; Guardiola *et al.* 2016; Klunder *et al.* 2020). In contrast, studies targeting bacteria and foraminifera, particularly those focussing on the input of organic and living material from above, more frequently use the spatula approach (Stoeck *et al.* 2018; Cordier 2020; Pawlowski *et al.* 2016). However, microbiota are also present deeper within the sediment and contaminants or organic matter from the sediment surface can be mixed deeper into the sediment through bioturbation. Bioturbation is the disturbance of sediment by living organisms, and macrofauna bioturbation has been found to be a key driver of microbial community structure down to 14 cm below the sediment surface (Chen *et al.* 2017). Meiofauna operate at a larger spatial scale than microorganisms do, being more mobile within the sediments on both the horizontal and vertical planes. It therefore makes sense to collect deeper cores of sediment to target infaunal communities of meiofauna and some macrofauna and to retain the surface layer where the most recent microbiota dominate. In this case, modified plastic syringes make ideal mini-corers for sampling for the following reasons:

1. The volume is marked on the outside, allowing standardisation of sampling depth
2. They are transparent, enabling visual inspection of the core to assess whether the sample includes different stratifications of sediment (e.g. both oxygenated and anoxic layers), and particular layers can be discarded or retained if desired.
3. The plunger creates suction which helps to extract an intact core when the sediment is wet.

Communities in the anoxic layer of the sediment have been found to differ from those in the oxygenated layer above (Moodley *et al.* 1997; Spedicato *et al.* 2020; Laroche *et al.* 2020b). The transition to the anoxic layer occurs at different depths and can often be recognised visually by an abrupt colour change to a much darker in colour. In some circumstances it is possible to selectively discard the anoxic portion of a core, but this will not be possible where the profile of the sediment is disrupted during sampling. Whilst the distinction between the anoxic and oxygenated sediment layers is rarely made in the sampling protocol (Fais *et al.* 2020; Lanzén *et al.* 2016), this can be negated by targeting only a shallow layer of surface sediment (Cordier *et al.* 2019; Laroche *et al.* 2018).

Most metabarcoding studies have targeted the surface layer of sediment (Cordier *et al.* 2019; Fais *et al*. 2020), as the sediment surface communities are most affected by pollution and disturbances as they are directly exposed. Furthermore, bacterial richness is highest in the seafloor sediment in the surface 1 cm and decreases with depth (Kerrigan *et al*. 2019). In deep-sea sediments, a small fraction of ASVs (10 % and 19 % for metazoans and non-metazoans, respectively) were found in both the top layer from 0 to 2 cm and in the 3-5 cm layer (Laroche *et al*. 2020b). In contrast, conventional benthic macrofauna assessments record all macrofauna in the sample but exclude pelagic taxa from analyses of diversity (Worsfold *et al*. 2010). Furthermore, taxa that are considered sessile (Protozoa, Proifera, sessile colonial Cnidaria, Entoprocta, Cirripedia, Sessile parasites, Bryozoa, Ascidiacea, Plants and algae and deposited eggs of invertebrate or vertebrates) are recorded as present, but are typically excluded when considering biomass (Worsfold *et al*. 2010). However, given the restricted dispersal ability of microorganisms and meiofauna (on account of small body size), the effect of transitory organisms on the sediment surface is likely negligible, and the eDNA traces from larger organisms will have minimum impact on diversity metrics. It therefore makes sense to include the surface layer for potential microbial assessments (bacteria and single-celled eukaryotes).

To allow for a broadly applicable protocol, the target taxa should be kept as broad as possible to allow for the greatest possible biotic information to be recovered. Mini-corers are most commonly used where multiple sediment taxa are considered (Cronin-O'Reilly *et al*. 2018), sometimes with the different sediment layers separated (Laroche *et al*. 2020b).

For coarse and mixed sediments, it may not be feasible to obtain a subsample using corer as for soft sediment as there will be no suction. In this case, if using a Shipek or a Day grab, a plastic scoop could be used to a depth of 5 cm avoiding contact with the sides and edges of the grab. This is similar to the procedure for contaminant sampling (Whomersley 2014; Mason 2016).

If using a Hamon grab, the depth of subsampling cannot be controlled as the sample will be partially mixed. In this case, subsampling could still be done using a scoop but it would be advisable to completely mix the sample prior to subsampling to make it as homogenous as possible and to avoid inadvertently sampling from particular, unknown sediment depths. This would ensure some level of consistency between samples.

### 2.2.2 Contamination Considerations

When collecting samples for DNA analysis, it is important to consider potential sources of DNA contamination. By taking steps to minimise sources of contamination, we can ensure that samples are representative of the DNA at each location. Sampling methods should therefore eliminate cross-contamination between samples or contamination from the sampling procedure.

To avoid sampling traces of DNA from previous sampling locations, subsamples should not be taken from against the wall of the grab/core (He *et al*. 2021; Chariton *et al*. 2015). To

minimise any potential subsample contamination from the floor of the grab it should also be noted that the minimum acceptable sediment sampling depth attained from a grab deployment should be deeper than the subsampling depth, which we recommend being standardised to 5 cm. The grab itself can be washed between deployments with saltwater to reduce contamination between samples (He *et al*. 2021) and should be visually inspected to ensure all sediment has been removed. The visual inspection of a Hamon grab or Day grab for any traces of sediment from the previous deployment should be particularly thorough as much of the sediment sample is likely to come in contact with the sides of the grab during sample collection. Equipment can also be washed with a bleach solution then rinsed with sterile distilled water between samples (Wei *et al*. 2018; Laroche *et al*. 2020a; Holman *et al*. 2019), although other, more environmentally friendly sterilisation solutions such as ELIMINase can be used (He *et al*. 2020). Careful consideration should be given to the sterilisation solution used, as bleach can corrode metal causing damage to the equipment and making it more difficult to decontaminate in future.

Low level cross-contamination is unlikely to affect overall diversity metrics or index scores but could be significant if the samples are used for biosecurity surveillance where the presence of trace levels of DNA from notifiable species could trigger costly management interventions. It is recommended that standardised sampling protocols allow for both community baselining and detection of notifiable species.

Other commonly used precautions include:

- wearing sterile single-use gloves which are changed between samples (e.g. Stoeck *et al*. 2018)
- double wrapping each sediment sample (e.g. Steyaert *et al*. 2020)
- using sterile equipment for subsampling (e.g. Holman *et al*. 2019)

### 2.2.3 Sample Preservation

Samples must be kept stable between sampling and DNA extraction. The purpose of sample preservation is twofold:

- To prevent degradation of DNA, which reduces the quality of results
- To kill or deactivate organisms to prevent changes in community occurring between the time of sampling and analysis.

A range of preservation methods are available (Figure 4). Sediment samples collected for physico-chemical analysis are usually frozen to prevent chemical reactions from occurring between the time of sampling and laboratory analyses (Kirby *et al*. 2018). Where logistical considerations allow, freezing at 20 $^{o}$C (Corinaldesi *et al*. 2005; Pawlowski *et al*. 2011; Lanzén *et al*. 2020) or at 80 $^{o}$C (Zheng *et al*. 2020; Laroche *et al*. 2020a) is a preferred option for preservation of sediment for DNA analysis. However, this also carries risks since thawing during transit will compromise DNA quality. This is a particular concern if the samples are being transported internationally and may be subject to delays in customs.

A major benefit of storing samples frozen is that any downstream DNA extraction processes can be employed, whereas storage in certain liquid preservatives may not be compatible with particular DNA extraction protocols.

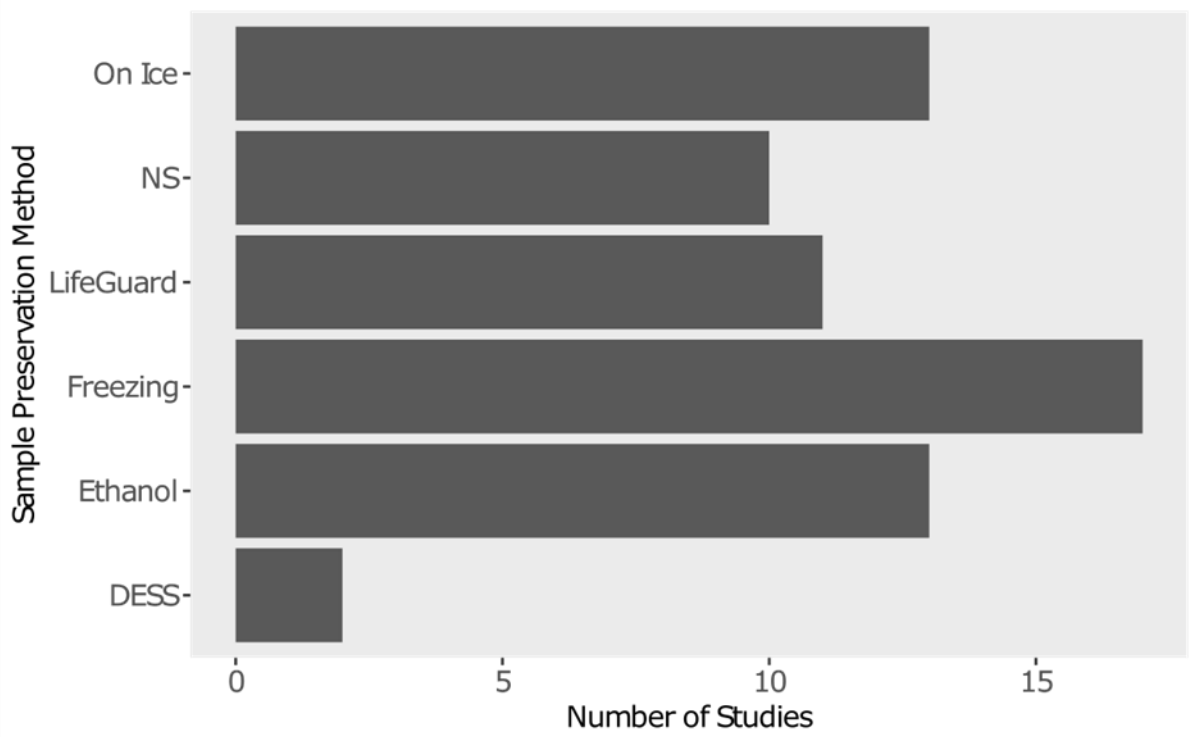


**Figure 4.** Sample preservation methods in the reviewed DNA-based benthic sampling literature (NS = not specified).

Where an onboard freezer is not available, DNA sediment samples can be kept chilled on ice (Wei *et al*. 2018; DiBattista *et al*. 2019; Pochon *et al*. 2015; Chariton *et al*. 2015) or dry ice (Fais *et al*. 2020; Cronin-O'Reilly *et al*. 2018). However, this does limit the time between sampling and samples being sent to the laboratory (Chariton *et al*. 2015).

Some studies may wish to also preserve RNA for analysis of functional gene expression. RNA degrades more readily than DNA, so flash freezing followed by storage at -80 oC is recommended (López-Escardó *et al*. 2018; Pochon *et al*. 2015). This typically involves the use of liquid nitrogen or dry ice (Laroche *et al*. 2020b), which is beyond the capacity of most survey vessels. A thorough review of methods to sample sediment for RNA-based analysis is outside the scope of this review.

When cold storage is not possible, the other option for sample preservation is the use of liquid preservatives. The formaldehyde solution used for preservation of morphological characteristics is not suited to DNA preservation, therefore alternative liquid preservatives must be considered. These fall into two categories:

1. Pure preservatives, which preserve the DNA in the form it is sampled in (i.e. inside organelles, cells, tissues or whole organisms). Examples include:
    a. Ethanol (Krolicka *et al*. 2020; Lanzén *et al*. 2016; Guardiola *et al*. 2016; He *et al*. 2020). This is generally an effective preservative of DNA but it poses health and safety and logistical challenges due to it being a flammable liquid, and requiring strict storage and transportation conditions. Furthermore, ethanol-preserved samples are more labour-intensive to process in the lab because ethanol can interfere with the chemistry of the DNA extraction kits and must therefore be eliminated from the sample prior to DNA extraction. Moreover, ethanol is not suited for the preservation of morphological characteristics of all macrofauna.

   b. RNAlater is designed to preserve both DNA and RNA (Kearns *et al*. 2016). It is commercially available but relatively expensive, which makes it only a viable option for small-volume samples. It can be prepared non-commercially in larger volumes, but this would not be guaranteed DNA / RNA free (which can potentially pose a contamination risk). Note that the manufacturer does not recommend use of RNAlater for RNA preservation in soils, and its use for RNA preservation in sediments has not been well tested.
   c. LifeGuard is a commercially available sample preservation liquid specifically designed for the preservation of both DNA and RNA in soil and sediment samples. Due to its relatively high cost, it is only viable for small-volume samples (Laroche *et al*. 2016; 2018; Cordier 2020; Dully *et al*. 2021) and cannot be produced non-commercially.
2. Lysis agents such as DESS (Tatangelo *et al*. 2014; Yoder *et al*. 2006; Lallias *et al*. 2015; Fonseca *et al*. 2017) and Longmire's solution (Longmire *et al*. 1997), which disrupt animal cells and consequently release the DNA into solution, and simultaneously prevent its degradation. However, in the reviewed literature Longmire's solution has not been used in sediment sample preservation. Note that lysis solutions do not break down the cell walls of many bacterial groups and single-celled eukaryotes such as diatoms; these groups require mechanical grinding to break open the cells and release the DNA. Lysis solutions can be expensive to purchase commercially but can be non-commercially made, although (as for RNAlater) this would not be guaranteed DNA / RNA free.

It is important to be aware that the sample preservation strategy needs to be carefully integrated with the DNA extraction process since the different solutions can interact in different ways with the chemistry of the extraction process. Furthermore, where a preservative solution is used, the sample needs to be shaken well after the addition of preservative to ensure thorough mixing of sample and preservative.

More comparative studies of preservation methods have been carried out for soil samples rather than for marine sediment samples. After testing how storage methods impact the fungal and bacterial community composition of soils compared to the assumed best method of immediate storage in liquid nitrogen, Delavaux *et al*. (2020) found that initial storage using ice packs and subsequent storage at -20°C did not alter the microbial community composition. Whereas storage at room temperature or in RNAlater® (Ambion) changed the community composition. Lauber *et al*. (2010) found temperature to have minor effects on the soil bacterial community composition using 454 pyrosequencing after 14 days at 20°C, 4°C, -20°C and -80°C. Rubin *et al*. (2013) also tested soil storage at room temperature, 4°C, -20°C for 14 days. Their findings supported those of Lauber *et al*. (2010) that differences in preservation time and temperature show minor changes in community composition but suggest best practise for soil preservation should include freezing. Tatangelo *et al*. (2014) also found that bacterial community composition of soil samples stored at room temperature with no preservation buffer were not significantly different from samples analysed immediately. However, they did note that the storage temperature in their study was similar to the environmental temperature where the samples were collected. The change in environmental conditions to which a marine sediment sample would be subjected would be much greater, both in terms of temperature and oxygen availability, and therefore would be more likely to experience changes in bacterial community composition during storage if bacterial activity is not prevented.

One study within our literature review compared sediment sample storage methods for nucleic acid preservation and bacterial community structure (Rissanen *et al*. 2010). The preservation methods tested were storage without a preservation solution, with ethanol, RNAlater® or phenol–chloroform–isoamyl alcohol (PCIAA) at +4°C, -20°C and -80°C. RNA and DNA yields from sediments stored in ethanol and RNAlater® were considerably lower

than the fresh control samples at all temperatures, whereas samples stored in PCIAA, or without preservation solution maintained similar nucleic acids yields to the control, irrespective of freezing temperature. However, samples preserved at +4°C with no PCIAA did show a slight reduction in nucleic acid concentrations. Ethanol and RNAlater® also caused changes in the bacterial community structure, whereas samples stored without a preservation solution or in PCIAA did not vary or varied insignificantly from the control, irrespective of temperature. Although, PCIAA performed well as a storage solution at all temperatures, this is a hazardous solution and is therefore not a practical storage solution for use in the field. Therefore, freezing samples at -20°C is the preferred method.

A recent review article (van der Loos & Nijland 2020) identified that use of DESS for sediment sample preservation has increased recently, and it also has the advantage that is can be stored at room temperature, is not considered to be hazardous, and can successfully preserve morphological characteristics (Yoder *et al*. 2006). However, the usefulness of DESS may be limited at cool temperatures because the saturated salt in the solution precipitates and DMSO, which makes up 20% of the solution, starts to freeze at 19 °C (Sharpe *et al*. 2020). It therefore may not be the most suitable solution for offshore sampling in the British climate.

### Recommendations

Because of the challenge of standardising sampling methods across sediment types and disturbance levels, discarding anoxic sediment portions is not recommended but any colour changes observed in the cores should be recorded so that this can be taken into account during analysis. It is recommended that the sampling depth is standardised to 5 cm (Guardiola *et al*. 2016; Cronin-O'Reilly *et al*. 2018). This will encompass the 2 cm depth of samples commonly considered in chemical analysis (Mcniven & Gilchrist 2017; Kirby *et al*. 2018), and assessment of oxidation-reduction potential (measured at 4 cm depth) for sediment anoxia (Environment Agency 2016) and particle size analysis from samples taken to a minimum of 5 cm depth (CEFAS 2012).

For Van Veen and Day grabs and cores, collecting composite samples comprising one mini-core sub-sample from each quadrant of the grab or core, for a total of four sub-samples mixed together, is recommended to capture as much variation as possible within the grab. While collecting sub-samples, contact should not be made with the edges or the bottom of the grab/core device. For a Shipek grab, a plastic scoop is recommended instead of the mini-core. For a Hamon grab, the grab sample should be mixed thoroughly then sub-sampled using a plastic scoop.

The sediment grab or core device should be rinsed thoroughly between deployments to remove residual sediment from the surfaces. Single use sterile items (such as gloves and mini-corers) should be discarded after each sample. Any reusable equipment must be cleaned between samples with a decontaminant such as 10% bleach solution. When sub-sampling from a grab or core device, sediment that has been in contact with the edges of the non-sterile sampling device should be avoided. Samples should be stored in two sterile snap lock bags for containment in case of leakage and to prevent cross-contamination.

If samples are to be delivered to the laboratory within a couple of days of sampling, they can be stored in a fridge, although immediate freezing is preferred. 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 cause DNA degradation. For transportation to the laboratory, samples should be packed in a cool box with ice packs leaving minimal headspace. We recommend thorough mixing of the sub-samples prior to freezing as mixing afterwards requires the sample to be thoroughly thawed and exposure to light, warmth, and oxygen

should be minimised prior to DNA extraction. If neither freezer nor short term cold sample preservation is feasible, DESS sample preservation is recommended.

## 2.3 Macrofauna

Macrofauna are currently the primary taxonomic component of sediment samples used for many common biotic indices and habitat classification systems. Sieving a sediment sample to remove extraneous material and keep fauna that are larger than the mesh size is a common approach for marine benthic macrofauna monitoring, with common mesh sizes being 0.5 – 1 mm (Environment Agency 2016). This approach is not suitable for DNA-based analysis targeting smaller fauna and/or microorganisms. As noted earlier in this review, and repeated here for context, a disadvantage of non-sieved samples is that they may be more difficult to compare directly with the more familiar macrofauna data from conventional assessments. Steyaert *et al*. (2020) compared sieved and non-sieved samples using both metabarcoding and morphotaxonomy. They found that sieving reduced the number of invertebrate species identified regardless of the taxonomic approach used. They also found that while the metabarcoding approach and the morphotaxonomy approach yielded similar numbers of the taxa, many were exclusively found in only one approach. Being able to characterise faunal diversity without the necessity of sieving would greatly increase the utility of DNA metabarcoding in benthic marine sampling. However, as further research is needed in this area to ensure backward compatibility and calibration, we are considering both sieved and unsieved sampling approaches for this review.

### 2.3.1 Sieving and Subsampling

There are various approaches taken with regard to how sediment samples are sieved for conventional morphotaxonomy. Whole grabs or large sub-samples can be sieved on board the sampling vessel to reduce the volume of sample to be stored for transport to the laboratory (Aylagas *et al*. 2016; Klunder *et al*. 2020). This onboard sieving approach is commonly used by NE and JNCC in subtidal benthic MPA monitoring surveys. Alternatively, samples can be kept on ice for transport to the laboratory for sieving and decanting (Steyaert *et al*. 2020). In some cases, samples are sieved initially on board and subsequently more thoroughly sieved in the laboratory. Studies targeting meiofauna have also used this size fractionating methodology (Fonseca *et al*. 2017; Lallias *et al*. 2015) and it has been implemented within macroinvertebrate metabarcoding studies (Aylagas *et al*. 2016; Lobo *et al*. 2017).

If only a partial grab is being sieved, this subsampling should be from evenly homogenised grab contents to ensure maximum representativity. This also facilitates the inclusion of sediment samples that have been mixed during collection, making a wider choice of equipment such as Hamon grabs a practical option.

For coarse sediment samples, a large volume of sediment frequently remains after sieving, resulting in large containers (10 L buckets) being used for conventional macrofauna sampling out of necessity. For DNA-based analysis it is recommended that the benthic megafauna >10 mm (Stratmann *et al*. 2020) are separated by using a second sieve. These can be:

- preserved in a formaldehyde solution for the conventional morphological identification;
- photographed and small subsamples of tissue taken for inclusion in the DNA macrofauna sample;
- preserved in a second, larger container with 96% ethanol for DNA barcoding;
- Sieving sediment samples has some drawbacks for DNA-based analysis as the process;

- has a higher risk of contamination between samples given the need to reuse equipment;
- requires more time, effort and equipment;
- results in loss of taxa smaller than the selected sieving mesh, resulting in inherent biases and reduction in both the number and diversity of sequence reads (Steyaert *et al*. 2020);
- has increased downstream effort due to the large size of the samples and the dominance of thick exoskeletons that require grinding and homogenisation prior to DNA extraction.

### 2.3.2 Contamination Considerations

For the purposes of bacterial and meiofauna analyses onboard sieving greatly increases the contamination risk as the availability of equipment for sterilisation of sieving apparatus may be limited. However, if these sieved samples are used uniquely to target macrofauna, the biomass of macrofauna would result in a signal outweighing any cross-contamination. It is recommended that plastic reusable equipment is rinsed in seawater to remove sediment, then soaked in 10% bleach solution for at least five minutes. 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. Rinsing in seawater will not sterilise equipment to a level suitable for bacterial sampling but should reduce cross-contamination of macrofauna between samples. It is therefore recommended that if bacterial DNA analyses are required in addition to macrofauna, that the bacterial sediment samples are collected separately before sieving.

### 2.3.3 Sample Preservation

It is recommended that macrofaunal samples should be preserved in cold ethanol (Aylagas *et al*. 2016). For preservation of DNA, 96% ethanol is most frequently recommended (e.g. Aylagas *et al*. 2016), but it should be noted that this may preclude morphological analysis as it can damage specimens (for which 80% is recommended) (Glover *et al*. 2016). It may be possible to obtain DNA from bulk macroinvertebrate samples by immersing them in a storage buffer (Linard *et al*. 2016) or even by filtering the water used to rinse the sample, but this has not yet been well trialled in the marine environment. Evidence from freshwater bulk invertebrate samples indicate that although fewer taxa are recovered by extracting DNA from a fixative, compared to grinding the sample, this can be a useful approach when the integrity of the bulk sample needs to be preserved (Martins *et al*. 2019).

Ethanol preservation of macrofaunal samples allows for morphological identification of most taxa (with the possible exception of molluscs), which can also be separated out so that they can be used for generating DNA barcodes (Glover *et al*. 2016). These barcodes could be added to DNA sequence databases, allowing for higher taxonomic resolution identification and a more direct comparison with conventional morphological macrofauna. Over time, this would also identify any biases in the metabarcoding method (Faria *et al*. 2018; Huang *et al*. 2020) and result in a robust and well-supported adoption of metabarcoding in the marine environment for monitoring purposes (Aylagas *et al*. 2020).

#### Recommendations

Sieving samples is generally not recommended so that analyses on samples can be applied for a broader range of taxa. However, if bulk macroinvertebrate sampling is required to enable comparison with conventional morphological monitoring at a site, sieving of the whole grab/core sample should be conducted in accordance with existing protocols (1 mm mesh) after any bacterial or meiofaunal samples have been taken). For this, the sieving equipment should be cleaned thoroughly between samples using a DNA decontaminant solution, such

as bleach, and then rinsed with water. If sieving is not conducted on board the vessel, and only subsamples are being retained then a large subsample (minimum 1000 $cm^3$) should be collected.

Sieved macrofaunal samples should be preserved in 96% ethanol at cold (<6°C) temperature.

Future research could explore:

- Subjecting macrofauna samples to both morphotaxonomic and DNA-based analysis.
- Targeting macrofauna from both sediment directly and from sieved portions of grab samples to determine whether macrofauna diversity is adequately captured using sediment.
- Investigation of the number of sediment subsamples to take from each grab to characterise the macrofauna community.
- Investigation of the number of macrofauna subsamples (where necessary) to take from each grab to characterise the macrofauna community.
- Comparison of available buffers for storing macrofauna samples as ethanol is hazardous.
- Determining the best method for collecting epifauna attached to pebbles and cobbles in sieved samples.

## 2.4 Sample size

Microbial communities are typically assessed from very small volume sediment samples (Cordier *et al*. 2019) of 0.2-0.5 g as this is the maximum volume that can be processed in standard microcentrifuge tube DNA extraction kits. Given the small spatial scale at which microbial communities operate and the density at which they exist in the sediment, this is sufficient for community characterisation. Small-volume samples also carry several advantages:

- They are easy to store and transport in large numbers and can be processed in high-throughput workflows for DNA extraction, using standard liquid handling robotics.
- They can be preserved in small volumes of liquids, so even expensive solutions (e.g. LifeGuard for RNA preservation) are a viable option.
- DNA extraction methods are highly efficient, involving mechanical grinding of the whole sample.

For larger, multicellular organisms, which move over larger distances and are more widely dispersed, studies have found that DNA extractions from 0.2-0.5 g of sediment are insufficient to capture a representative community and that larger volumes of sediment (at least 10 g) need to be processed (Fais *et al*. 2020). Options are more limited for processing these larger-volume samples:

- The largest volume that can be processed in a commercially available (and therefore consistent) extraction kit is 10 g (e.g. Qiagen PowerSoil Max kits), although larger volumes of 20 g have been used in certain studies (Laroche *et al*. 2020b). These are more expensive than the small-volume kits per sample due to the higher volumes of reagents required.
- Commercially available solutions that preserve RNA as well as DNA (e.g. LifeGuard) are more expensive to use in the volumes required for preserving a 10 g sample.

### Recommendations

For samples to be analysed for DNA only, taking a large sample volume (> 30 g) is recommended to ensure that both infauna and bacteria can be considered for analysis. However, if samples were also to be analysed for RNA, and bacteria was the only target of interest, a smaller subsample of a composite sample could potentially be preserved for downstream RNA analysis.

## 2.5 Field Control Samples

None of the sediment sampling studies reviewed explicitly described field control samples, nor are they explicitly described for the monitoring of contaminants (Kirby *et al*. 2018). However, we understand, particularly given the novelty and potential sensitivity that the use of field controls would be useful, as is used for water sampling.

There are several types of samples that are often used for field sampling quality control for evaluating cross contamination. These are not specific to DNA sampling and were primarily developed for contaminant sampling but have the potential to be adapted for DNA sampling purposes. Types of field blanks include:

- Preservative blanks: if samples are to be stored in a preservative buffer solution, such as DESS, a sterile sample of the solution (from the same batch as is being used for the samples) is taken to the field where it is opened then poured into a sterile sample pot. This will detect any potential contamination in the preservative and in the sampling environment.
- Equipment rinsate blanks: For equipment that is decontaminated between samples a rinsate blank can be performed to evaluate how successful the cleaning process between samples was. This involves pouring sterile water over a piece of equipment after it has been cleaned and then collecting the water into a sterile sample pot.

DNA can then be extracted from these blank samples to identify potential contamination within the samples. The problem with the equipment rinsate blank, is that unless you collect a blank alongside every sample, any contaminant taxa detected in the blank will only be representative of the contamination in the sediment sample that was collected immediately afterwards. It does not mean that those taxa were contaminants in every sample. Also, as both of these blanks give you a liquid sample, they need to be subjected to a different DNA extraction protocol to the sediment which could also induce biases.

One possibility is the use of sterile sand for the negative control, a substance used for mechanical breakdown of DNA samples (Yee *et al*. 2018). This could be transported into the field in a sterile container and then the preservative blank or equipment rinsate blank poured into the same container. Further testing would be required to assess the validity of this suggested method.

## 2.6 Record keeping

An adapted version of current JNCC metadata recording form and the MEDIN grab/core forms are available in the associated protocol. 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.

# 3 Conclusions and Recommendations

Different sampling strategies are required for different projects and areas. DNA benthic sampling should be aligned with pre-existing sampling strategy for benthic fauna where possible. For MPA surveys, this would most frequently be either stratified random sampling or systematic grid sampling. Where investigating point sources of pollution or similar impacts, it is recommended to include a reference station and to have stations in the form of a cruciform or in a transect aligned with the prevailing water currents (along an impact gradient). When sampling broader areas as part of coastal and estuarine monitoring, it is recommended that stations are placed along salinity gradients and in a representative range of habitat types. Where habitat classifications / biotopes are unknown and the purpose is to identity these, the spatial scale of sampling is very important to ensure that the required granularity is achieved and not to miss important protected benthic habitats. This must be determined by topography and coastal processes of erosion and deposition and will be influenced by the likely level of habitat heterogeneity in the area.

At each station, taking three to five replicate samples is recommended, based on both the existing literature and statistical considerations. It is possible that as a better evidence base is generated using this standardised sampling method that valid statistical analyses can be carried out with fewer replicates.

Any sampling equipment used should minimise both mixing and disturbance of the surface layer of sediment. The use of a box core is recommended where possible, although a van Veen or Day grab is recommended where a broader array of sediment types are being sampled, provided the grab has observation doors for subsampling. Where sampling coarse sediments a Shipek grab is recommended to minimise mixing and enable the same subsampling policy to be used. If a Hamon grab is the only available option, as mixing is inevitable, complete homogenisation of the grab prior to subsampling is recommended to ensure consistency. The grab or core should be rinsed with seawater and visually inspected to remove as much sediment before the next deployment. Sampling at greater depths is most commonly achieved using a multicorer or can be done using a remotely operated vehicle (ROV). It is considered that the use of ROVs to be a fruitful area of research and development in sampling, as are suction based samplers for communities on hard substrates. Further innovation in sampling equipment could improve ease of deployment, automation of sampling and lead to cost benefits.

To ensure a representative composite sample of the grab for bacteria and meiofauna, subsampling in each grab or core quadrant is recommended using a sterile plastic syringe mini-core to a minimum depth of 5 cm. Any colour changes in the core associated with anoxia should be noted. Subsampling should be done while wearing sterile single-use gloves. Samples should be double-contained to prevent cross-contamination and stored at 4 °C for two days or less, or -20 °C if stored for longer. For transportation to the laboratory, samples should be packed in a cool box with ice packs leaving minimal headspace. Where cool storage is not feasible, a DESS preservation buffer should be used.

For sampling for macrofauna, the whole grab or core should be used if sieving on board the vessel and equipment rinsed thoroughly and visually inspected between samples. If sieving is not conducted on board the vessel, a minimum sediment sample of 1000 $cm^3$ should be collected. Sieved macrofaunal samples should be preserved in 96% at cold (6°C) temperatures and can subsequently be used in DNA barcoding campaigns, which would help to overcome one of the major challenges of DNA metabarcoding.

The sampling protocols established from this review should provide a clear pathway for regular DNA sampling as part of biomonitoring programmes. The resulting samples can be used for both DNA metabarcoding of a range of differently sized taxa, fulfilling the aims of

the marine scientists and managers. This should enable practical application of standardised DNA sampling by the SNCBs across a range of benthic habitats both within and beyond the UK's Marine Protected Area network.

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