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## Environmental DNA metabarcoding for benthic monitoring: a review of sediment sampling and DNA extraction methods

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## **Abstract**

Environmental DNA (eDNA) metabarcoding (parallel sequencing of DNA/RNA for identification of whole communities within a targeted group) is revolutionizing the field of aquatic biomonitoring. To date, most metabarcoding studies aiming to assess the ecological status of aquatic ecosystems have focused on water eDNA and macroinvertebrate bulk samples. However, the eDNA metabarcoding has also been applied to soft sediment samples, mainly for assessing microbial or meiofaunal taxa. Compared to classical methodologies based on manual sorting and morphological identification of benthic taxa, eDNA metabarcoding offers potentially important advantages for assessing the environmental quality of sediments. The methods and protocols utilized for sediment eDNA metabarcoding can vary considerably among studies, and standardization efforts are needed to improve their robustness, comparability and use within regulatory frameworks. Here, we review the available information on eDNA metabarcoding applied to sediment samples, with a focus on sampling, preservation, and DNA extraction steps. We discuss challenges specific to sediment eDNA analysis, including the variety of different sources and states of eDNA and its persistence in the sediment. This paper aims to identify good-practice strategies and facilitate method harmonization for routine use of sediment eDNA in future benthic monitoring.

## **1. Current status of benthic monitoring**

### **1.1. Importance of sediments for the assessment of ecological status**

In aquatic habitats, soft sediments play a critical role in biogeochemical cycling and other benthic processes (Boulton et al., 1998; Gray and Elliott, 2009; Hauer et al., 2018; Snelgrove et al., 2018). Sediments can also act as reservoirs of contaminants discharged into surface water (Matsuyama et al., 2018). This is particularly evident in estuaries, coastal lagoons and other transitional ecosystems (Cuevas et al., 2018) that accumulate large amounts of silt, clay and organic matter, on which many contaminants (e.g. metals, PCBs, PAHs) adsorb (Cibic et al., 2019; Fazi et al., 2020; Quero et al., 2015). These sediment-bound pollutants can have damaging ecotoxicological effects both locally and at the hydroecosystem level via sediment transport and release of contaminants, for example during high water events (Vivien et al., 2020a). One of the most consistent effects of contamination in sediments is a change in the biological communities that inhabit them, as pollution-sensitive species are lost and replaced by more tolerant ones (Clark et al., 2020; Nepote et al., 2017). This makes the benthic communities themselves powerful indicators of pollution. Assessment of sediment quality based on benthic bioindicators is included in all governmental regulations that aim to achieve environmental objectives, such as the Water Framework Directive (WFD), Marine Strategy Framework Directive (MSFD), Clean Water Act, among others. Yet, in practice, routine monitoring focuses mainly on surface waters that are known to serve as the main recipients of chemicals discharged into the environment from industrial and agricultural sources, wastewater and other processes (Förstner and Wittmann, 1981; Stehle and Schulz, 2015). While the quality of many European surface waters has significantly improved over the last decades, contaminated sediments represent one of the major obstacles to the achievement of quality goals (Gieswein et al., 2019), and the assessment of sediment quality is considered to be of major concern (Brils, 2020).

Ecological status of contaminated sediments has been assessed in various ways since the 1970s (Burton, 1991 and references therein). Integrated and hierarchical “weight of

evidence” (WoE) approaches (e.g. the Sediment Quality Triad, Long and Chapman, 1985) are recommended for meaningful and comprehensive assessment of both freshwater (Pesce et al., 2018) and marine (Chapman et al., 2017) environments. These assessments combine multiple lines of evidence derived from different ecotoxicological disciplines (i.e. chemical analysis, toxicity testing, and community ecology) to provide a holistic view of sediment quality. Drawing on these integrated WoE approaches, priorities have been set out for the quality elements used to assess the ecological status of water bodies (*Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy*, 2000), and these include biological indicators (Moreno et al., 2011; Poikane et al., 2016; Rosenberg et al., 2004; Tweedley et al., 2012). By providing information on the *in situ* effects of pollutants present in soft sediments, biological indicators efficiently complement other types of ecotoxicological tests performed in the laboratory.

## **1.2. Limitations of morpho-taxonomic surveys of benthic macroinvertebrates**

A plethora of indices exist for benthic quality assessment in soft sediment habitats. These are based primarily on benthic macroinvertebrate communities in both marine (Borja et al., 2008) and freshwater ecosystems (Poikane et al., 2016; Rosenberg et al., 2004; Tweedley et al., 2012). Indices typically take into account the diversity, abundance and ecological adaptations of taxa and have been adapted for a wide variety of habitats ranging from freshwater (Vitecek et al., 2021) to estuarine, coastal and deep-sea ecosystems (Borja et al., 2015; Rumohr, 2009). While most indices consider a broad range of taxonomic groups, some focused on a particular group. For instance, indices based on the community structure of oligochaetes have been developed and standardized for biological quality assessment of soft sediments in streams and lakes (AFNOR., 2016; Lafont et al., 2012, 2010). Oligochaetes are abundant and diverse in fine/sandy sediments and include a large number of species encompassing various levels of

sensitivity to pollutants (Rodriguez and Reynoldson, 2011; Vivien et al., 2020a).

The prevalence of assessment methods based on macroinvertebrates is the result of a long tradition and extensive taxonomic and ecological knowledge of these groups (Pearson and Rosenberg, 1977; Rosenberg and Resh, 1993). The benthic macroinvertebrates show consistent responses to anthropogenic pressures (Dale and Beyeler, 2001), allowing the development of reliable indices (Diaz et al., 2004; Rosenberg et al., 2004), and they also can be categorized by a variety of functional traits and adaptations to environmental changes. In addition, they comprise a broad range of taxonomic groups, widely distributed in benthic ecosystems, and are mostly characterized by low mobility, making them relatively easy and cheap to sample.

However, morpho-taxonomic inventories of benthic macroinvertebrates are vulnerable to certain technical and operational hurdles that may prevent timely and cost-effective responses by environmental managers (Hering et al., 2018; Nygård et al., 2016). These include the separation of the specimens from the substrate, their sorting and morphological identification, a time-consuming process demanding a high level of taxonomic expertise (Haase et al., 2006; Rumohr, 2009). Moreover, the large volume of these samples make them difficult to archive for ulterior analyses. These operations inherently result in low throughput for processing of environmental samples, which may limit the spatial and temporal frequency of morpho-taxonomic surveys (Cahill et al., 2018; Ferraro et al., 1989). This, in turn, limits the resolution and statistical power of datasets and their usefulness for deriving confident conclusions as to variations in ecosystem quality.

The level of taxonomic discrimination required to detect pollution is a subject of recurrent discussions (Terlizzi et al., 2003; Warwick, 1988). Although family level identifications are commonly used for river assessments (Birk et al., 2012), species- or genus-level data are generally considered more sensitive for evaluating pollution impacts, particularly in multi-



pressure marine environments (Borja et al., 2011), and 74% of assessment methods employed in the EU require species-level identifications (Birk et al., 2012). In practice, this level of taxonomic accuracy is difficult to achieve reliably due to morphological variability and subjective interpretation of morphological features (Haase et al., 2010). In addition, taxonomic ambiguities and uncertainties often arise from the complex life stages that may not be morphologically distinguishable, or from organisms being damaged during sampling and preservation. This scenario may be further complicated by the presence of cryptic diversity, defined as two or more distinct species or local variants that were classified as a single one due to morphological similarity, which can highly bias morpho-taxonomic surveys (Bickford et al., 2007; Macher et al., 2018; Trontelj and Fišer, 2009).

### **1.3. The use of meiofauna and microorganisms for biomonitoring**

Despite their dominant use in biomonitoring and bioassessment, macroinvertebrates represent only a portion of the benthic community biomass (Giere, 2009), which also includes a great diversity and abundance of meiofauna and microorganisms (Patrício et al., 2012).

Ecologically, meiofauna serve as an important link between the microbial food web (i.e. prokaryotes and microbial eukaryotes) and higher trophic levels (i.e. macroinvertebrates and fishes) and are also major contributors to benthic energy flow (Bergtold and Traunspurger, 2005; Danovaro et al., 2002; Majdi and Traunspurger, 2015; Reiss and Schmid-Araya, 2010). Since most meiofauna spend their entire life cycle within the sediments, these organisms are directly influenced by sediment characteristics and contaminants (Traunspurger and Drews, 1996). Additionally, because of the shorter generation time and life cycles, meiofaunal communities can be more responsive to pollutants compared to macrofauna (Balsamo et al., 2012; Coull and Chandler, 1992). Finally, their tiny body size and restricted motility mean that a representative community might be recovered from smaller volume of sediment samples. These characteristics make them potentially suitable for the assessment of sediment

quality.

Indeed, several meiofaunal groups have been used in experimental and observational studies related to environmental biomonitoring. Among them, nematodes have been demonstrated as bioindicators in both marine and freshwater ecosystems (Höss et al., 2017; Moreno et al., 2011). In addition to being the most abundant and diverse meiofaunal taxon (Giere, 2009), nematodes are sensitive to a wide range of impacts, including organic matter enrichment, hydrocarbons, and heavy metals (Höss et al., 2017; Zeppilli et al., 2015). At least for freshwater environments, they are considered the most informative meiofaunal group for sediment quality assessment (Höss et al., 2017). In marine benthic habitats, the most commonly used bioindicators are Foraminifera (Frontalini et al., 2018a, 2018b), Copepoda (Giere, 2009) and Ostracoda (Ruiz et al., 2005). Foraminifera are meiofauna-size protists, whose cells can be enclosed in calcareous or agglutinated tests that can be preserved within the sediment and may provide useful information for the definition of baseline ecosystem conditions (Jesus et al., 2020). Harpacticoid copepods, the second most diverse and abundant component of the meiofauna (Giere, 2009), are known to be highly sensitive to low oxygen concentrations (De Troch et al., 2013) and riverine inputs (Frontalini et al., 2011). Meanwhile, marine ostracods have also been used as bioindicators of water (Frenzel and Boomer, 2005) and sediment quality (Ferraro et al., 2017; Ruiz et al., 2005) showing high sensitivity to heavy-metal pollution, oil discharges and anoxic conditions (Ruiz et al., 2005).

The microbial benthic community represents another potentially powerful yet relatively under-explored source of sediment quality bioindicators (Sagova-Mareckova et al., 2021). Microorganisms play a pivotal role in biogeochemical cycles, carbon sequestration and mitigation of pollutants by metabolizing allochthonous organic and inorganic compounds (Azam and Malfatti, 2007). All these processes sustain the ecosystem functioning and provide fundamental services for human well-being (Kremen, 2005). The community

composition and metabolic activity of microbes are directly influenced by changes in physical-chemical parameters, making them highly sensitive to local and global pressures. Short generation times mean that community responses occur rapidly, making microbial communities good indicators of current conditions (Parmar et al., 2015). Furthermore, the microbial community responses are reported to be highly specific to a contaminant type (Xu et al., 2019; Yakimov et al., 2007).

Many environmental genomic studies have analyzed the impact of pollutants on microbial communities (Fabiano et al., 1994; Handley et al., 2017; Mahmoud et al., 2018; Nawaz et al., 2018; Smith et al., 2015; Sohlberg et al., 2015) with several demonstrating strong, predictable responses to stressors (Cordier et al., 2020; Dewle et al., 2015; Fazi et al., 2020; Lanzén et al., 2020; Laroche et al., 2018b; Mahmoud et al., 2020; Stoeck et al., 2018a). A bacterial index (microgAMBI) has been developed to assess marine sediment quality using microbial diversity inferred from metabarcoding data (Aylagas et al., 2017) and its efficiency in detecting impacts has been tested around the world (Borja, 2018). Similarly, microbial biotic indices have been used for assessment of freshwater ecosystems (reviewed in Sagova-Mareckova et al., 2021). These and other studies (e.g. Cordier et al., 2020, 2019) provide increasingly strong evidence that microbial environmental genomics represents a powerful new tool for routine biomonitoring across multiple habitats. However, current legislation does not yet include any microbial component in sediment quality assessment methods.

## **2. Environmental DNA metabarcoding**

### **2.1. Overview of sediment eDNA metabarcoding studies**

The rapid development of high-throughput sequencing technologies has enabled the use of molecular taxonomy as a viable alternative to morpho-taxonomic inventories. Underpinning the use of these DNA-based methodologies is the assumption that taxa can be reliably identified on the basis of a particular fragment of its genome, referred to as a “DNA barcode”

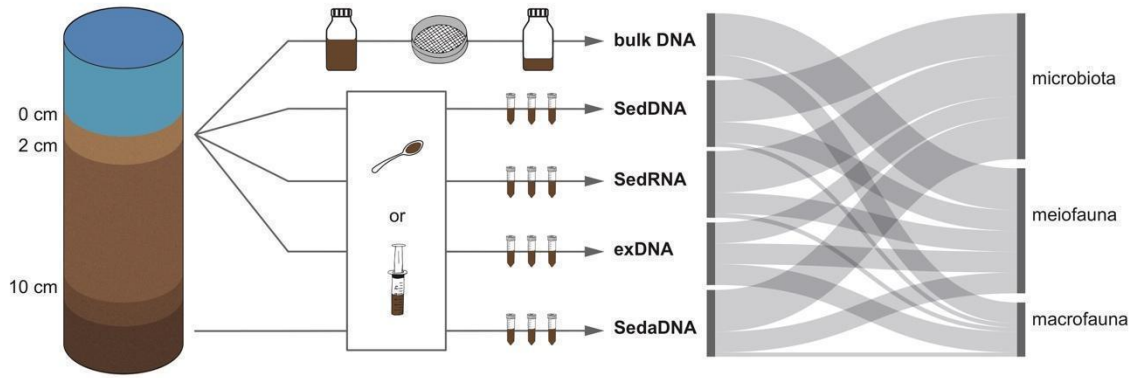
region. When high-throughput sequencing of a DNA barcode region is used to identify many species in parallel from complex samples, this is known as metabarcoding. Bulk metabarcoding refers to the analysis of samples that consist of mixed collections of organisms, while eDNA metabarcoding refers to the analysis of environmental samples such as sediments or water (Taberlet et al., 2012).

The direct extraction of nucleic acids from sediments revolutionized the study of microbial diversity in benthic ecosystems, since only a very small fraction of fungi, archaea and bacteria can be cultivated using standard techniques (Roose-Arnaleg et al., 2001). Since then, metabarcoding of marine and freshwater sediment eDNA has revealed huge and largely undescribed diversity of viruses (Tangherlini et al., 2020; Zheng et al., 2020), archaea (Corinaldesi et al., 2019; Hoshino et al., 2020), bacteria (Zinger et al., 2012), fungi (Grossart et al., 2019; Manohar and Raghukumar, 2013; Panzer et al., 2015; Picard, 2017; Wurzbacher et al., 2016) and protists (Forster et al., 2016; Pawlowski et al., 2011; Scheckenbach et al., 2010). The vast majority of these taxa are new to science, enormously increasing our knowledge of prokaryotic and eukaryotic microbial diversity (Thompson et al., 2017). The novel lineages revealed by sediment eDNA metabarcoding has contributed to radically change our view of the evolution and diversity of some microbial groups (e.g. Lokiarchaeota, Spang et al., 2015; Chlamydiae, Dharamshi et al., 2020). It has also improved our understanding of the ecology of benthic microbial communities, particularly in poorly-explored extreme environments, such as the deep-sea (Bienhold et al., 2016; Danovaro et al., 2017, 2015; Pawlowski et al., 2011), the pools affected by hydrothermal fluids (Crognale et al., 2018), soda lakes (Fazi et al., 2021), and polar regions (Fang et al., 2019).

Similarly, sediment eDNA metabarcoding has transformed our understanding of benthic meiofauna. Numerous molecular studies have demonstrated the huge diversity of nematodes (Brannock et al., 2014; Dell'Anno et al., 2015), copepods (Hirai et al., 2017), foraminifera

(Lejzerowicz et al., 2021; Pawlowski et al., 2014) and other meiofaunal taxa (Bellisario et al., 2021; Creer et al., 2010; Fais et al., 2020a, 2020b; Fonseca et al., 2017, 2014, 2010; Lejzerowicz et al., 2021), which are difficult to assess using morpho-taxonomic approaches. Some of these studies were based on specimens separated from the sediment via pre-processing steps (Brannock and Halanych, 2015; Creer et al., 2010; Dell'Anno et al., 2015; Fonseca et al., 2017, 2014, 2010), while others extracted DNA directly from the sediment (Bellisario et al., 2021; Fais et al., 2020a, 2020b; Lejzerowicz et al., 2021). Sediment eDNA metabarcoding has been used to reveal previously unknown meiofaunal diversity in the deep-sea (Laroche et al., 2020; Lejzerowicz et al., 2021; Sinniger et al., 2016), and coastal marine ecosystems (Bellisario et al., 2021; Chariton et al., 2015, 2014; DiBattista et al., 2020; Fais et al., 2020a, 2020b; Guardiola et al., 2016; López-Escobar et al., 2018).

Another major research field involving sediment eDNA metabarcoding is paleogenomics. Although this field is not the focus of the current review, it is worth mentioning that sedimentary ancient DNA (sedaDNA) can be used to reconstruct past communities and their changes through time (Capo et al., 2021; Domaizon et al., 2017; Dommain et al., 2020). Paleogenomic analysis of lake sediments has been used to trace the impact of human activities on freshwater ecosystems (Domaizon et al., 2017; Giguet-Covex et al., 2019; Keck et al., 2020), while analysis of marine sediment cores has revealed change in benthic and planktonic communities associated with urbanization and agriculture pollution (Siano et al., 2021) and the impact of shipping on algal bloom distribution (Shaw et al., 2019).



**Figure 1.** Different types of genetic material isolated from sediments and their application to monitor different groups of organisms. The depths (cm) are based on numbers used in reference studies (Table 1). The abbreviations refer to the origin of genetic material extracted from sediment samples: bulk DNA = preprocessed samples of meio- or macrofauna, SedDNA and SedRNA = surface sediment samples, exDNA = extracellular material, SedaDNA = historical sediment samples.

## 2.2. Current applications of sediment eDNA to benthic monitoring

### 2.2.1. Environmental impact assessment of marine industries

Marine industries such as coastal aquaculture and oil and gas operations impose a high pressure on benthic ecosystems. Routine benthic monitoring is mandated as a part of environmental impact assessment for these industries and used to inform management processes that help maintain healthy ecosystems together with associated ecosystem functions and services. This is highly based on the use of macroinvertebrate bioindicators, which, as above-mentioned (section 1.2), imposes significant limitations on the scope and scale of monitoring. This in turn limits the usefulness of the data to guide adaptive management of the benthic ecosystem and timely response to pollution events.

Many studies have now explored the potential of sediment eDNA for routine monitoring of marine industries, with the aim to replace or complement the macrofauna-based benthic

monitoring currently employed in accordance with regional and national legislation (Cordier et al., 2019; Lanzén et al., 2016, 2021; Laroche et al., 2018b; Lejzerowicz et al., 2015; Pochon et al., 2020). In the case of marine aquaculture, a number of different taxonomic groups has been targeted by metabarcoding studies, ranging from selected groups of protists such as Foraminifera (Frontalini et al., 2020; He et al., 2019; Pawlowski et al., 2014; Pochon et al., 2015) and Ciliophora (Forster et al., 2019; Stoeck et al., 2018b) to the whole domains of Eukarya (Cordier et al., 2018; Keeley et al., 2018) and Bacteria (Cordier et al., 2018; Dowle et al., 2015; Keeley et al., 2018; Stoeck et al., 2018). Most of these studies used sediment eDNA metabarcoding to generate biotic indices that were correlated with those derived from benthic macroinvertebrates as well as with physical and chemical factors. Despite being only semi-quantitative, metabarcoding data has often provided very similar patterns to traditional methods (Lejzerowicz et al., 2015). Stronger results have generally been obtained from studies that target a broad taxonomic spectrum (eukaryotes or bacteria) compared with those targeting more specific taxonomic groups (Cordier et al., 2018; Keeley et al., 2018). Taxonomy-free approaches, particularly those that combine metabarcoding data with machine learning models (e.g. random forest), have been shown to outperform taxonomy-based indices to predict ecological status from eDNA data (Cordier et al., 2019, 2018, 2017; Frühe et al., 2020; Lanzén et al., 2021, 2020).

Similar findings have been reported in the case of sediment eDNA metabarcoding studies assessing environmental impacts from oil and gas operations (Cordier et al., 2019; Lanzén et al., 2016, 2021; Laroche et al., 2018b; Mauffrey et al., 2020). Lanzén and co-authors (2016) showed that total eukaryotic metabarcoding revealed relatively subtle impacts from parameters such as barium (drilling fluid) and heavy metal contamination. The authors identified novel indicator taxa, among which many protists that are not targeted by routine monitoring programs (Lanzén et al., 2021). This study was complemented by Krolicka and

co-authors (2020), but with a focus on the microbial community. They found that both relative (metabarcoding) and absolute abundances (qPCR) of bacterial taxa correlated with the level of petroleum-related compounds such as barium and PAH. In an extensive study, including both eDNA and eRNA from several groups of benthic organisms, Laroche and co-authors (2018b) showed that bacterial communities exhibited the strongest response to drilling and extraction impacts, followed by foraminifera, while macroinvertebrates (identified using morphology) were the least responsive.

Other applications of sediment eDNA metabarcoding to assess the impact of marine industries, included (1) baselining of prospective exploitation areas, e.g. deep sea mining; (Laroche et al., 2020), (2) monitoring the effects of exploratory drilling (Nguyen et al., 2018), (3) assessing the impacts of oil spills (Bik et al., 2012; Xie et al., 2018), (4) assessing the long-term biodiversity effects of leaving decommissioned platforms as artificial reefs (Klunder et al., 2018). In addition, sediment eDNA metabarcoding has also been used for ecological assessment of coastal marine environments exposed to anthropogenic pressures, such as estuaries (Chariton et al., 2015, 2010) and coastal lagoons (Behera et al., 2020). Eukaryotic diversity derived from metabarcoding data was shown to correlate strongly with environmental variables in estuarine environments exposed to rapid coastal development (Chariton et al., 2015). Fazi and co-authors (2020) used metabarcoding to assess the influence of the freshwater runoff and the impact of extreme flood events on benthic prokaryotic communities in coastal environments.

### **2.2.2. Biomonitoring of freshwater ecosystems**

Compared to the marine environment, the use of sediment eDNA for monitoring freshwater ecosystems has been relatively limited. Freshwater research has focused primarily on the use of aquatic eDNA (DNA from water samples) for monitoring communities of fish and other vertebrates (Handley et al., 2019; Sales et al., 2021) and detecting invasive or endangered



species (Valentini et al., 2016). Metabarcoding of benthic diatoms from biofilm samples has proved to be an effective way of deriving water quality indices in rivers and streams (Apothéloz-Perret-Gentil et al., 2020; Rivera et al., 2021; Visco et al., 2015; Zimmermann et al., 2015). Recently, metabarcoding of bacteria from biofilms has also been applied to develop new molecular indicators (Pin et al., 2021). Freshwater sediment eDNA metabarcoding has mainly been used for analyses of microbial communities in sites exposed to strong anthropogenic pressures, with several studies demonstrating the response of benthic bacterial and eukaryotic communities to industrial and agricultural chemical pollutants (Xie et al., 2018, 2017, 2016). Sediment eDNA metabarcoding could be pivotal in providing early warning indicators of human pressures, with the potential to be linked with surveillance for pathogenic bacteria and protists (VanMensel et al., 2020).

Freshwater sediment metabarcoding has also been used to analyze macro- and meiofaunal communities, focusing on oligochaetes and nematodes, respectively. Vivien and others (2019) compared a morphology-based Oligochaete index to a genetic index derived from total sediment, sieved sediments and mixed specimens (bulk DNA). They found that bulk DNA metabarcoding provided a more representative assessment of the local biodiversity than eDNA metabarcoding of sieved or total sediments. Although community structures obtained from morphological and metabarcoding analyses differed in terms of species composition and abundances, the ecological assessments based on these two approaches largely agreed (Vivien et al., 2019).

Freshwater nematodes have been well studied at the molecular level (Derycke et al., 2013; Holovachov, 2016), but their potential as molecular bioindicators has rarely been exploited (Gardham et al., 2014). Schenk and co-authors (2020) evaluated the performance of a nematode-based sediment quality index (NemaSPEAR) by metabarcoding of nematode communities along a chemical pollution gradient. Metabarcoding-derived NemaSPEAR[%]

results were similar to those obtained from conventional morphology-based assessment. NemaSPEAR was also calculated as a part of a sediment eDNA metabarcoding survey conducted as part of the 4<sup>th</sup> Joint Danube Survey (Weigand et al., 2021).

### 2.2.3. Biosecurity

Sediment eDNA metabarcoding holds great potential for the early detection of invasive alien species and harmful organisms (e.g., toxic algae, pathogenic bacteria and viruses). This is particularly relevant in closed aquatic systems (e.g., ports), where there is a high risk of non-native species establishing (e.g., ballast waters, biofouling). It is also a significant concern in aquaculture, where non-native species are deliberately introduced for farming, but may escape and establish in the wider environment, along with their parasites and other associated organisms. Unlike in the case of biomonitoring for assessment of ecosystem health, confident detection and accurate species-level identification are critical for biosecurity, since the management response can be extremely costly. In most cases, monitoring for pathogens and invasive species takes the form of targeted surveillance using species-specific primers and probes to determine their presence or absence. Such assays are highly sensitive but require extensive validation to ensure species specificity and can only be used to screen for known taxa.

The advantage of metabarcoding for biosecurity surveillance lies in the potential to co-detect a broad range of species, including those that are not anticipated. This was demonstrated by Holman and co-authors (2019) who carried out sediment metabarcoding in UK ports, and detected multiple non-indigenous species (NIS), including several new records that were later validated using visual survey methods. Koziol and others (2019) compared metabarcoding-based species detection in different substrate types and detected the invasive species *Sabella spallanzanii* only in sediment samples, showing that sediment eDNA can be particularly relevant for the detection of benthic NIS and those found in ballast water sediments (Shang et

al., 2019; Shaw et al., 2019). However, extreme caution must be exercised in using metabarcoding data for NIS surveillance (reviewed in Duarte et al., 2021; Zaiko et al., 2018) given: (1) the inability of some commonly-used gene regions to reliably separate taxa at species level, (2) the incompleteness of reference databases for marine benthic organisms and errors that may occur in the reference data (Duarte et al., 2020), and (3) the tendency for a lack of scrutiny in species-level identifications of metabarcoding data. These challenges are extensively reviewed by Darling and colleagues (2020), who highlight the need for those generating metabarcoding data to be aware of the potential policy triggers that could be activated by recording species of concern within biodiversity datasets.

### **3. Challenges and opportunities specific to sediment eDNA**

The use of sediment eDNA in biomonitoring presents specific challenges related to the capacity of sediments to accumulate DNA from different sources and to preserve it for long periods of time (Coolen and Overmann, 2007; Corinaldesi et al., 2008). Sediments act as a repository of both intra- and extracellular DNA, and large-scale studies have shown that the majority of DNA in marine benthic ecosystems is represented by extracellular DNA rather than living biomass (Corinaldesi et al., 2005; Dell'Anno and Danovaro, 2005). This makes it difficult to differentiate between living and dead organisms, or between species that live *in situ* versus those that have settled out from the water column. While this can be an advantage for certain applications, since combining the planktonic and benthic domains provides a wider view of the overall biodiversity of the region, it is largely viewed as a limitation from a sediment biomonitoring perspective. At the same time, the limited volume of sediment that can be used for DNA studies (generally in the order of a few grams) hampers its application to detect larger-sized organisms. Various solutions have been proposed to overcome these challenges and to fully make use of sediments as a source of biological information.

#### **3.1. Using eRNA to distinguish between living (active/inactive) and dead organisms**

One of the key issues in sediment eDNA analysis is the distinction of living organisms that are part of the active benthic community from those organisms that are represented either by inactive dormant stages or by DNA traces originating from tissue fragments, faeces, secretions, among others, of mega- and macrofauna (Deiner et al., 2017; Thomsen and Willerslev, 2015). *A priori*, this is not a problem when studying large size organisms because they are rarely physically captured in the small volume of sediment usually taken for DNA extraction. Note that distinguishing living from non-living (recently dead) organisms can also pose a problem when applying morpho-taxonomic identification, because death can occur during collection (e.g., by decompression or thermal shock) or processing (e.g. during sieving) of the sample.

Targeting living organisms is even more challenging in the case of microbial and meiofaunal species. These smaller organisms may be physically present in sediment eDNA samples and it is virtually impossible to determine whether or not they were living at the time of sampling. Similarly, it may not be possible to distinguish between species living in the sediment and those originating from the water column that have sunk to the bottom. Some taxa are benthic throughout their whole life cycle (e.g., nematodes) or have easily recognizable benthic and planktonic species (foraminifera). However, many benthic taxa have planktonic larval stages, and some planktonic species have resting stages that can remain in sediments for long periods of time. In general, the ecology and life cycle of microbial and meiofaunal taxa are not sufficiently known to unambiguously assign taxa to functional groups.

The use of environmental RNA has been proposed as a solution to this issue. RNA molecules are generally considered to be less stable, more fragile and faster to degrade after the organism's death, and it is therefore expected that they persist in the sediment for shorter periods of time. Several metabarcoding studies have analysed eRNA in parallel with eDNA for bioassessment (Birrer et al., 2018; Brandt et al., 2020; Dowle et al., 2015; Keeley et al.,

2018; Laroche et al., 2016, 2017, 2018b; Lejzerowicz et al., 2015; López-Escardó et al., 2018; Pawlowski et al., 2014, 2016; Pochon et al., 2015; Visco et al., 2015). Most of these studies concluded that the indices inferred from eRNA datasets more accurately reflected those inferred from morphological data. However, some (Birrer et al., 2018; Brandt et al., 2020; Laroche et al., 2018b) found that eDNA provided better accuracy compared to eRNA, and it is logistically simpler. eRNA datasets usually contain fewer OTUs (Lejzerowicz et al., 2021; Pawlowski et al., 2014), although this is not always the case (e.g. Brandt et al., 2020). Retaining only those taxa detected in both eDNA and eRNA datasets has been proposed as an effective way to increase the accuracy of metabarcoding data analyses (Laroche et al., 2017; Pawlowski et al., 2014), particularly for biosecurity applications, where detecting viable non-indigenous organisms is critical (Pochon et al., 2017).

Nevertheless, working with eRNA presents some technical challenges that limit its practical applicability to routine monitoring programs (Wood et al., 2018). First, the collection and preservation of samples for RNA analysis requires much more strict storage conditions (e.g. deep-freezing or the use of specific preservative solutions). Second, the extraction of eRNA and its transcription into complementary DNA (cDNA) is expensive and time-consuming compared to the extraction of eDNA. Moreover, RNA degradation in environmental samples might not be as fast and complete as generally assumed, with one recent study showing similar decay rates of eDNA and eRNA (Wood et al., 2020). This is consistent with observed similarity of macroinvertebrates communities in paired eDNA/eRNA datasets, since these are assumed to originate mainly from trace eDNA/eRNA rather than from capture of the organisms themselves in the small-volume sediment samples (Lejzerowicz et al., 2021, 2015). Although eRNA may not represent a robust solution for distinguishing between active and inactive organisms, eRNA studies offer new opportunities for integrating functional genomics into environmental monitoring (Cristescu, 2019), particularly for microbial groups.

### 3.2. Extracellular DNA

The extracellular DNA pool in marine sediments derives from the input of pelagic organisms and their remains that reach the seafloor by vertical fluxes and lateral advections (Dell'Anno et al., 2005; Dell'Anno and Danovaro, 2005), and from *in situ* production by benthic processes that include: i) exudation and excretion from viable cells/organisms; ii) natural and/or human-induced processes; iii) predation, and iv) virus-mediated induced mortality (Corinaldesi et al., 2014; Danovaro et al., 2016; Dell'Anno et al., 2015). Thus, DNA extraction from sediment samples cannot exclude DNA originating from dead organisms (Corinaldesi et al., 2018).

Targeted analysis of extracellular DNA can be useful in biomonitoring studies to help discriminate between what is potentially alive from what is certainly dead (Corinaldesi et al., 2018). This is achieved by comparing the total eDNA sequences of living organisms with those obtained from the extracellular fraction. This requires a selective extraction of extracellular DNA using specific laboratory procedures (Danovaro, 2010), a number of which have been published (Alawi et al., 2014; Corinaldesi et al., 2005; Lever et al., 2015; Ogram et al., 1987).

The most difficult tasks for the isolation of extracellular DNA in sediments are: (i) avoiding extracellular DNA contamination by intracellular DNA, (ii) separating intracellular DNA from the extracellular DNA pool, and (iii) obtaining DNA that is pure enough for subsequent molecular studies. Once extracted, extracellular DNA can be analyzed using the same downstream protocols as used for other eDNA analyses (Corinaldesi et al., 2018; Dell'Anno et al., 2015; Pearman et al., 2015).

### 3.3. Sediment eDNA vs bulk DNA

The detectability of benthic taxa in sediment eDNA depends on their size and biomass. A standard DNA extraction is performed on a maximum of a few grams of sediment, in which

the number of living meiofaunal specimens is relatively low and macroinvertebrates are practically absent (except their eggs, larvae, moults/debris or tiny specimens). Macrofauna and to lesser degree meiofauna are instead represented by DNA traces originating from tissue fragments, secretions, free cells, organelles or extracellular DNA molecules. Such material is fragmentary and might not be adequate for benthic community surveys. Therefore, metabarcoding analyses of benthic macroinvertebrates are typically based on bulk DNA, which consists of mixed specimens sorted from large samples of pre-processed sediment. Meiofauna can be analysed using either bulk DNA or direct eDNA extractions from sediment, while microbial communities are almost always targeted by direct sediment eDNA extractions.

For bulk DNA metabarcoding of macroinvertebrates, organisms can be preprocessed and separated from the sediment by sieving, before or after their fixation in the field (Aylagas et al., 2018; Vivien et al., 2019). This can be labour-intensive and limits the potential for parallel processing of large numbers of samples. However, DNA extracted from sediment samples is highly inefficient for describing macroinvertebrate diversity since their DNA typically represents only a tiny fraction of the data obtained from sediment metabarcoding (< 0.1% in Lejzerowicz et al. (2015) and reveals only a limited subset of the diversity obtained through conventional assessments (Aylagas et al., 2016).

Bulk DNA metabarcoding is also sometimes used for meiofaunal organisms (e.g. Schenk et al., 2020). Separating the organisms from the sediment can considerably increase the abundance and richness of meiofauna compared to direct sediment extraction (Brannock and Halanych, 2015; Carugati et al., 2015), largely due to the larger volume of sediment that can be processed in this way. Nevertheless, separating organisms from the sediment is time-consuming and not amenable for large-scale monitoring. Recent studies have shown that directly extracted sediment eDNA can reliably characterise meiofaunal diversity and

community composition (Bellisario et al., 2021; Fais et al., 2020a, 2020b; Lejzerowicz et al., 2021; Nascimento et al., 2018).

### **3.4. Sediment structure and physical-chemical characteristics**

Soft sediments are highly variable in their physical and chemical properties, and this has implications in terms of both expected community diversity and the technical efficiency of the sediment eDNA analysis.

Sediment texture is a major determinant of benthic microbial community structure (Böer et al., 2009; Wang et al., 2013; Zheng et al., 2014), since it influences the capability of bacteria to adhere to sediment particles (Dang and Lovell, 2016). The surface structure of coarser sediment grains offers better adhesion possibilities for bacteria than do the smoother surfaces of fine particles, so bacterial diversity and abundance is often highest in sediments with higher median grain size (Alsaffar et al., 2017; Wang et al., 2013), and is negatively associated with the proportion of fine particles (< 63 µm, silt and clay minerals; Stoeck and Albers, 2000, 1999; Stoeck and Kröncke, 2001). Grain size is also associated with factors such as porosity, permeability, nutrient availability and dissolved oxygen, all of which can affect benthic community composition (Freitag et al., 2003). Therefore, grain size should be recorded and taken into account as a potential explanatory variable in monitoring programs that may span diverse benthic habitats.

Vertical sediment gradients play a strong role in structuring benthic microbial communities, since factors such as wave action can force organic material into deeper sediment layers (Freitag et al., 2003). This is highly relevant when collecting surface sediments for biomonitoring because while the “surface benthic community” may be restricted to only a few millimeters at one sampling site, it may extend as deep as a few centimeters on another sampling site.

Sediment chemistry can affect the efficiency of both DNA extraction and PCR amplification,



especially in polluted sediments, where organic and inorganic compounds bind to the sediment. This can interfere with DNA extraction through inhibiting enzymes used for cell lysis, and also causes PCR efficiency to be reduced by enzyme inhibition (Fortin et al., 2004). Inhibition may cause PCR to fail completely or to be inefficient. It is the latter scenario that poses the greatest risk to biomonitoring projects since the partial inhibition may not be recognized if it is not explicitly tested for (e.g. using internal positive controls IPC; Krolicka et al., 2020). Thus, low diversity obtained in metabarcoding output could be erroneously attributed to a genuine loss of diversity in the benthic community when it is actually the result of inefficient amplification due to inhibition. This is a particular risk given that the sediments that are likely to contain the most inhibitors are also those that could be expected to have negatively impacted the benthic communities through pollution (Krolicka et al., 2020), so it is vital that these two factors are separated. It is also important to notice that the methods used to overcome PCR inhibition such as DNA template dilutions (Castle et al., 2018; Schrader et al., 2012), additional DNA purification steps and changes in number of PCR cyclers (Kelly et al., 2019) may also be associated with adverse effects on the final results of metabarcoding analyses.

#### **4. Review of methods**

##### **4.1. Sampling**

Sampling methodologies for sediment eDNA recovery vary depending on factors such as water depth, bottom characteristics and target organisms (Table 1). Compared to water eDNA sampling, collecting sediment eDNA samples is much more demanding. For example, sampling deep-sea bottom sediments requires long shipping time and therefore the number of available samples is usually limited. In general, only a few cores or grabs are taken from each sampling station in marine environment, but this number can be higher in estuaries and other shallow water settings (Bellisario et al., 2021; Fais et al., 2020b). The number of replicates

and sample size also depends on the type of sediment. Soft sediments are easy to sample and provide large amounts of eDNA, while coarse sediments or hard bottom are more difficult to sample for eDNA studies. Innovative technologies such as the Substrate-Independent Benthic Sampler (Cahill et al., 2018; Keeley et al., 2021) offer solutions that add to the growing array of observing devices that are critically needed to sample remote habitats such the deep sea (Levin et al., 2019), and that can be coupled with eDNA readouts. Finally, a sampling strategy needs to be adapted to its biological targets. The optimal size for a sediment sample depends on the size and biomass of target taxa. Thus, a small sediment volume (0.2 - 0.5 g) is sufficient for microbial diversity measurements (Xie et al., 2017) as studies have shown that increasing sample size has little effect on results for microbes (Dopheide et al., 2019; Penton et al., 2016). In contrast, larger volume samples are necessary to accurately recover the community structure of metazoans, which thrive at larger spatial scales and are more widely dispersed in the sediment (Dopheide et al., 2019; Fais et al., 2020a; Nascimento et al., 2018).

Table 1. Summary of major methodological details of sediment sampling, storage and DNA extraction in different aquatic environments targeting (●) prokaryotes; (○) microbial eukaryotes; (□) meio- and (Δ) macrofauna. 1 symbol = up to 5 study cases. For details and references, see Supplementary Table 1

Sampling equipment	Grab	●●●●○○□Δ
	Corer	●○○□□Δ
Sediment depth	Top 2 cm	●●○○□□
	Top 5 cm	●○○□Δ
	Top 10 cm	●●○○□Δ
	>10 cm	●
Number of sites per study	1-5	●○○
	6-10	●○○Δ
	11-15	●○○□Δ
	>15	●○○□□Δ
Replicates	≤3	●●●●○○○○□□□Δ
	>3	●○○□
Sample preservation	Frozen -20	●●○○□Δ
	Frozen -80	●●○○□□Δ
	Storage solutions	●○○□□

Sample size for extraction	$\leq 0.25$ g	●●○□
	0.25 – 0.5 g	●○□
	0.5 – 2 g	●●○○□△
	2 – 10 g	●○□△
Extraction methods	Commercial kits	●●●○○○○□□□△
	Lab protocols	○△

The choice of equipment used for obtaining sediment samples depends on factors such as water depth and substrate type. The Van Veen grab is commonly used for sampling sediment from large rivers (Xie et al., 2017) as well as coastal and other shallow marine substrates at depths of less than a few hundred meters (Pawlowski et al., 2014; Stoeck et al., 2018a). Alternatively, soft sediment samples can be taken using different types of cores, including simple tubes with diameter ranging from 4 to 13 cm, or more complex multicores, boxcores or megacores, which are used principally for sampling lakes and the deep-sea bottom (e.g., Brannock et al., 2018; Cronin-O'Reilly et al., 2018; Fonseca et al., 2010; Guardiola et al., 2016; Lejzerowicz et al., 2021; Lindh et al., 2017; Sinniger et al., 2016). Cores are generally preferable to grabs because they better preserve the vertical sediment profile, allowing more standardized and targeted subsampling of specific sediment depths, especially for the meiofauna (Lins et al., 2021).

If primary sediment samples are collected using a grab or large corer, the surface sediment are usually subsampled. Subsampling allows to increase the number of biological replicates, which are necessary to ensure the accuracy at detecting changes of benthic communities. For example, the reliable assessment of the richness at a deep-sea bottom, would require just about three or at least 20 samples to detect a 50 % change for the meio- and macrofauna, respectively (Lins et al., 2021). Similar results were obtained by Hestetun and others (2021) using eDNA metabarcoding of relatively undisturbed sediments adjacent to an oil drilling platform in the North Sea, indicating that five physical subsamples from a van Veen grab, with five DNA extraction replicates from each subsample, roughly doubled the richness

obtained.

Subsampling can be achieved by scooping the surface sediments (usually top 1-2 cm) with a spatula or sterile spoon (e.g., He et al., 2019; Hestetun et al., 2021; Keeley et al., 2018; Laroche et al., 2018b, 2018a; Lejzerowicz et al., 2021, 2015; Pawlowski et al., 2016; Stoeck et al., 2018b, from Tab. 1) or using minicores or syringes (Guardiola et al., 2016; Lindh et al., 2017). The edges of the grab or large core sample should be avoided during subsampling because the vertical profile is disrupted here. In very shallow water, subsamples may be collected by SCUBA diving directly from the bottom.

The choice of subsampling method depends on which part of the vertical profile of the sediment is targeted. Scoops and spoons maximise recovery of the surface sediment, which represents the interface between benthic and pelagic environments and includes surface biofilms. In some cases, it may be preferred to eliminate the very surface layer of the sediment in order to minimize the contribution of DNA from pelagic sources and biofilms. In this case minicores are more suitable since they allow the surface layer to be cut off when the sediment is removed from the core. Corers also facilitate sampling to a more precise and consistent depth, and transparent plexiglas corers let the user observe features such as transitions from oxic to anoxic layers, which are characterized by colour changes. This enables selective sampling of particular layers if desired by discarding certain portions of the core as it is ejected.

The subsamples can be combined and homogenized, and then re-sampled to achieve a small-volume sample that is representative of a wider area and accounts for fine-scale heterogeneity (Hestetun et al., 2021; Loos and Nijland, 2020). An implicit trade-off with this approach is a gain in estimating total richness at lower cost, at the expense of the loss of fine-scale spatial resolution, the distortion of abundance profiles requiring careful compositional data analysis practices (Gloor et al., 2017; Quinn and Erb, 2020), and the likely drowning of the signal of

rare taxa that might be represented by few cells with low target-gene copy number. For large ecosystems such as the deep-sea, rare meiofaunal taxa can be extremely numerous locally (Gooday et al., 2021; Lejzerowicz et al., 2014), and even for the macrofauna, small-scale variability may determine landscape and connectivity patterns (Smith et al., 2020). Therefore, it is important to consider the specific features of ecosystems and targeted taxa when establishing a monitoring programme.

#### 4.2. Pre-processing

As discussed above, some sampling protocols include a pre-processing stage, usually with the aim of isolating meio- or macrofauna from larger volumes of sediment. Separating meiofauna from sediment samples involves pre-processing by sieving, decanting, elutriation, repetitive centrifugation in Ludox solution, or combinations of these methods (Brannock et al., 2014; Creer et al., 2010; Faria et al., 2018; Fonseca et al., 2017, 2014, 2010; Lallias et al., 2015). Decantation and homogenization are also proposed as an efficient method for benthic macroinvertebrate metabarcoding (Aylagas et al., 2016), while melting seawater-ice elution has been used to enrich the benthic protists communities (Reñé et al., 2020).

To extract macroinvertebrates from sediments (for bulk DNA analysis), the sieving can be performed in the field (e.g. Aylagas et al., 2018) or in the laboratory after fixation of organisms in the field (e.g. Vivien et al., 2019). In the latter case, if the soft bodied organisms, such as oligochaetes, are to be sorted, it is recommended to fix samples with neutral buffered formalin rather than with ethanol. It has been shown that oligochaetes can be fixed in situ using neutral buffered formalin, which does not prevent subsequent genetic analyses if the specimens are preserved in this medium for a short time, maximum 4 weeks (Vivien et al., 2020b, 2019, 2018). The sieve mesh size is very important to consider for the extraction of macroinvertebrates. Mesh sizes greater than 0.25 mm (until 1 mm) are sometimes used for sieving live macroinvertebrates. However, such mesh sizes are not

adapted to extract small organisms, such as oligochaetes (Heuschele, 1982; Vivien et al., 2020c). To extract live oligochaetes for example, sieve mesh sizes of maximum 0.2-0.25 mm are recommended (Vivien et al., 2020c). When organisms are fixed before sieving, mesh sizes of 0.25-0.5 mm are acceptable and allow to retain most organisms.

### **4.3. Sediment sample preservation**

Sediment samples are typically preserved either by freezing or by immersion in a preservative solution. When cold storage is used, sediment samples are either frozen immediately after collection or transported on ice and then frozen in the laboratory (Table 1). Samples are stored at either -20°C or -80°C depending on the availability of equipment and the analysis targets. Deep freezing at -80°C is often used when bacteria and archaea are targeted (Dowle et al., 2015; Lindh et al., 2017) and it is required when eRNA is to be analysed (Lejzerowicz et al., 2015) or for long-time storage.

An alternative to freezing is to store the sediment samples in solutions that preserve nucleic acids, such as ethanol or LifeGuard (Cordier, 2020; Guardiola et al., 2015; Lanzén et al., 2016; Lejzerowicz et al., 2021). Ethanol is a good preservative of DNA, but it interferes with the chemistry of the DNA extraction process, meaning that additional cleaning steps are required prior to DNA extraction when ethanol is used, and this increases the labour cost of sample analysis. In addition, ethanol is difficult to ship because it is classed as dangerous goods (UN1170, class III, packing group II), and its storage in significant volumes aboard a ship poses notable health and safety risks. Therefore, in practice ethanol may often not be a viable option for large-scale routine monitoring programmes and it is important to explore logistical feasibility before committing to its use as a preservative.

In meiofaunal studies, where large volumes of sediment are collected, samples have sometimes been preserved in DESS, a solution of 20% DMSO, 0.25 M disodium EDTA, saturated with NaCl (Yoder et al., 2006), which inhibits nuclease activities. Samples

preserved in large volumes can be washed through sieves to separate the meiofaunal fraction. Weigand and co-authors highlighted recently the use of propylene glycol as an effective, safe and low-cost preservative solution for bulk invertebrate samples (Weigand et al., 2021), although further research is required to assess its suitability for use with sediment samples and to understand how it interacts with the DNA extraction process. Some commercial forms of propylene glycol are viscous and difficult to work with in the laboratory.

#### **4.4. Sediment DNA extraction**

Different DNA extraction methods are appropriate for targeting different states of DNA, especially because the chemistry influences the extent to which particle-bound DNA is released into solution (Pearman et al., 2020). For biomonitoring purposes, it is usual to target total eDNA of intra- and extra-organismal origin, so we do not consider here the extraction methods designed specifically to recover extracellular DNA.

It is widely recognized that the choice of DNA extraction method can affect microbial community profiles (Carrigg et al., 2007). Many studies have compared different sediment eDNA extraction methods and their impact on diversity and community structure, with several showing that choice of extraction method has a significant effect on diversity obtained (Hestetun et al., 2021; Lekang et al., 2015; Mateus-Barros et al., 2019; Pearman et al., 2020). This highlights the importance of methodological consistency within studies where results need to be comparable between samples, and it represents an argument in favour of using commercial extraction kits, which are generally the most standardized option.

Physical disruption (e.g., bead beating) is required for efficient DNA extraction from sediments, particularly when microorganisms are targeted, since many have cell walls that will not be disrupted by chemical lysis (Teng et al., 2018). Hestetun and others (2020) showed that moderate bead beating also increases the completeness of the community recovered in an individual DNA extraction more efficiently than the more intensive bead

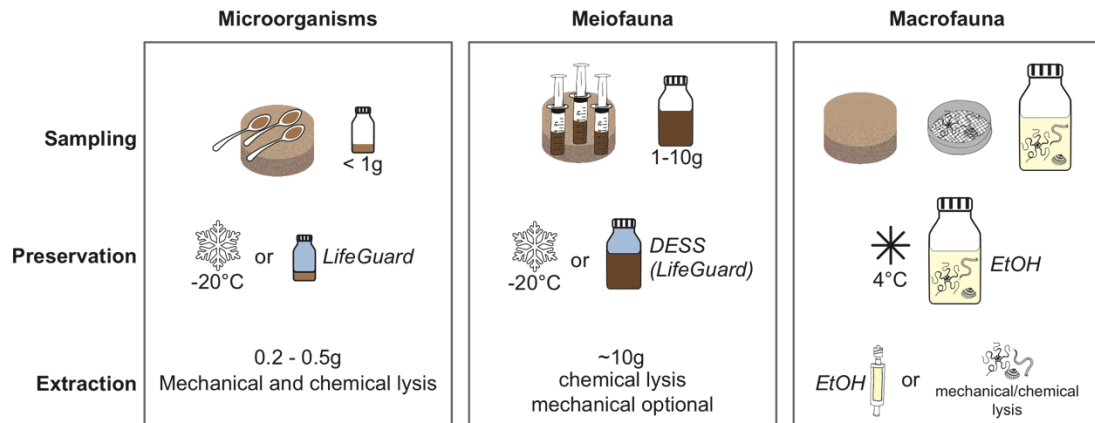
beating. Thus, the performance of commercial DNA extraction kits may be improved with the addition of an extra bead-beating step.

Because of the presence of organic and metal compounds in aquatic sediments, any DNA extraction method must incorporate removal of inhibitors to ensure that efficient amplification can be achieved. Commercially available kits designed for DNA extraction from soils and sediments do typically integrate inhibitor removal during the extraction process. The most commonly-used commercial DNA and RNA isolation kits are indicated in Table S1. For example, the Qiagen's PowerSoil DNA isolation kit adapted for use with magnetic bead plates is recommended by the Earth Microbiome Project (Marotz et al., 2017). It is also considered as a universal eDNA extraction method for different sample types (including soil and sediment) and various taxa (Hermans et al., 2018; Mateus-Barros et al., 2019).

A key factor that drives choice of DNA extraction method is the volume of sediment to be processed. The PowerSoil DNA and RNA isolation kits mentioned above are designed for small-volume samples (0.2-0.5 g) and are generally used in microbial studies. The PowerSoil RNA extraction kit (in combination with the DNA elution buffer) takes in a larger volume of 2 g of sediment, which proved efficient to capture the eDNA and eRNA of a range of micro and meiofaunal organisms (Lejzerowicz et al., 2021). The PowerMax DNA isolation kit represents the only commercially available kit that is designed to process up to 10 g of sediment and it is commonly used for the assessment of meiofaunal diversity (Bellisario et al., 2021; Fais et al., 2020b; Nascimento et al., 2018). This kit is identical to PowerSoil in terms of chemistry, but it is more than four times the price of PowerSoil kits, presenting a potential barrier to large-scale use. All these kits are based on bead-beating and silica columns, which for marine sediments was found to reduce the yield and molecular weight of microbial DNA and RNA extracts (Lloyd et al., 2010).



The accuracy of benthic diversity metrics can be improved by increasing the number of replicates of sediment eDNA extractions to counteract small scale spatial heterogeneity and improve inferences of diversity and composition from metabarcoding studies (Ficetola et al., 2015; Hestetun et al., 2020, 2021; Lanzén et al., 2017; Lejzerowicz et al., 2014). Hestetun and coauthors (2020) showed that separate extractions from three 0.5 g replicate samples could yield equivalent metazoan diversity to extraction from a single 5 g sample, likely because more complete sample homogenisation can be achieved on a small-volume sample. Using bead-beating (Precellys) combined with extraction replicates of smaller volume is also time and cost efficient, since it allows for the use of 1.5 mL tubes that is more easily subjected to automatisation using robotics (Hestetun et al., 2020). The replication of sediment eDNA samples has also been proposed to analyse the spatial patchiness of deep-sea benthic foraminifera (Lejzerowicz et al., 2014). However, in this case, as well as in many other studies (Weigand and Macher, 2018), the sediment samples were not homogenized before, making each sample an independent replicate. These different types of replicates can be pooled at later steps of the metabarcoding workflow to reduce the biases associated with DNA extraction (Feinstein et al., 2009), or to target metabolically active organisms (e.g., by combining DNA and RNA datasets, Pawlowski et al., 2014). Further, Hestetun et al., (2020) also noted that pooling extraction replicates after PCR yielded more diverse results compared to pooling pre-PCR. However, PCR artifacts are likely to explain a part of this discrepancy.



**Figure 2.** Recommended methods for sampling, preservation and extraction of sediment DNA depending on the target group of organisms.

## 6. Implementation of sediment eDNA: challenges and future developments

A substantial body of research supports the use of sediment eDNA metabarcoding for characterising benthic communities and assessing the ecological status of benthic habitats. Depending on the context, this has the potential to either complement or replace conventional survey approaches based on morpho-taxonomic assessment of benthic macroinvertebrates (reviewed in Cordier et al., 2020; Lanzén et al., 2016; Laroche et al., 2018a; Lejzerowicz et al., 2015). The adoption of sediment eDNA metabarcoding as a tool for environmental impact assessment is rapidly growing, especially in marine industries, driven by advantages in terms of time and cost effectiveness (Aylagas et al., 2020). Nonetheless, wide uptake of this new approach remains constrained by certain technical and regulatory factors. In this last section, we discuss some of these factors and present new developments that might speed up the process of implementing sediment eDNA analysis into routine benthic monitoring programmes around the world.

From a technical perspective, the basic workflow to generate sediment eDNA metabarcoding data is well established. However, technical challenges remain in the optimization of

sampling strategy and early steps of sampling processing and analysis. More extensive testing of sample size, depth profile of subsamples, and replication level is needed to improve the ecological relevance of generated data, taking into account the temporal and spatial variations of benthic taxa. In marine environments, there is a pressing need for technological solutions that enable multiple samples to be collected from bottom sediments without the need to lower and raise equipment from surface to seafloor for each individual sample. The increasing use of remote underwater vehicles and submersible robotics seems to offer great promise in this regard and will enable much more intensive sampling by reducing the cost of sample acquisition. Sample preservation remains a significant challenge, with the need to achieve reliable performance alongside affordability and logistical feasibility (freezing capacity or regulations around use and transport of flammable liquids). We urge researchers to consider and address these industry-relevant constraints when designing experiments. Finally, developing alternative methods for extracting eDNA from larger volumes of sediments would reduce the costs of currently available kits and enable more comprehensive assessment of benthic communities from a single sample. Further research is required to meet these challenges, but in the meantime it is essential to standardize the existing workflow in order to enable its use in benthic monitoring.

From a regulatory perspective, the lack of standardized protocols is one of the principal factors limiting the adoption of eDNA-based technology. Standardization helps to build confidence among regulators and end-users that eDNA data have been generated based on good scientific practice, are broadly comparable and can be interpreted in a consistent way. Our paper presents the first attempt to define best practices and standard conditions for sampling and extracting sediment eDNA from various environments. The recommendations listed in Table S2 provide a basis for a proposal that will be submitted to the CEN TC230 Working Group on DNA and eDNA methods (WG28). A similar proposal on sampling and

processing of eDNA from water samples is currently under consultation (New Work Item Proposal: CEN/TC 230 N 1229), while CEN technical reports have already been published for the sampling and preservation of diatoms for molecular analysis (CEN, 2018) and further efforts are under way to standardize the laboratory analysis procedure for diatom metabarcoding. The standardization of sediment eDNA methods will fill an important gap in the foundations for future regulated use of eDNA in benthic monitoring. Moving beyond the sampling and DNA extraction protocols presented here, future efforts will need to address the standards for analysis of the extracted DNA and the associated quality control frameworks that need to be implemented in laboratories where the analysis is carried out. However, we consider that the early stages of the process discussed in this paper are the most critical to standardize. This is because while DNA can be archived for repeat analysis at a later date if methods change, it is never possible to go back in time to repeat sampling.

It is important to emphasize that metabarcoding presents only one of the omics techniques that can be used for DNA-based benthic monitoring. Going beyond taxonomic surveys, it is likely that future benthic assessments will also integrate an element of functional genomics (Achermann et al., 2020; Corábar et al., 2020; Cristescu, 2019), especially as sequencing cost reduces. Functional profiles of microbial communities are thought to be highly correlated with environmental variables, with functional community structure considered to be more stable than taxonomic variability (Laroche et al., 2018a; Louca et al., 2016). Functional profiles can be inferred from taxonomic identity or measured directly through the expression of functional genes characterized via metatranscriptomics (e.g. Di Cesare et al., 2020). The latter enables the contribution of taxonomically unassigned taxa, which often represent a significant portion of diversity in metabarcoding datasets.

Targeted assays using approaches such as qPCR or ddPCR to detect the presence of particular indicator taxa represent an alternative approach for monitoring benthic communities

(Krolicka et al., 2020) and are easier to implement in the field for real-time surveillance. Bagi and coauthors (2019) have reviewed several promising technological advances in genosensing for marine monitoring, including methods based on thermocycling PCR amplification (qPCR, ddPCR; Scholin et al., 2017), loop-mediated isothermal amplification (LAMP; Lee et al., 2019), and direct hybridization approaches such as surface plasmon resonance (SPR; Bagi et al., 2018). Environmental biosensors using microarray technology (PhyloChip, GeoChip) have been successfully tested for the detection of fecal (Dubinsky et al., 2016), toxic algae (Medlin et al., 2020), and profiling of microbial communities in water (Herfort et al., 2016; Ottesen et al., 2011) and sediment (Zhou et al., 2015) samples. Phylogenetic microarrays have also been tested for marine sediments (Lekang et al., 2020).

To conclude, we expect that these and other developments will continue to produce new eDNA-based tools that will collectively revolutionize the field of biomonitoring. However, all require a common foundation in the form of standardized principles for reliable and consistent field sampling and DNA extraction. We therefore believe that the present review and synthesis of protocols and methods is important for establishing the foundations for rapid and effective implementation of sediment eDNA analyses in routine benthic monitoring.

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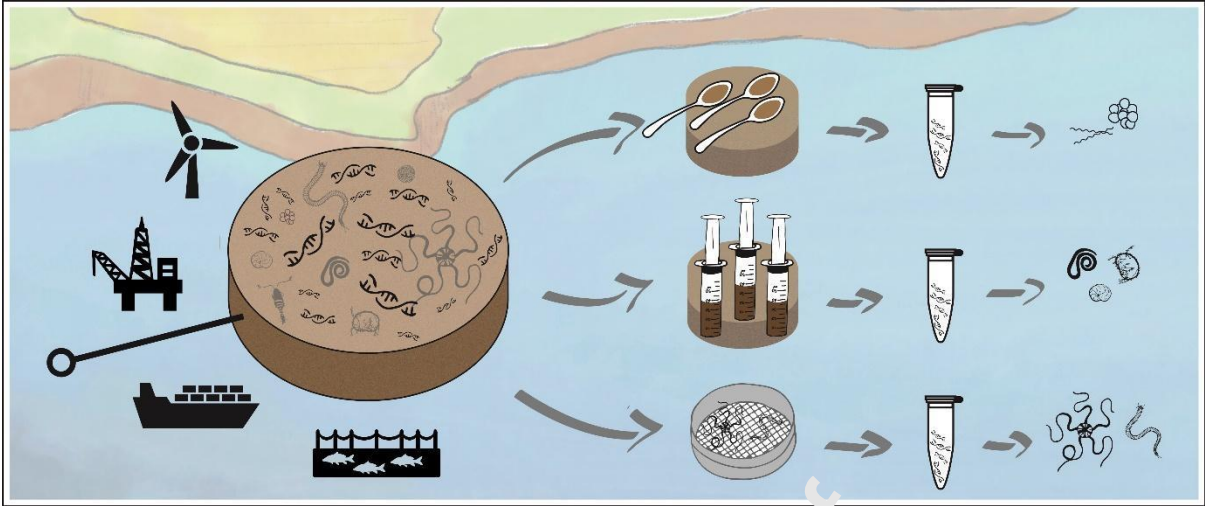
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### **Author contributions**

The work largely benefited from the discussions with DNAqua-Net members during DNAqua-Net workshop held in Rome, on June 10-12, 2019, as other DNAqua-Net conferences. JP, KB, PK, SF wrote the first draft of the manuscript. All Authors wrote sections of the manuscript, contributed to manuscript revision, read and approved the submitted version.

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## Highlights

- eDNA metabarcoding is revolutionizing the field of aquatic biomonitoring
- The methods and protocols for sediment eDNA metabarcoding can vary considerably
- Available information on metabarcoding applied to sediment samples were reviewed
- Challenges specific to sediment eDNA analysis were discussed
- The aim was to define best-practices and recommend potential standard procedures

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