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MetaMon final project report

High-throughput metabarcoding of eukaryotic diversity for environmental monitoring of marine sediments

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Summary

MetaMon (2018-2021) was a project at the NORCE Molecular Ecology Research Group aimed at maturing eDNA for sediment offshore environmental monitoring. Findings include (i) guidelines for sampling and processing, (ii) a 97 station metabarcoding study showing metabarcoding *de novo* biotic indices performed comparable to the morpho-taxonomic NSI index and co-occurrence networks for impacted and non-impacted sites. Two ddPCR assays were developed and tested as a proof of concept. Finally, (iii) a gap analysis was done for online databases, (iv) several species were individually sequenced, and (v) COI metabarcoding of bulk animal tissue evaluated. With these deliverables together with an ambitious program for communication and outreach MetaMon has significantly advanced progress towards use of metabarcoding data in offshore environmental monitoring, yet findings need to be consolidated with further data and time series validation.

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Executive summary

The Norwegian offshore oil and gas industry conducts extensive seafloor monitoring surveys in 12 survey regions (I-XII) on the Norwegian Shelf as part of compliance with Norwegian environmental regulations. In addition to chemical parameters related to hydrocarbon and metal impact, monitoring surveys include the 1 mm sieved fraction of softbottom seafloor organism communities. Such morphological taxonomy, while a good impact parameter, is also time consuming and reliant on available specialized taxonomic expertise. Environmental DNA (eDNA) based methods, including metabarcoding, have the potential to enable faster and more affordable analyses and provide a more complete and accurate picture of seafloor communities and functional relationships. In order to increase the maturity level of metabarcoding and other eDNA-based methods for environmental monitoring, three knowledge gaps were identified:

- Standardized guidelines for sampling, lab and bioinformatic processing.
- Temporal and geographical eDNA metabarcoding baseline data.
- Direct comparisons between morphological and metabarcoding data in detecting environmental impact.

MetaMon (2018-2021) is a project coordinated by the Molecular Ecology Research Group (MERG) part of NORCE Environment, funded as a knowledge-building project by the Norwegian Research Council (NRC PETROMAKS2, grant no. 280919) and industry operators Equinor and Total. Scientific collaborators include AZTI (Spain), the University of Bergen and Auburn University (USA). MetaMon builds on previous projects at MERG, partially funded by Equinor (Statoil), including the Research Council of Norway RCN Environmental Monitoring Programme (EMP) project, the Norwegian Deep-Water program and a MetaMon pre-project.

The main aim of MetaMon is advancement towards implementation of eDNA-based techniques, with a special emphasis on metabarcoding, in future routine monitoring of seafloor organisms for environmental impact assessment of petroleum extraction activities, through the following secondary objectives:

- Determine how metabarcoding qualitatively compares to morphological taxonomic analysis for biodiversity assessment in marine sediments and its potential for assessing changes in abundance of indicator species.
- Explore benthic taxa, including taxa only identified from molecular data, with verified potential as biological indicators of environmental disturbance related to oil drilling activities.
- Develop specific, preliminary guidelines for metabarcoding methodology, encompassing all stages from study design, sample collection, processing, DNA sequencing, sequence data analysis, data archiving and deposition, statistical assessment and reporting.
- Calculate estimates of taxonomic gaps in the Barents Sea for marine benthic species identification using two different metabarcoding approaches (18S SSU rRNA and COI).
- Obtain and submit to online databases COI and 18S barcodes from collected specimens representing the most common taxa in the O&G monitoring programs.
- Actively communicate results and insights with stakeholders such as industry, management and consultancy companies.

To achieve these secondary objectives, the MetaMon research tasks were divided into three work packages:

- WP1: Comparative analysis of morphologic and metabarcoding-based monitoring
- WP2: Standardization of sediment sampling methodology
- WP3: Assessment of the archive and taxonomic gaps

Field work and collected samples

An important aspect of MetaMon is the direct comparison of metabarcoding data with existing environmental monitoring parameters. This required samples from existing monitoring stations, obtained as part of the in-kind contribution to the project by Equinor, which facilitated sampling. Thus, sediment samples were taken during the routine 2018 region II and 2019 region III and IX spring monitoring campaigns, collected and analyzed for existing monitoring parameters by Stim Miljøtjenester AS, DNV-GL and Akvaplan-niva, respectively. For the MetaMon metabarcoding sampling, 399 sediment samples were collected, frozen at -20 °C on board, and subsequently sent to the MERG lab in Bergen for further processing. For the 2019 campaigns, an additional 33 sieved macrofauna samples were also collected and preserved in ethanol for WP3 comparative morphological and animal tissue eDNA analysis.

Standardization of sediment sampling methodology (WP2)

WP2 results serves as the basis for WP1 and WP3 results and are thus presented first here: Accurate use of metabarcoding relies on several possible choices in sampling design and processing. In a monitoring setting, this requires that best-practices and solutions for each part of the process are identified and implemented in a consistent manner, in order to produce standardized and consistent data. Lab related processing and protocol optimization constituted most of the work from the fall of 2018 to 2019 and included choosing and optimizing PCR and library preparation reagents and protocols, molecular metabarcoding markers (18S V1-V2 and COI), mock communities, and sediment DNA extraction protocols. Research tasks in WP2 were divided into two sets of experiments to (1) optimize sediment homogenization during DNA extraction and (2) investigate the extent of variation of samples taken at different parts of the same sediment sample and grab, as well as between different grabs from the same station.

Here, we found (1) **that an intermediate homogenization program using a Precellys homogenizer significantly increased the total amount of DNA that could be extracted from each sediment sample and improved the accuracy (homogeneity) of the benthic community data obtained**. This experiment also validated the chosen pre-PCR pooling approach for DNA extracts and helped gauge an optimal number of replicates per grab and station. Furthermore, (2) **sampled variability increased gradually with increased spatial distance**. It was feasible to capture a large majority of single celled organism diversity with a modest sampling effort, but findings also highlighted higher variability between samples for the COI macrofauna data.

Comparative analysis of morphologic and metabarcoding-based monitoring (WP1)

In order to demonstrate the efficacy of metabarcoding for routine monitoring, it is necessary to compare resulting data to currently used physicochemical parameters that correspond to negative environmental impacts, such as hydrocarbon and metal concentrations. It is also important to compare generated metabarcoding results to those based on morphological taxonomy data. To this end, sediment samples from 97 monitoring stations (regions II, III and IX) were subjected to COI and 18S metabarcoding. Stations were chosen to also include known previously detected impact. Most of the lab processing for this dataset was done in the fall and winter of 2019, and data analysis during 2020. The resulting data was supplemented with standard monitoring parameters for each of these stations, downloaded from the DNV GL MOD database.

The main aims of this study were to examine the correlation between metabarcoding and current monitoring practices to identify and classify sensitive and tolerant organisms in the metabarcoding dataset. This included the use of already known bioindicator species included in the Norwegian Sensitivity Index (NSI) biotic index, currently used for morphological species data. As a part of this work, we also produced new biotic indices for the two markers (18S and COI) from the molecular data directly, to improve on existing ones such as NSI that have been developed using data from morphological taxonomy. Finally, in order to examine the correlations between species (or higher taxa) we used an association network approach. **The main findings were that the newly developed molecular COI indices could predict environmental impact almost as accurately as the existing biotic index NSI (based on morphological taxonomy as currently used), and that COI metabarcoding agreed well with morphological data when both were applied to the existing NSI index.** Predictions based on a newly developed 18S based index, including mainly non-metazoan indicators such as protists, also correlated significantly with impact index and morphotaxonomy / NSI-based values. An alternative approach, using supervised machine learning to predict environmental impact, did not yield significant results. The co-occurrence approach showed that the connections between individual taxa were different in impacted vs non-impacted sites, and that impacted sites resulted in a more fragmented association structure. However, it became clear that the dataset targeted was not optimal for association network analysis. Instead, a higher level of spatial or temporal replication from sites subjected to more similar impact levels, would be required. WP1 also included the quantification of two OTUs with especially clear correlation to environmental impacts, assessed as possible indicator species using ddPCR: Capitella sp. and a haplosporidian species. The Capitella results were very promising, while we found that the generally low abundance of the haplosporidian makes this species less consistent as a bioindicator. The results clearly showed the viability of the approach used, but the findings need to be validated, and we recommend increasing the amount of available data for more robust identification of new indicator species, especially with regards to sites with known contamination.

Assessment of the archive and taxonomic gaps (WP3)

An advantage of targeting animals (macrofauna) rather than microorganisms is that a much larger fraction of sequences can be identified to genus or even species level since macrofauna is much better represented in online databases such as GenBank or the Barcode of Life Database (BOLD) than microorganism groups. This approach relies on using such databases to identify the sequences present in the metabarcoding data as accurately as possible.

In this work package, we started out by assessing the percentage of macrofauna reported from a specific monitoring region, here monitoring region IV, that was present in online sequence databases GenBank, BOLD and SILVA, to identify remaining database gaps. Based on sieved ethanol samples, we performed morphological taxonomy of individual animals, and sequenced species lacking in the databases. Finally, to get a better understanding of the markers used in MetaMon, we investigated the metabarcoding data from homogenized bulk tissue samples with a known species composition based on morphological identification.

Visibility and outreach

In addition to scientific publications, MetaMon findings have been communicated at a variety of venues during the project, including International Workshop on Environmental Genomics (IWEG) annual meetings 2018-2021, the 2019 International Barcode of Life (iBOL) conference, the World Conference of Marine Biodiversity (2020), the 1st DNAqua-Net International Conference (2021), and at several smaller symposia and meetings with stakeholders and policymakers.

Conclusions and future recommendations

The MetaMon work packages have significantly advanced progress towards implementation of eDNA as a tool for offshore environmental monitoring, but results need to be further validated using more data in order to reach a sufficient maturity level for regulatory acceptance:

- Eukaryote microorganism (18S) distribution showed great promise for consistent results due to the more even distribution of these smaller organisms, and we suggest testing additional microorganism 18S and prokaryote 16S markers. An additional advantage of 16S is that prokaryotes may react more quickly to impact.
- *De novo* biotic indices are very promising but need more data. Supervised machine learning predictions (AI) were not significant in the MetaMon dataset, but again, are limited by the current amount of data.
- Quantitative ddPCR is a promising approach, especially the *Capitella* assay. More data are needed to establish secure correlations to further bioindicator taxa.
- Time series are needed to validate metabarcoding data consistency over multiple sampling events and time.
- While uneven distribution is a concern for macrofauna, we recommend building upon MetaMon COI findings to investigate the extent of this increased dataset noise, and sieved bulk samples as a possible alternative metabarcoding method for this organism group.
- More direct involvement by policymakers would enable input on optimal direction for future routine monitoring.

MetaBridge

To build on the main findings from MetaMon, a proposal to a follow-up project was created in collaboration with Equinor, Total and Lundin as industry partners. The proposal was submitted to the Research Council of Norway in August 2020. This project, MetaBridge (2021-2024), was approved for funding in December 2020.

MetaBridge will expand the amount of data available to improve the foundation for developing biotic indices, individual species quantitative assays and a supervised machine learning algorithm. As in MetaMon, samples are collected as part of regular monitoring surveys, enabling comparison to parameters currently in use. In order to validate data consistency over time, MetaBridge collected material at the MetaMon 2018 stations in early 2021 and will revisit MetaMon 2019 stations in 2022. As additional deliverables, MetaBridge will also expand the number of molecular markers for both MetaMon and new MetaBridge samples, work to further refine macrofaunal methodology, investigate the feasibility of bottom water eDNA samples, work towards storage and curation solutions for physical samples and metabarcoding data, and drive a closer dialogue with stakeholders and policymakers as the eDNA field matures.

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1. Introduction

1.1. Background

The current state of marine biological research builds on the accumulation of information from studies of oceanographic data and open water and seafloor marine organism biodiversity from the pioneering studies of the late 19th century to modern projects and initiatives. The information gained through this research, including both oceanographic data and the biota itself, provides the building blocks for the current understanding of marine biodiversity, community patterns and ecological processes. Yet, due to the remote nature, large scale and huge costs involved with studies of the marine environment, marine habitats and biotopes remain among the least known on Earth, especially so for the deep sea (Ramirez-Llodra et al. 2010).

The ability to infer the composition, ecological connections and anthropogenic impact on marine organisms is dependent on direct and proxy measures, including but not limited to parameters such as sediment composition, chemical parameters, depth and salinity, in addition to information regarding the organisms themselves, such as collection of specimens or video analysis. Depending on the sampling equipment used, different parts of the biological communities can be collected, for instance in the case of soft bottom seafloor sediments, a bottom trawl or sledge will sample different organisms than grab or core samplers. Thus, any given sampling method provides a particular view of the examined biotope.

In the latter half of the 20th century, increased scientific interest in ecological processes and patterns provided the necessary insights for studies of anthropogenic impact on the marine environment (e.g. Daan et al. 1994; Gray & Pearson 1982; Pearson & Rosenberg 1978; Rygg 1985) that form the basis for current monitoring programs connected to regulations such as the Norwegian implementation of the EU Water framework Directive (Direktoratsguppen vanndirektivet 2018), and national monitoring programs such as the offshore oil and gas monitoring regime currently used on the Norwegian shelf (Norwegian Environment Agency 2020). This includes development of biotic indices that combines the sensitivity values of individual organisms at a community level, such as the AZTI Marine Biotic Index (AMBI) and Norwegian equivalents such as the Norwegian Sensitivity Index and Indicator Species Index (Borja et al. 2000; Rygg 2006; Rygg & Norling 2013).

1.2. Environmental DNA

The development of DNA sequencing systems and other molecular biological methods to directly examine DNA and RNA opens up a completely new set of potential parameters that can be used to examine both single species as well as entire marine communities (Schander & Willassen 2005). Due to the development of high throughput DNA sequencing systems and bioinformatic pipelines, it has become feasible to sequence and analyze a large amount of DNA directly from environmental samples such as soil, sediment or water, termed environmental DNA (eDNA). Environmental DNA thus represents a completely new alternative or complement to existing, morphology-based methods of investigating and characterizing organism communities or the presence (or absence) of specific organisms of interest (Bourlat et al. 2013; Taberlet et al. 2012).

Most fundamentally, eDNA can be divided into methods to either quantitatively detect traces of single species, or to characterize organism communities (Kelly 2016): In the first case, methods such

as quantitative, digital or droplet digital PCR (qPCR, dPCR and ddPCR) can be used to measure the number of DNA copies of a particular species or group of species from an environmental sample (Medlin & Orozco 2017). Such assays are very sensitive, and can be used to detect organisms of particular interest such as invasive or threatened species, or species that are indicators of impacted environmental conditions (e.g. Krolicka et al. 2020; Kutti et al. 2020; Uthicke et al. 2018). In the second case, methods such as metabarcoding, targeted enrichment or shotgun sequencing allow broad-scale ecological assessment of the biodiversity patterns of the sample organism community (Taberlet et al. 2012).

The potential of eDNA as a tool for new ecological insights as well as monitoring of anthropogenic environmental impact was quickly recognized by the scientific community (Aylagas et al. 2018; Aylagas et al. 2017; Baird & Hajibabaei 2012; Bik et al. 2012a; Chariton et al. 2014; Cordier et al. 2019; Leray & Knowlton 2016; Pawlowski et al. 2018):

1. eDNA allows the study of previously inaccessible parts of organism communities such as juvenile, minute and single-celled eukaryotes as well as prokaryotes such as bacteria and archaea.
2. The time and effort of sampling and sample processing can be significantly reduced compared to morphological methods.
3. Identification of organisms in the dataset can be done by matching sequence data to a database containing DNA sequences of previously identified organisms rather than examination of each organism by a taxonomic expert, a time-consuming bottleneck in current research and monitoring.

The ambitions of policymakers as asserted by the UN sustainability goal 14: Life below water[1] in the face of an increased anthropogenic footprint as well as climate change highlights the need for an increase in monitoring capacity and cost-effectiveness. As such, the potential advantages of eDNA in ecosystem mapping and monitoring may act as an important contribution to future good ocean stewardship.

While the prospective advantages of eDNA are clear, there are also several obstacles to widespread implementation, which are especially relevant for environmental monitoring applications, and for community-level methods such as metabarcoding:

1. The widespread collection and analysis of DNA from the environment represents a completely new ecological data type. Though the number of studies that include eDNA data have increased at a rapid pace, study eDNA data still often lacks necessary context. Interpreting molecular datasets can thus be a challenging exercise.
2. This challenge is compounded by the incomplete nature of the databases used to identify eDNA sequences (Hestetun et al. 2020; Lejzerowicz et al. 2015; Sinniger et al. 2016). The closest match to a given sequence might be only distantly related. Though databases such as the Barcode of Life System (BOLD) aim to provide species-level identification, organism coverage is low and biased towards certain groups and geographical areas. The legwork of collecting, identifying and sequencing individual organisms still needs to be done to provide the database DNA copy used to identify eDNA sequences.

[1] "Conserve and sustainably use the oceans, sea and marine resources for sustainable development."

3. The sampling design, lab processing and bioinformatic processing chosen to create an eDNA dataset are critical for the quality of the data obtained (Alberdi et al. 2018; Zinger et al. 2019). In the case of metabarcoding, where DNA sequences part of a specific gene (or "markers") are chosen across the organisms in the sample, the variation among targeted organisms in the marker binding site means that a particular marker always introduces a bias on the resultant data (Deagle et al. 2014; Tang et al. 2012). This means that multiple markers need to be used in concert for a more complete picture of the organism community. Finally, PCR as well as high throughput sequencing (HTS) are imperfect techniques in that they may bias results and introduce different types of erroneous sequence variants (Schirmer et al. 2015). Standardizing protocols is therefore necessary for meaningful comparison between samples, but such protocols depend on knowing which protocols produce best results, which is still an area of ongoing research.
4. Finally, depending on the type of samples that are collected, the sampling design must be sufficient to support the aims of the data. For environmental monitoring, this typically means that the data must be sufficient to counter uneven distribution of the targeted organisms in order to be representative of the organism community at the collection site, so that any environmental impact can be assessed (Hestetun et al. 2021a; Le et al. 2021).

The use of eDNA-based methods in scientific studies has seen a large increase due to decreased cost and increased availability of high throughput sequencing platforms and bioinformatic pipelines and is currently a field in rapid development (Makiola et al. 2020). For marine benthic communities, studies have variously examined the ecological composition of both shallow and intertidal communities (Brannock & Halanych 2015; Faria et al. 2018), as well as the deep sea (Guardiola et al. 2015; Sinniger et al. 2016). Most of marine benthic eDNA studies can be roughly divided into whether they extract DNA directly from sediment, or whether they concentrate the biomass of targeted organisms by sieving, floating or other methods, before extraction, so-called community or bulk samples (Andújar et al. 2018).

Community (bulk) samples are typically used when the targeted organisms are multicellular metazoans (animals). The metazoan barcode marker cytochrome oxidase subunit I (COI) is commonly used in studies targeting metazoans, as is a small selection of ribosomal small subunit (18S) markers such as the 18S V1-V2 region. Using COI for macrofauna (>1 mm), Aylagas et al. (2016a) showed that sieved bulk samples recovered around half of morphological species in a dataset from the Basque coast. Lobo et al. (2017) were able to identify up to around 80% of species identified from morphology in the same samples in a similar study. However, the total amount of unique species in the COI dataset was over twice as many compared to using morphological identification, showing how morphological methods miss much of the community diversity. As the number of species present in identification databases such as BOLD increases, performance of macrofaunal identification through COI is expected to increase further.

Smaller metazoans belonging to the meiofauna (<0.5-1 mm), which represent a particular challenge for traditional taxonomy, have also been the subject of numerous metabarcoding studies. Faria et al. (2018) found that metabarcoding could assess meiofaunal ecological patterns in a Brazilian bay. A study by Atherton & Jondelius (2020) showed the effectiveness of using a combined 18S and COI approach to assess biodiversity from a Swedish intertidal site, though both studies emphasized the need for further groundwork to include more meiofaunal taxa in identification databases to increase the resolution of meiofaunal datasets.

Sediment extraction allows collection of single-celled organisms and extracellular DNA, and has the advantage of a simpler and quicker processing. Bik et al. (2012b) were able to show broad-scale biogeographic patterns containing both cosmopolitan and regionally distinct taxa in microorganism composition in a dataset spanning both deep sea and shallow sediments. Ecologically meaningful patterns of microorganism community distribution are typically reported in similar studies (Guardiola et al. 2016; Lanzén et al. 2016). For single-celled benthic organisms, species level assignment is typically unfeasible, mainly due to the limitations of current reference databases. Rather, the ecological patterns of higher taxon groups represent primary results for analysis.

Both the bulk community and sediment eDNA approaches have been used to assess the suitability of eDNA in impact monitoring of softbottom communities: Metazoan-based studies of community samples have showed that macrofaunal COI data from this kind of samples can be used to calculate existing biotic indices such as variations of AMBI and the Norwegian indices ISI and NSI with similar performance to that of morphological datasets (Aylagas et al. 2016a; Cordier & Pawlowski 2018; Lejzerowicz et al. 2015). Similar results have also been shown for COI data directly from sediment samples (Hestetun et al. 2021a; Mauffrey et al. 2020).

However, to leverage the full potential of metabarcoding data, organisms not part of the current morphological monitoring must also be included in impact assessment. By correlating taxa in metabarcoding datasets to impact parameters, it is possible to create new (*de novo*) biological indices (Bis) that are not limited to morphospecies or even the taxonomic classification of metabarcoding data. Due to the need for extensive datasets to establish such correlations, this work is mostly at a proof of concept stage, but has been applied to aquaculture (Keeley et al. 2018), coastal environments (Aylagas et al. 2017) and offshore monitoring (Mauffrey et al. 2020). As an alternative approach, supervised machine learning (SML) can be leveraged to predict parameter values based on a training dataset (Cordier et al. 2017; Cordier et al. 2018). While both methods hold great promise, they need to be constructed from large datasets and are currently at a preliminary stage.

1.3. Oil and gas impact monitoring on the Norwegian Shelf

Offshore oil and gas activities on the Norwegian shelf are subject to an environmental monitoring regime based on several environmental regions I-XII from southern Norway to the Barents Sea. As part of this monitoring, large-scale seafloor environmental surveys are routinely conducted around offshore installations. These surveys are organized by the industry itself, conducted by a handful of accredited environmental consultancy companies, and overseen by the offshore section at the Norwegian Environment Agency (NEA) according to published guidelines (Norwegian Environment Agency 2020). Each year, a subset of regions is surveyed, so that each region is visited every three years. Reports from these surveys (in Norwegian) are made public on the NEA webpages[2], and environmental data is also stored and made accessible through a combined repository called the MOD database (DNV GL 2021).

The parameters included in these bottom surveys, collected using a van Veen grab, include grain size distribution, hydrocarbon measurements (TOC, PAH, NPD), a selection of metals and bottom fauna, sometimes extra parameters, such as radioactivity or video transects, may also be included.

[2] https://www.miljodirektoratet.no/om-oss/roller/miljoovervaking/overvakingsprogrammer/petroleumsovervaking/

Regional baseline stations, located away from the installations, are used to calculate local limits of contamination, and parameters, such as biota or PAHs, are only investigated where there is previous evidence of impact. Sampling stations are typically organized in a cross-like transect pattern from the monitored installation, and while individual stations are typically fixed, the number of stations and amount of parameters measured at particular stations are subject to change based on a number of factors including impact results from the previous survey (Norwegian Environment Agency 2020).

1.4. The MetaMon project

The Molecular ecology research group (MERG) at NORCE Environment (previously Uni Research) has a long-standing collaboration with Equinor (previously Statoil) through several previous eDNA studies with data from the Norwegian offshore monitoring program. This includes the RCN funded EMP (Environmental Monitoring Programme) project, Phylogenetic microarrays and high-throughput sequencing: A new tool for biodiversity assessment in Northern Norway, a project connected to the Norwegian Deepwater Programme (NDP) and a MetaMon pre-project. In all these projects, the ability to get softbottom sediment samples from regular monitoring survey stations made it possible to efficiently gain access to samples that could be processed for molecular analysis. Previous projects were dependent on earlier high throughput sequencing (HTS) platforms such as 454 pyrosequencing, which, due to relative costs and technical limitations, reduced the total scope of number of samples as well as the number of sequences from each sample (sequencing depth). As the eDNA field has matured, new HTS sequencing technologies such as the Illumina platforms and insights gained from initial studies now allow studies with a substantial expansion of dataset scope beyond prototype proof of concept studies.

Thus, the MetaMon project, conceived as a collaboration between MERG and industry partners Equinor and Total, serves as a logical continuation of the groundwork necessary to advance towards the use of eDNA, and metabarcoding, in offshore environmental monitoring on the Norwegian shelf. The ability to piggyback on existing environmental monitoring, including both ship time and direct comparison with data from currently monitoring reporting, represents a cost-efficient design extending the scope of the project: Chemical, sediment and biological datasets based on morphological taxonomy (i.e., species lists) are available for direct comparison with metabarcoding data from chosen sites; a unique advantage compared to many other eDNA studies.

The main objective of MetaMon as stated in the project description, is to unlock the potential of metabarcoding as routine technology for the monitoring of benthic community biodiversity in relation to environmental impact assessment of petroleum extraction activities.

Partial aims towards this objective include 1) determining how metabarcoding qualitatively compares to taxonomic analysis for biodiversity assessment in marine sediments and its potential for assessing changes in abundance of indicator species; 2) exploring characterized benthic taxa and uncharacterized molecular identifiers, with verified potential as biological indicators of environmental disturbance related to oil drilling activities; 3) developing specific, preliminary guidelines for metabarcoding methodology, encompassing all stages from study design, sample collection, processing, DNA sequencing, sequence data analysis, data archiving and deposition, statistical assessment and reporting; 4) calculating estimates of taxonomic gaps for marine benthic species identification using two different metabarcoding approaches (SSU rRNA and COI); and 5) submitting COI and 18S barcodes for the most common taxa in the O&G monitoring programs. To this end, the content of MetaMon is divided into four work packages:

WP1: Comparative analysis of morphologic and metabarcoding-based monitoring

This work package comprises a comparison of morphology-based and metabarcoding data from a set of monitoring stations from the Norwegian Shelf. The discriminatory power of metabarcoding is compared to that of the current morphological data, and to further assess development of metabarcoding best practices for monitoring. Tools such as co-occurrence networks, SML and identification of indicator species will be explored. A subset of identified indicator species will be chosen for quantitative detection using ddPCR.

WP2: Standardization of sediment sampling methodology

This work package comprises further experiments to assess cost-effective metabarcoding sampling and DNA extraction with a particular focus on the best way, either through increasing the number of replicates, or volume of sediment, and of mitigating incomplete sampling of the sediment organism community due to uneven distribution of organisms. This data is needed to provide evidence-based input with regards to best practices sampling for a future metabarcoding methodological standard.

WP3: Assessment of the archive and taxonomic gaps

This work package aims to document and partially mitigate gaps in species identification databases for marine benthic macrofauna on the Norwegian Shelf.

WP4: Organization and project management

In addition to the scientific content of the project, a fourth work package includes administrative as well as outreach activities.

1.5. Report structure

The aims of the methodological studies as part of WP2 were to investigate the impact of methodological choices on the resulting metabarcoding data. These methodological experiments provided the findings that informed the protocol for the main WP1 metabarcoding study, and as such constituted a basis and natural starting point for the MetaMon project. With regards to WP1, this work package included both a metabarcoding study as well as a ddPCR quantitative approach. While the ddPCR work builds on WP1 metabarcoding findings, the methodological approach is quite different. Finally, while WP3 comprised both an estimation of gaps in identification databases and a targeted barcoding project to increase the coverage of species found on the Norwegian Shelf, these two approaches are both concerned with macrofauna specifically, rather than the full range of eukaryote organisms in the metabarcoding dataset. To clearly present the scientific findings of MetaMon, the report thus has the following general structure:

- Methodological findings and guidelines (WP2)
- Sediment metabarcoding study (WP1)
- Quantitative ddPCR to determine indicator species abundance (WP1)
- Metabarcoding for macrofaunal identification and gaps in database taxonomy coverage (WP3)
- Summary of findings, conclusions and recommendations for future research

2. WP2 – Methodological findings and guidelines

Acquiring a metabarcoding dataset is a process that is dependent on a number of processing steps, including sampling design and DNA extraction protocol (what to sample, how much volume, how many replicate samples, which extraction protocol or kit to use, sample treatment during extraction etc.), which molecular marker, and primers, to use (18S, 28S, COI or others), PCR cycling conditions and reagents, sequencing platform, and how the raw sequence data are filtered and clustered in a particular bioinformatic pipeline (Fig. 1). No matter the exact processing method used, a particular set of biases are introduced to the data, meaning that it can be challenging to compare metabarcoding data from different studies, especially at a fine scale (Zinger et al 2019).

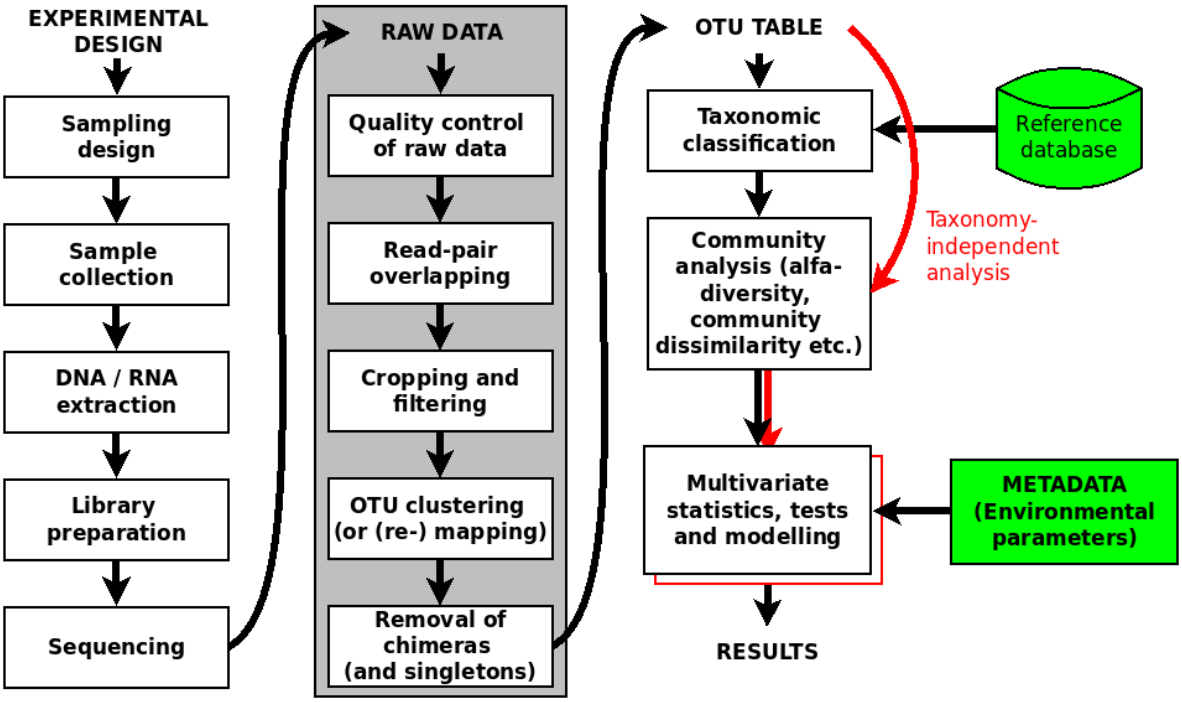


Figure 1. Schematic illustration of the steps required in sediment eDNA metabarcoding projects.

For currently used sediment morphological methods, a particular set of standards have been developed to keep processing as consistent as possible, ranging from sampling standards such as ISO 16665 (ISO 2014), to initiatives such as ring tests where identification accuracy is controlled by letting taxonomists at different institutions identify the same sample set of organisms.

Due to the recent state of eDNA-based methods, there is little consistency in the methods employed by various studies. In part, this is because best practices are still not established and must be developed through cumulative studies of different steps of the process. Given the number of possible strategies, finding an optimal methodology requires a significant effort with regards to methodological development.

While any method will produce a particular set of biases, a standardized methodology means this bias will also be consistent and results thus still comparable. At the same time, a chosen method needs to maximize cost-effectiveness in terms of ecologically relevant information to be used for monitoring of anthropogenic impact. In order to be used in a routine monitoring setting, metabarcoding thus needs to have a set of standardized guidelines so that results are comparable

from one study to the next. Part of the MetaMon project is thus concerned with combining existing knowledge with new experiments designed to provide such guidelines. The MetaMon pipeline, developed as part of this project, is shown as a graphic in Figure 2.

2.1. Previous findings

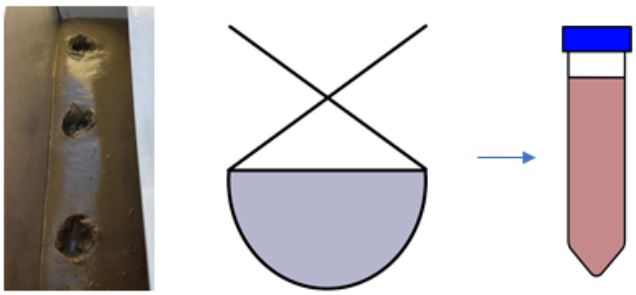
2.1.1. Sampling and DNA extraction

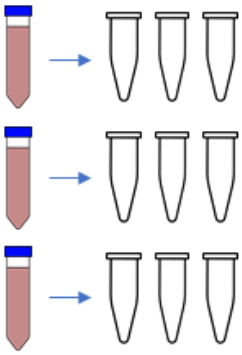
The methodological approach must be tailored to the specific habitat and type of samples being studied, in this case, marine sediments. Existing studies have examined technical steps in sediment metabarcoding processing:

Several studies have compared the use of extracellular DNA only (Pansu et al. 2021), direct sediment extraction and sieved community (bulk) samples (Brannock & Halanych 2015; Macher et al. 2018) and the effect of sediment volume subject to DNA extraction (Nascimento et al. 2018). Methodological work has also been a focal point for the MERG projects that preceded MetaMon: Thus, for instance, Hadziavdic et al. (2014) analyzed the small subunit 18S gene in depth for optimal primers for this gene. Lekang et al. (2015) compared the efficacy of different DNA extraction protocols on North Sea sediments, and found that the DNeasy PowerSoil Kit (Qiagen) provided high-quality data for a comparatively low amount of effort. Lanzén et al. (2017) investigated the effect of increased replicate samples and number of reads per sample on the overall richness and diversity; important measures to evaluate to which degree the underlying organism community is sufficiently represented in the data.

Field sampling:

- Three grabs per station.
- Sediment is collected from three spots in each grab (0-2 cm depth) and pooled in a single 50 ml Falcon tube for each grab, 20-30 g in total.
- Sediment is frozen on board.



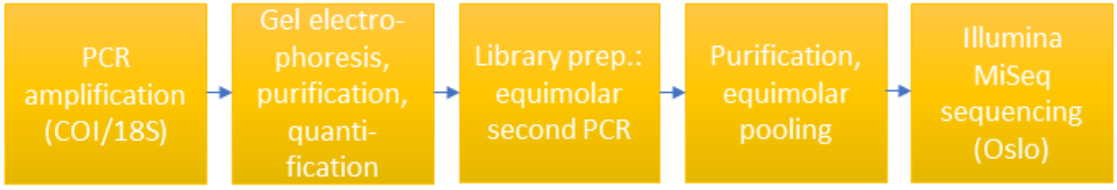


DNA-extraction:

- Sediment samples are thawed in a refrigerator, and pre-mixed before subsampling.
- Three 0.5 g replicate subsamples are collected for DNA extraction from each sediment sample ("grab").
- DNA extraction uses the PowerSoil kit with two modifications: Extra Precellys homogenizer treatment, and automated later steps using a QIAsymphony extraction robot.

PCR:

- Extract replicates are pooled prior to PCR amplification.
- One PCR/sequencing for each grab (three per station).
- Samples are diluted to similar (equimolar) DNA concentration throughout.
- Library prep. (second PCR) adds individual sample IDs and sequencing binding sites.



Data processing:

- Slightly modified SLIM pipeline
- Final filtering includes sequences in negative controls, crosstalk, and may include abundance cutoff, removing taxonomic groups.

Figure 2. Schematic representation of the MetaMon sampling and processing pipeline.

2.1.2. Marker choice and targeted organisms

The majority of sediment eDNA studies have coalesced around a handful of genes that have been found to give consistent results, depending on which part of the organism community that is targeted: For macrofauna (animals >1 mm), the Barcode of life (BOL) standard metazoan gene marker, cytochrome oxidase subunit I (COI), is common, using the so-called "Leray primers" (Leray et al. 2013) or variations thereof (Wangensteen et al. 2018); again a part of the standard barcode sequence derived using the "Folmer primers" (Folmer et al. 1994). This marker is for the most part sufficiently variable to identify individual animal species and has the highest database coverage (number of organisms in reference databases) (Andújar et al. 2018). Still, it also has several drawbacks: Since it is a protein-coding gene, it has more sequence variation in the primer binding sites than found in ribosomal genes, which means that it does not bind equally well to all metazoan groups and thus is biased against many marine taxa (Leray & Knowlton 2017; Zhan & MacIsaac 2015). As a mitochondrial gene (given the origin of the mitochondrion as an ancient proteobacterium), it is also very susceptible to non-target amplification of bacterial sequences (Collins et al. 2019). Still, while not perfect, COI remains the best option for targeting metazoans due to its ability to resolve sequences to species level.

For other groups, such as meiofauna (animals <1 mm) and single-celled eukaryotes, ribosomal markers are typically used, with a particular emphasis on the ribosomal small subunit (18S) (other markers include 28S and ITS). Owing to their structure and function, ribosomal sequences contain both highly variable and highly conserved (slowly evolving) sequences, a feature not found in protein-coding genes such as COI. It is thus easier to design universal primers targeting a broader range of organisms by placing primers in conserved areas, while still being able to discriminate between sequences based on variable areas. Different regions of 18S are used, most commonly the V1-V2 (Faria et al. 2018; Martínez et al. 2020), V4 (Lanzén et al. 2016; Laroche et al. 2020), V7 (Guardiola et al. 2015; Wangensteen et al. 2018) or V9 (Brannock & Halanych 2015) regions, which each provide a different bias in the parts of the eukaryote community they target best. Specific groups, such as Foraminifera, can also be targeted (Cordier et al. 2017; Keeley et al. 2018). Finally, for prokaryotes, the prokaryote 18S analogue 16S is targeted (Aylagas et al. 2017; Lanzén et al. 2020).

2.1.3. PCR, sequencing and bioinformatic filtering

Copying mistakes during PCR amplification and read artifacts during sequencing add noise to a metabarcoding dataset. Such errors can range from single base pair errors and sequences that are a mix of two other sequences (chimeras) to mistakes in barcode tags that wrongly attributes sequences to the wrong sample. A range of bioinformatic tools are used to filter and cluster together closely related sequences including, at different steps in the analysis, DADA2, VSEARCH, SWARM and LULU. While the effect of choices made during this processing is not as severe as that of sampling and DNA extraction (Brannock & Halanych 2015), bioinformatic considerations still need to be taken into account for data analysis and comparison, since results that have been treated and filtered differently are not necessarily directly comparable.

2.2. MetaMon lab processing

2.2.1. Primer choice

Two primer pairs were chosen based on a survey of the literature: COI and 18S V1-V2. While slightly biased towards meiofauna, the 18S V1-V2 primers are extensively used in metabarcoding studies and can pick up a broad range of both metazoans and other single-celled eukaryote organisms. Specifically, it provides less coverage of many pelagic microalgae, which were considered non-target for the aims of the project. The primers SSU_F04mod (5'-GCTTGWCTCAAAGATTAAGCC-3') (Cordier pers. comm.), originally from Blaxter et al. (1998), and SSU_R22 (5'-CCTGCTGCCTTCCTTRGA-3') (Sinniger et al. 2016) were chosen for this marker.

While COI is a more challenging marker than 18S in metabarcoding studies, this marker allowed coverage of macrofaunal taxa, allowing easier comparison with morphological methods as well as enabling increased species level identification of recovered sequences. A slightly modified version of the forward Leray primer, mlCOIintF-XT (5'-GGWACWRGWTGRACWITITAYCCYCC-3') (Wangensteen et al., 2018) and modified Folmer reverse primer jgHCO2198 (5'-TAIACYTCIGGRTGICCRAARAAYCA-3') (Geller et al. 2013). For both 18S and COI, the modifications to the forward primer aimed to increase the number of organisms that would be picked up by the chosen markers.

A small test was done in order to assess the feasibility of using an alternate set of primers for amplification of COI. These primers, BF2 (5'-GCHCCHGAYATRGCHTTYCC-3') and BR1 (5'-ARYATDGTRATDGCHCCDGC-3'), were chosen based on results from Elbrecht & Leese (2017). A PCR run including this primer pair was made for testing purposes, but with poor results, and no further optimization was attempted.

2.2.2. DNA Polymerase

Sediment DNA extracts may contain significant impurities that can inhibit the PCR reaction. Different DNA polymerases and buffer solutions perform better than others when exposed to such conditions, and thus three different polymerases were tested in order to find optimal conditions for PCR amplification: the Qiagen Hotstart, KAPA3G Plant PCR kit and DyNAzyme II DNA polymerases. Some reports indicate an elevated number of difficult-to-detect artifacts using high-fidelity polymerases. As such, none of these DNA polymerases were included in the comparison. DNA Polymerases were evaluated based on comparison of gel electrophoresis band strength following amplification of the same set of samples from all DNA polymerases. Electrophoresis gel band strength was significantly higher for the KAPA3G DNA polymerase than other DNA polymerases in this experiment. These findings fit well with the description of this DNA polymerase as developed to be resistant to a range of inhibitors, and this DNA polymerase was thus selected for all further PCR amplification in the project.

2.2.3. Optimizing PCR conditions

Based on a survey of literature using the chosen COI and 18S primers as well as PCR phase times recommended by the KAPA 3G documentation (Kapa Biosystems 2016), initial programs were selected for both markers and subjected to tests in order to improve performance. Temperature gradients were used in order to find optimal annealing temperature for both markers. We found an optimal annealing temperature of 57°C for the 18S SSU F04mod and R22 primers. While programs

using so-called touchdown profiles are common in the literature for Leray COI primers, we found no increased performance using either touchdown or "touchup" programs, mirroring the results of Aylagas et al. (2016a), and thus used a single annealing temperature of 45°C for the Leray-XT and Geller reverse primers.

As the COI primers include several ambiguous bases due to the variable nature of the binding sites, we hypothesized that we would need to increase the COI primer concentration compared to that of 18S. As we did not want to increase primer concentrations more than necessary to avoid problems in subsequent library preparation and sequencing, we did not want to increase primer concentrations beyond 4x of the 18S concentration. Tests using 1x, 2x and 4x primer concentration confirmed that increased primer concentration gave higher product concentration, and thus a 4x COI primer concentration compared to 18S was used in all subsequent amplification. Bovine serum albumin (BSA) has been shown to increase reaction effectiveness (e.g., Farell & Alexandre 2012) and was added to all PCR reactions.

2.2.4. Library preparation and sequencing

Initial PCR was performed using adapter-linked primers including 12 random bases to aid amplicon sequencing. Illumina dual index TruSeq i5/i7 barcodes were used for library preparation with equimolar PCR product concentration for each sample, and extraction and PCR negative controls were used to detect contamination during processing. Sequencing was performed on an Illumina MiSeq instrument using v3 with 300 bp chemistry at the Norwegian Sequencing Centre (University of Oslo, Norway).

2.2.5. Bioinformatic pipeline

Merging of the overlapping sequencing reads (forward and reverse direction) was carried out using the program VSEARCH (Rognes et al. 2016), allowing for up to 40 mismatches. Further quality filtering of sequencing reads was carried out using VSEARCH and cutadapt (Martin 2011) and included length-based filtering (330 – 450 bp for 18S and 274 – 333 bp for COI), removal and verification of a correct primer sequence. This was followed by clustering into unique operational taxonomic units (OTUs) using SWARM (Mahé et al. 2015). Thereafter, singleton OTUs (those represented by only one read in total), and potential chimeric OTUs resulting from PCR artefacts (see section 2.1.3), were removed using VSEARCH. Remaining OTUs were subjected to post-clustering correction using the program LULU (Frøslev et al. 2017), in order to join intra-specific sequence variants or remaining artefacts.

Taxonomic assignments were made using CREST (Lanzén et al., 2012). For COI, the BOLD database was used (Ratnasingham & Hebert 2007; accessed February 2018) and adapted to CREST (https://github.com/lanzen/CREST), while for 18S, we used SilvaMod v128 as reference. Likely contaminant OTUs were identified and removed based on both suspicious sample OTU abundance patterns and PCR and extraction blanks, for each plate, using decontam (Davis et al. 2018). Further, filtration was carried out based on taxonomic assignments in order to remove OTUs of likely pelagic origin. Cross-contamination was reduced by setting OTU abundances to zero where it occurred in a sample at very low abundances compared to its average abundance across samples (<1%), like the UNCROSS algorithm (Edgar 2016).

Alpha diversity estimates (rarefied, i.e. expected richness at minimum read depth, and Shannon diversity) were calculated using the R package vegan (Oksanen et al. 2019). Bray-Curtis pairwise

dissimilarities were calculated based on relative OTU abundances, filtered to compensate for differences in sequence depth and random sampling effects.

All scripts used for sequence data processing and analyses have been made publicly available through the GitHub repositories https://github.com/lanzen/Metamon1 and https://github.com/lanzen/Metamon2 (doi: 10.5281/zenodo.4826641).

2.2.6. Mock communities

To assess primer bias for the 18S and COI marker genes, two mock communities (extract templates with a known composition) were made by picking and sequencing 21 clones for each marker from metabarcoding samples. For 18S **the Hadziavdic et al. (2014)** primers F-40 (5'-AAGATTAAGCCATGCATG-3') and R-1797 (5'-TGATCCTTCTGCAGGTTCACCTAC-3') were used; for COI the **Wangensteen et al. (2018)** mlCOIintF-XT (5'-GGWACWRGWTGRACWITITAYCCYCC-3') and **Geller et al. (2013)** jgHCO2198 (5'-TAIACYTCIGGRTGICCRAARAAYCA-3').

Cloning was performed using the StrataClone PCR Cloning kit, using the kit standard protocol; the same protocol as used in the MetaMon pre-project. In brief: Ligation reactions (one per PCR sample) were prepared using ampicillin and 5-bromo-4-chloro-3-indolyl-β- D-galactopyranoside (X-gal) and then incubated. Transformant plates were incubated agar side up at 37°C overnight. Sterile toothpicks or pipette tips were touched against individual colonies, resuspended into PCR reactions and run with the standard kit M13F/M13R primers. Sanger sequencing was used to obtain sequences of individual clones after PCR, using BLAST to identify clonal sequences.

Given recent articles outside the scope of MetaMon covering primer biases for the markers in question (e.g., Elbrecht & Leese 2015; Elbrecht & Leese 2017; Wangensteen et al. 2018), and the higher relevance of other aspects of MetaMon to the final deliverables of the project, the mock communities were not investigated further.

2.2.7. MetaMon sampling design findings

A major aim of MetaMon was increased cost-effectiveness of sampling and processing of ecological samples. As such, a direct sediment rather than bulk community approach was chosen for most samples in the project. To enable collection of many such sediment samples, they were achieved as part of the standard environmental monitoring survey, using a van Veen grab, as this allowed collecting sediment for metabarcoding at the same time as chemical and morphological biology samples were taken as part of the normal survey.

While larger volume sediment samples (10 g) on a one-to-one basis have been shown to outperform smaller volumes and can be processed using specialized DNA extraction kits such as PowerMax (5 g of sediment), extraction using these kits is manual, and the per-sample processing time significantly higher than that of smaller sediment volumes (0.5 g). Following and building upon the results of Lanzén et al. (2017), MetaMon thus employed a sampling and extraction approach that combined multiple small volume replicates that could be processed using a semi-automated pipeline, in order to maximize sampling cost-effectiveness. To increase the amount of the total organisms found in each grab, sediment was collected from three different parts of each grab and pooled together in the field. Sediment samples were frozen on board and kept at -20°C until processing in the lab.

For the technical experiments specifically, extra sediment was collected from five grab parallels belonging to monitoring region II station GK-DA-01 (Gina Krog installation). This sampling design was chosen to allow a large number of extraction replicates to be made from the grab replicates from this station, allowing direct comparison of technical parameters associated with metabarcoding processing.

Two main research questions were the subject of experiments connected to MetaMon WP2:

1. The impact of the degree of sample homogenization during DNA extraction, that is what effect the intensity of the shaking during extraction had on the composition of the resultant DNA extract.
2. Comparing the differences in organism composition from samples from different parts of the same and different grabs, to investigate the level of sampling needed to obtain adequate representation of the organisms at the station.

Both cases represented areas where little to no previous research was available yet would provide valuable information for developing specific sampling and processing recommendations for standardized guidelines.

2.2.8. Extract homogenization

DNA extraction is the first laboratory step in the processing of metabarcoding samples and involves extracting DNA from the source sample into a buffer solution for PCR amplification. The DNA composition of this extract has a large effect on metabarcoding results. A previous MERG study (Lekang et al. 2015) compared the performance of several different kits and protocols of extracting DNA from sediment samples. Overall, the Qiagen DNeasy PowerSoil kit was found to provide an optimal trade-off between workload and performance. PowerSoil belongs to a type of extraction kits in which sediment is mixed with specialized beads and rapidly shaken (homogenized) in order to release DNA from sediment particles as part of the DNA extraction process. Intuitively, the amount and intensity of such homogenization might influence how much of the sediment DNA is retrieved in the resultant DNA extract.

The MERG lab had already developed a hybrid protocol incorporating a QIAsymphony extraction robot with PowerSoil reagents in order to speed up DNA extraction. For MetaMon, a series of experiments were designed to investigate the effects on the number of unique sequences (richness) and number of each unique sequence (abundance) in the metabarcoding data from samples subjected to different homogenization intensity during the DNA extraction process, and compare any such homogenization effect with an increased volume of sediment. An additional goal was to compare the richness increase with additional extraction replicates for the different homogenization treatments, the similarity between replicates, and a separate experiment comparing the effects on the metabarcoding data of pooling extracts before or after PCR amplification.

The intent of these experiments was to optimize the methodology in order to maximize the cost-effectiveness of the metabarcoding pipeline. The results from these experiments have been published in *Environmental DNA* (Hestetun et al. 2021b).

2.2.9. Homogenization results

In all, 34 sediment subsamples were taken from a single large sediment sample from van Veen grab 4, station GK-DA-01, during the 2018 monitoring cruise to monitoring region II and subjected to different homogenization treatment using either 1) a high intensity homogenizer treatment (Precellys 1), 2) a medium homogenizer treatment (Precellys 2), 3) Vortexing only (lower intensity) or 4) Vortexing only, but with 10x (5 g) increase in sediment volume (using the PowerMax kit) during DNA extraction. Extracts were then subjected to PCR amplification and sequencing using the 18S and COI markers.

To evaluate the effect of homogenization treatment on the resulting metabarcoding data, we compared the alpha diversity (number of unique sequences and their *within*-sample distribution) and beta diversity (similarity *between* samples) for the different homogenization treatments. Alpha diversity was measured using rarefied richness, which is the number of unique sequences where all samples have been standardized to the same number of sequence reads (e.g., all samples downscaled to for instance 70,000 unique sequences), and Shannon diversity, which is an index number considering both the number of unique sequences, but also their relative abundance (Fig. 3).

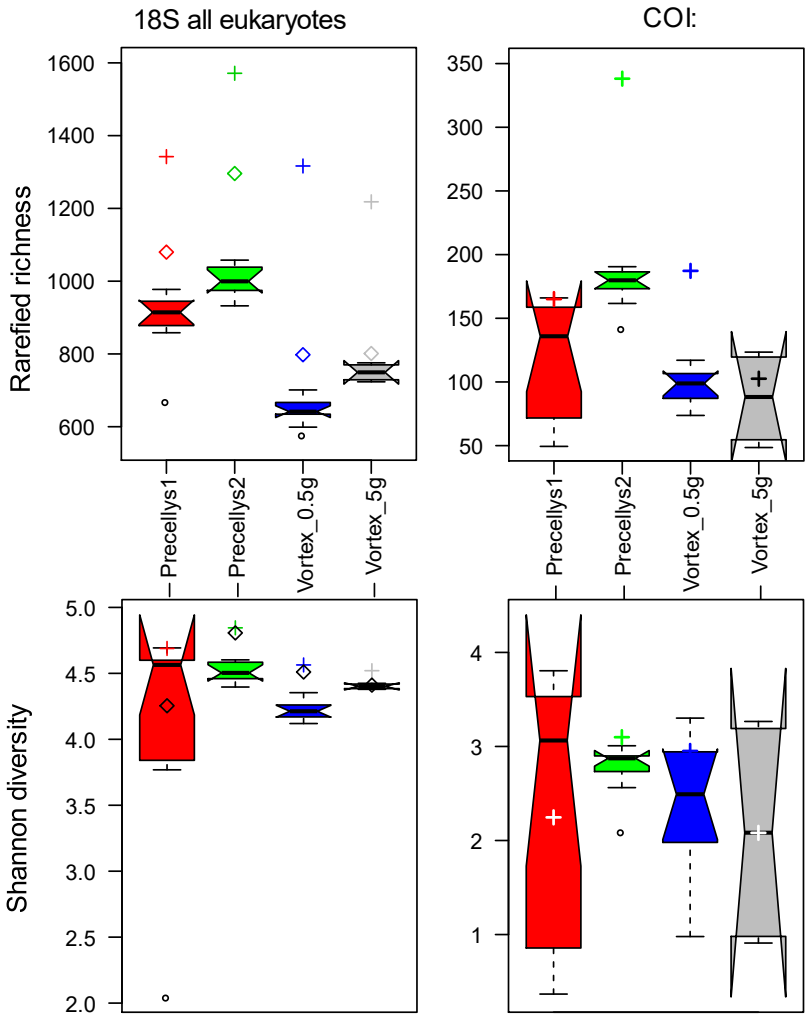


Figure 3. The effect of homogenization treatment on alpha diversity measured as richness and Shannon value.

Alpha diversity results showed significant differences between samples: The intermediate Precellys treatment had the highest rarefied richness values, while Shannon values were a bit more mixed. COI had much higher variability between extracts, highlighting the more uneven distribution of metazoans specifically compared to the 18S dataset, which also included single-celled eukaryotes.

In addition, we included pooling of all extracts prior to PCR and sequenced together (diamonds) and the rarefied richness/Shannon diversity of all samples together (*in silico* pooling, plus signs) for comparison. Here, we noted that pre-PCR pooling led to higher diversity than single extracts, but not to the extent of the rarified sum of all single extracts (i.e., diamonds higher than single extracts, lower than plus signs).

Similarity between extracts, a measure of beta diversity, shows the consistency of individual extracts, and how well they can represent the community from which the sample is drawn, here shown using Hellinger-transformed Bray-Curtis dissimilarities (Fig. 4). PCR replicates of the same extract were used as a baseline. In total, both the intermediate Precellys treatment and the 5 g PowerMax treatment came out ahead (lower scores are better) for 18S data (both the whole dataset and metazoans only) while the 5 g treatment did not show increased performance from increased sediment volume for the COI marker.

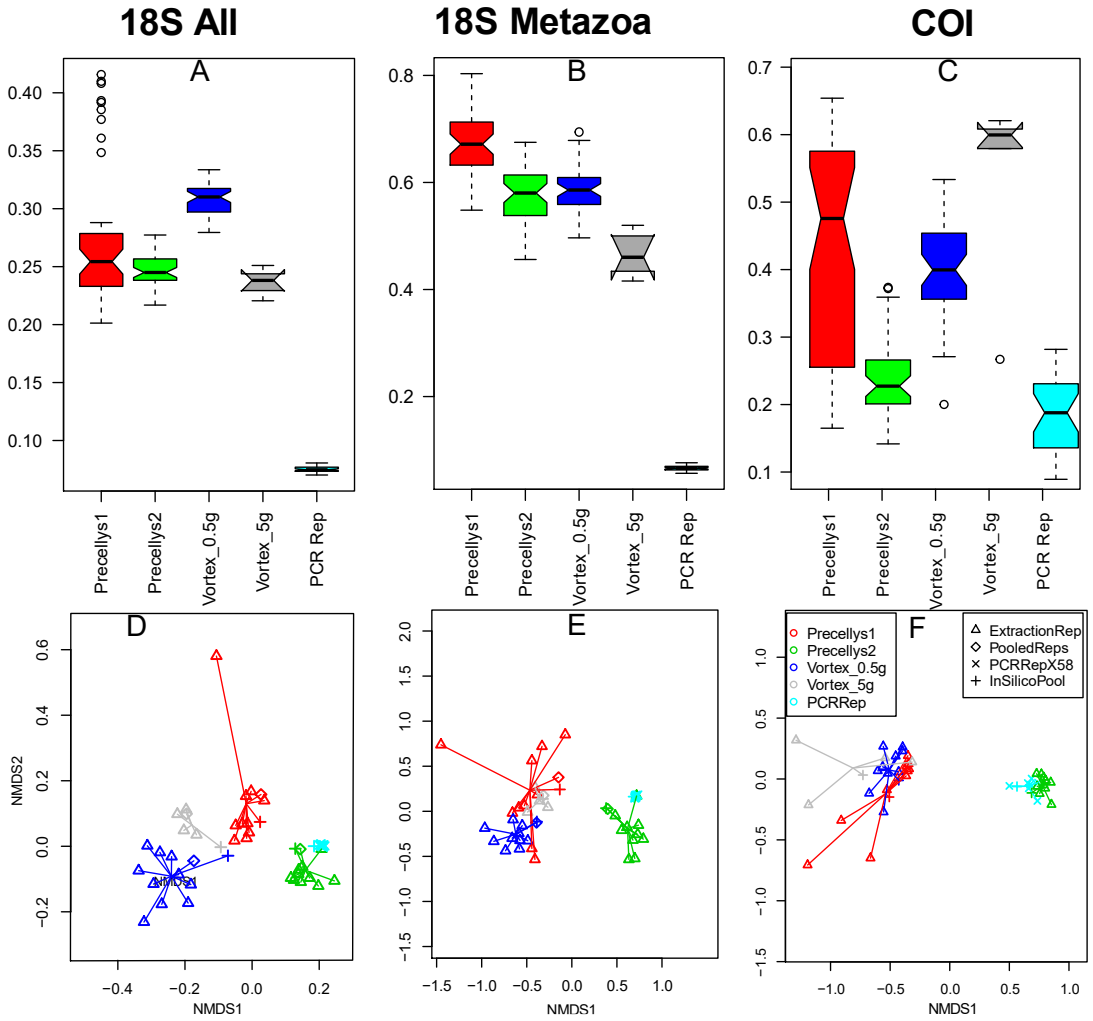


Figure 4. Similarity between extract replicates for each treatment shown as box and NMDS plots for the complete 18S, 18S metazoan and COI metazoan datasets.

For 18S, we also calculated the median rarefied richness increase for each additional extract for the four homogenization treatments, using repeated randomized sub-sampling. This showed a modest decline in additional richness per simulated extract for all treatments, but more so in the case of the total eukaryote dataset compared to the metazoan part of the 18S dataset only (Fig. 5).

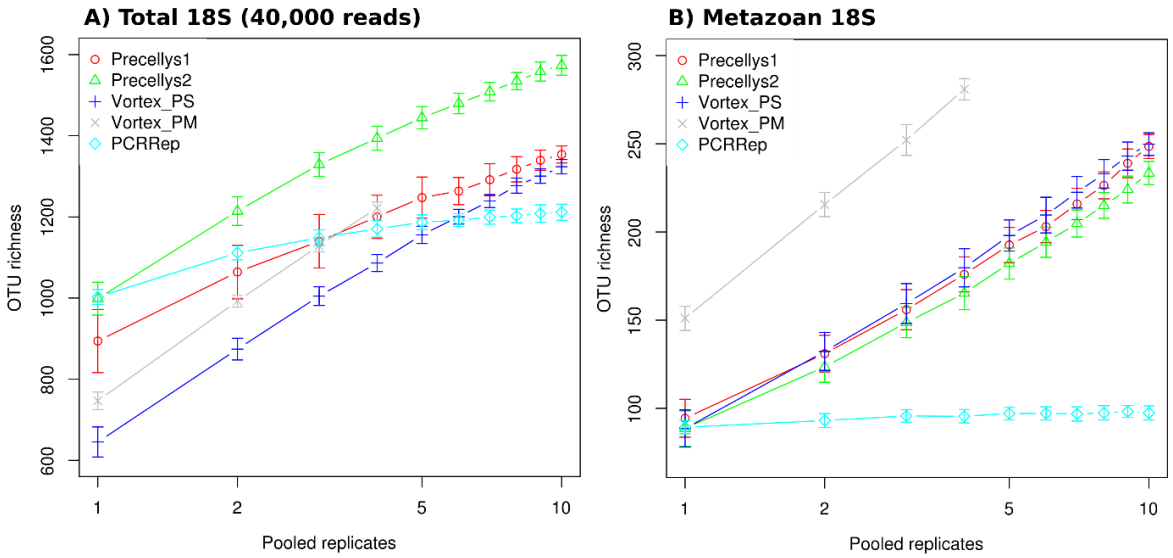


Figure 5. Additional median rarefied richness with increased number of replicates for the 18S total and metazoan only dataset.

The main conclusions of the work done for this article were that A) choice of homogenization protocol does have a significant impact on the amount of the sediment DNA that is obtained during DNA extraction, and that an intermediate homogenizer treatment gives optimal results, B) an increased number of small volume extract replicates is better than fewer large volume replicates in terms of cost-effectiveness and also dilutes the impact of large organisms in the source sediment, C) pre-PCR pooled extracts significantly outperforms individual extracts, and represent a feasible strategy both for cost-effective processing, and minimizing PCR-based artifacts, and D) COI metazoan data are significantly less consistent than 18S due to uneven organism size and sediment distribution. Together with random PCR effects due to less conserved primer sites, this introduces a large amount of noise in COI datasets compared to total eukaryote 18S data.

2.2.10. Spatial distribution experiment

Metabarcoding and other eDNA methods use sediment source volumes that are magnitudes smaller than the whole grabs that are sieved in morphological sampling. A second set of sampling design experiments were made in order to get a better understanding of the relative degree of difference between samples taken at different spatial scales, in order to understand how different sampling designs with regards to sediment from the same or different grab parallels, influence how representative the resultant data is for the sampling station as a whole.

Here, for three separate van Veen grab samples from the GK-DA-01 station, sediment samples were taken individually, not pooled, from five separate places from the each van Veen grab. For each of these 15 sediment samples, five DNA extract replicates were made: in all 75 DNA extracts (Fig. 6).

These extracts were sequenced individually or pooled prior to PCR for a total of 30 18S and 30 COI metabarcoding data samples.

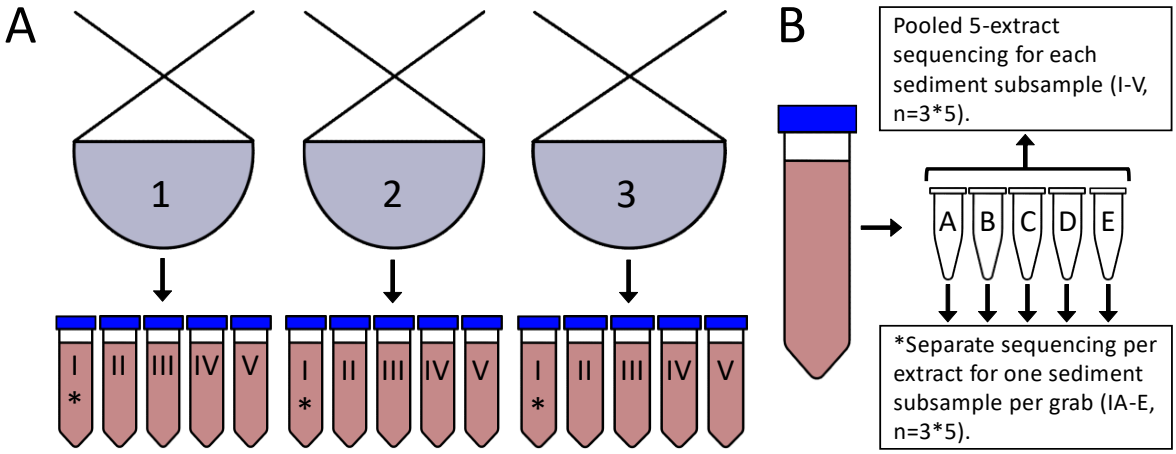


Figure 6. Sampling design for the study of metabarcoding data spatial variation.

The aim of this study was to compare the amount of the total diversity of the station (as represented by the sum of all metabarcoding data collected in the study) that could be obtained for individual extracts, sediment samples and grab samples, as with the previous study, all extract combinations were sequenced using the 18S V1-V2 and COI markers.

Table 1. The absolute and percentages of the total three-grab dataset for each marker obtained for individual extracts, sediment samples and grabs.

18S absolute counts							
	Single extracts			Intra-grab, 5 pooled extracts			Whole grab
	Min	Max	Median	Min	Max	Median	Value
Grab 1	641	666	659	622	812	752	959
Grab 2	642	656	646	711	782	775	933
Grab 3	666	706	679	695	779	711	933
18S percentage of total (n=1030)							
	Single extracts			Intra-grab, 5 pooled extracts			Whole grab
	Min	Max	Median	Min	Max	Median	Value
Grab 1	62%	65%	64%	60%	79%	73%	93%
Grab 2	62%	64%	63%	69%	76%	75%	91%
Grab 3	65%	69%	66%	67%	76%	69%	91%
COI absolute counts							
	Single extracts			Intra-grab, 5 pooled extracts			Whole grab
	Min	Max	Median	Min	Max	Median	Value
Grab 1	35	48	39	37	63	60	83
Grab 2	45	55	49	47	67	48	84
Grab 3	52	59	56	38	59	51	87
COI percentage of total (n=108)							
	Single extracts			Intra-grab, 5 pooled extracts			Whole grab
	Min	Max	Median	Min	Max	Median	Value
Grab 1	32%	44%	36%	34%	58%	56%	77%
Grab 2	42%	51%	45%	44%	62%	44%	78%
Grab 3	48%	55%	52%	35%	55%	47%	81%

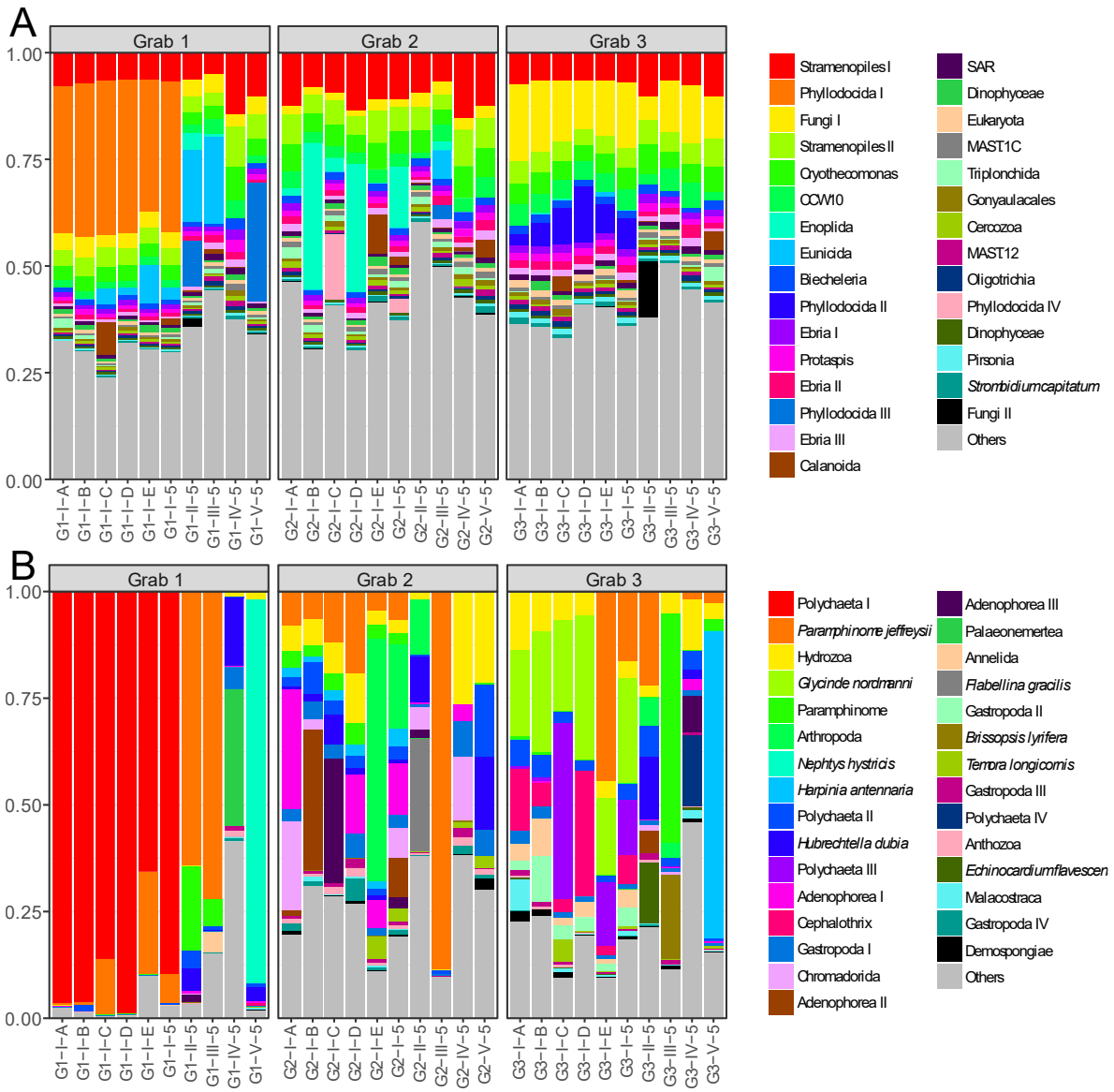


Figure 7. Relative read abundances between sediment and grab replicates for the 18S (A) and COI (B) datasets in the spatial variability study gives an intuitive picture of the differences in read counts for main taxa in these datasets. Not the higher variability of the COI data compared to 18S.

The read abundances of the different data are shown in Fig. 7. The degree to which different samples were able to represent the total three-grab dataset is shown in Table 1. To examine differences between the sample metabarcoding data, beta diversity Bray-Curtis dissimilarities were calculated, shown as box plots and NMDS plots (Fig. 8). For COI, we also calculated commonly used current biotic indicators, based on the parts of the COI data that could be identified to species level.

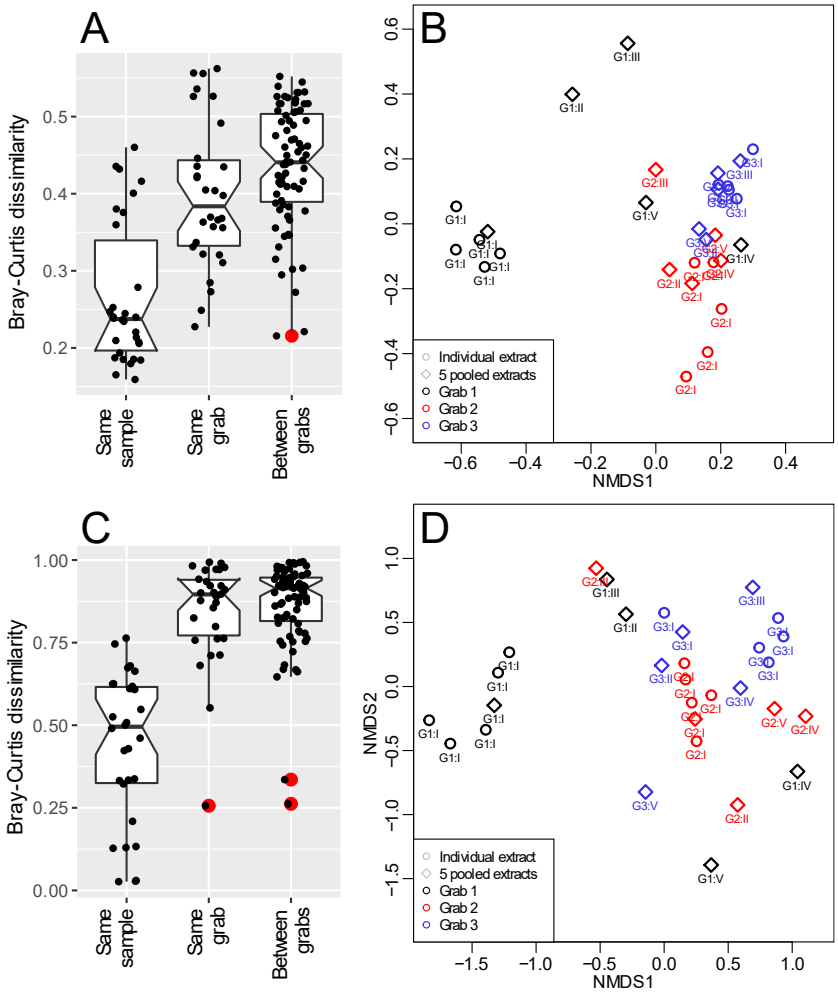


Figure 8. Bray-Curtis dissimilarity between samples for 18S (A-B) and COI (C-D) represented by box and NMDS plots.

The main conclusions from this work, published in *PeerJ* (Hestetun et al. 2021a), showed that even a small amount of source material from a manageable number of replicates was well able to represent total eukaryotic diversity at grab or even multi-grab level, showing the presence of an eukaryotic core community. There was a decrease in data similarity with distance (extract < sediment samples < grab level), and as seen in the homogenization study, individual extracts and even sediment samples were vulnerable to high read abundances from single metazoan OTUs. This means that both extract and spatial replicates should be used to increase coverage of the organism community at the site.

COI results were less consistent than 18S, again highlighting the increased heterogeneity of metazoan distribution compared to single-celled eukaryotes. Yet, COI results become more representative when represented as index values from e.g., the ISI or NSI index, showing that these index values are more resilient than direct alpha diversity values such as richness or Shannon values. A likely explanation here is that the species present in the indices are found among the more abundant OTUs in the dataset, and thus exhibit less variability than rarer OTUs in the data.

3. WP1 – Comparative study of metabarcoding and morphological data

Anthropogenic impact from offshore industrial and oil and gas extraction activities has the potential to negatively influence nearby seafloor organism communities. Such impact can change the composition of these communities, and the extent of such impact can thus be detected through investigating these communities directly. In the context of offshore monitoring on the Norwegian Shelf, this is currently done through sieving the 1 mm fraction of whole van Veen grab samples, retaining all collected animals, and identifying them to the lowest taxonomic group possible in order to provide species lists that are used to calculate ecological parameters, including alpha diversity parameters such as the Shannon index, and biotic indices that take into account the sensitivity to impact of the recovered organisms: Impacted localities typically have a lower number of species (e.g. lower diversity), and the species that are present have low sensitivity (e.g. are tolerant to impact).

Metabarcoding involves the amplification of part of a specific gene (marker) from a wide selection of organisms found in the source sample. Differences in gene sequence are used to identify taxa or taxon groups, depending on method used described as operational taxonomic units (OTUs) or amplicon sequence variants (ASVs), and the resulting list of OTUs or ASVs, a so-called OTU or ASV table, is in many ways analogous to a species list derived from morphological identification. As such, metabarcoding can represent a potential alternative or complement to the currently used morphological methods to investigate community composition at a given locality.

As reviewed more in depth in the general introduction and methods chapters here, however, the metabarcoding approach is not always straightforward. The most important question may be if the information regarding community composition provided by the metabarcoding data be used to determine whether the source community has been impacted? If so, how does this ability compare to the currently used morphological methods? Finally, are there any molecular markers, such as 18S, COI or even bacterial 16S, that can detect such impact better than others?

There are two main conceptual approaches to using metabarcoding data to infer anthropogenic impact:

1. By using a marker (COI) that can target the same organisms that are examined in current methods, i.e., macrofauna, it is possible to detect the presence of sensitive or tolerant organisms that are used in current biotic indices such as AMBI or NSI/ISI. This approach is highly dependent on the ability to identify genetic sequences in the data to a low level, preferably species level, and thus relies on the relatively high taxonomic coverage of COI in online databases. The advantages of the COI macrofauna approach is that it is more closely related to the current monitoring system. As such, the data can to a certain extent use the extensive knowledge of the ecology of, and historical data from, the sampling area macrofauna, to help infer impact status. Thus, it provides more of a continuation of existing methodology. Disadvantages include the increased sampling needed to adequately represent larger organisms compared to e.g., single-celled eukaryotes due to uneven sediment distribution.

2. By using markers that target a wider variety of organisms, it is instead possible to also include those groups in impact assessment. Ribosomal markers (e.g., 18S, 28S, 16S) are generally easier to work with, as a combination of conserved primer sites and a more even distribution in the sediment of single-celled organisms. Using this approach, however, the resolution of taxonomic information obtained is much lower: most sequences in the data can only be roughly identified as belonging to one of several high-level groups, and there is yet little metabarcoding available for comparison of impacted vs non-impacted conditions. As such, biotic indices that depend on sensitivity need to be remade from scratch (*de novo*). Alternatively, taxonomy can be scrapped altogether using e.g., a supervised machine learning (SML) approach that has the potential to perform well even with diverse and complex input data (compared to the creation of novel biotic indices). However, SML is reliant on large training datasets in order to make accurate predictions for new sites.

3.1. MetaMon metabarcoding study

In order to examine the ability of metabarcoding data to detect anthropogenic impact from offshore oil and gas extraction, it needs to be compared against the existing parameters that are used in the environmental monitoring program. In practice, this means establishing correlations between metabarcoding data and parameters such as the morphological taxonomy datasets and abiotic parameters such as hydrocarbon or metal concentrations.

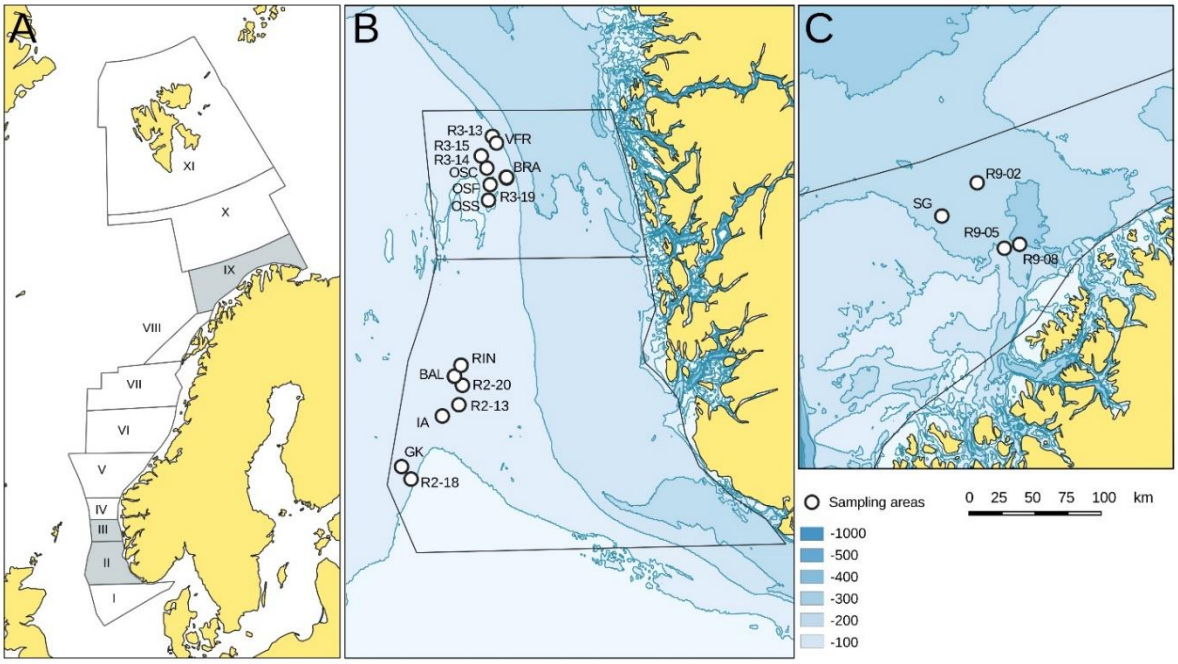


Figure 9. An overview of the offshore installations included in the MetaMon WP1 metabarcoding study.

As part of MetaMon work package 1 (WP1), sediment from several stations from monitoring regions II, III and IX were sampled for metabarcoding amplification and sequencing (Fig. 9). The metabarcoding sampling included 97 stations with three grab replicates from each, in all 291 sediment samples. Metabarcoding sample processing followed the protocol established by WP2, with three parallel DNA extractions made from each sediment sample using the MERG semi-

automated pipeline, thus encompassing 873 separate DNA extracts. The small subunit 18S V1-V2 and COI markers, were sequenced as described in the previous chapter.

The stations chosen for metabarcoding sampling were all stations that also were subjected to regular monitoring during the 2018 and 2019 campaigns. As such, sampling was designed to allow comparison of metabarcoding data with morphological taxonomy and abiotic parameters, downloaded from the DNV GL MOD database (DNV GL 2021). The metabarcoding bioinformatic pipeline was similar to that of the methodological studies and is described further in the previous chapter.

The metabarcoding comparative study aims included:

1. Creating an aggregated physicochemical pressure index (PI) combining the THC, PAH16, Ba and Cu parameters from the relevant stations.
2. Examining the correlation between this PI and morphological and COI-based NSI biotic index values.
3. Using correlation between PI values and individual OTU abundance to identify the sensitivity to impact of OTUs in the 18S and COI data, to create a proof-of-concept *de novo* biotic index for each marker.
4. Examining the accuracy of the developed *de novo* indices through cross validation.
5. Examining co-occurrence networks of OTUs for impacted and non-impacted sites to investigate ecological relationships of individual OTUs in the dataset.

The following section is a short summary of the main study findings from this study, published as Lanzén et al. (2021).

3.1.1. Developing the pressure index

A pressure index (PI) is a way of combining several abiotic impact measures together into one parameter (Aylagas et al. 2017). Calculated PI values then serve as a ground truth against which to compare metabarcoding and morphotaxonomy data. The PI developed here includes total hydrocarbons (THC), polyaromatic hydrocarbons (PAH16), barium (Ba, as a proxy for drilling activity) and copper (Cu). Possible PI values range from 0 to 6 (where 0 represents no impact) and where a value of 2 corresponds to the highest allowed values classification as good environmental status. Thus, the values of the PI were set to correspond to the ecological status groups of the NSI as well, with 0—1 representing very good status, 1—2 good, 2—3 moderate impact, 4—5 poor status and 5 and above very poor status.

3.1.2. Correlation between alpha diversity and PI values

Alpha diversity of the metabarcoding and morphological data was examined both in terms of rarefied richness (number of unique OTUs per standardized sequence read number) and as Shannon diversity (Fig. 10). Key results include:

- COI and 18S rarefied richness correlated strongly between samples (i.e., low COI richness correlated with low 18S richness and vice versa).

- Shannon values of the morphological dataset correlated stronger with 18S rarefied richness, compared to with COI. The correlation between morphology and metabarcoding-based Shannon values was consistently weaker than to metabarcoding-based rarefied richness.
- PI values had moderate correlation with morphological Shannon values and 18S richness, and low correlation with COI richness.

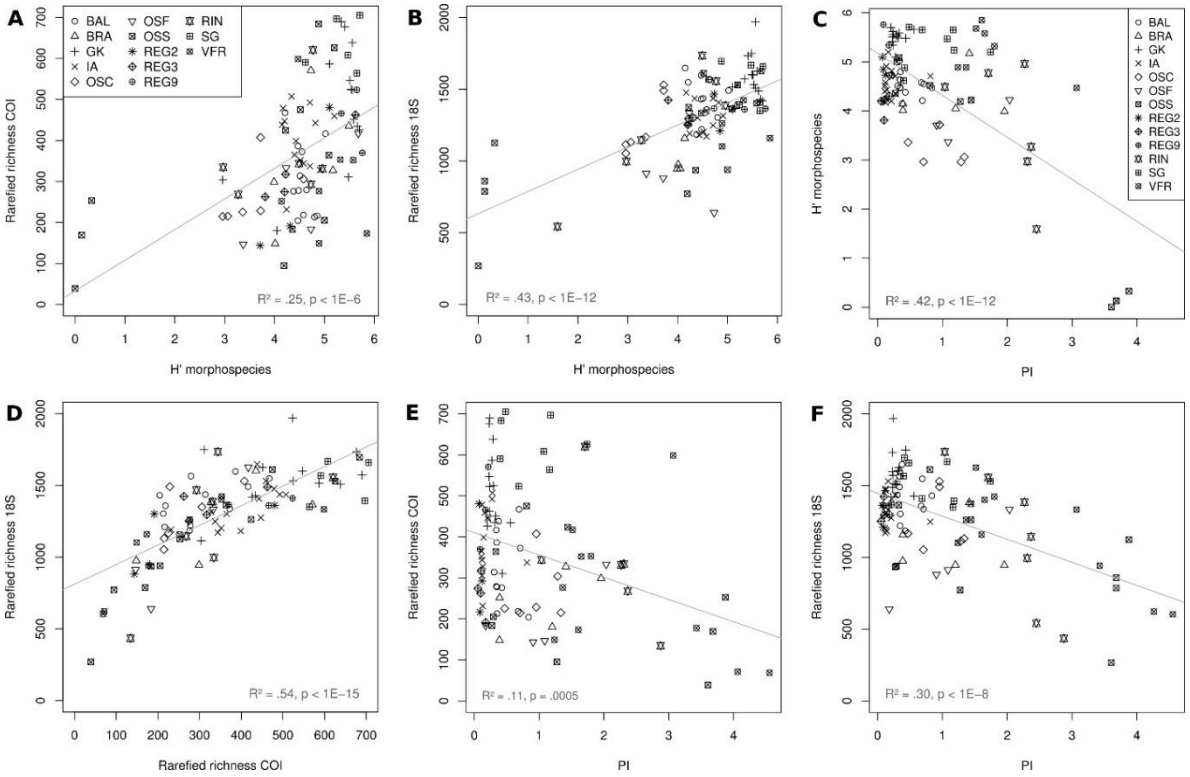


Figure 10. Plots showing the correlation between alpha diversity values of the different datasets (morphology, 18S and COI), including Shannon values (H') and richness (number of unique sequences/species).

3.1.3. Correlation between beta diversity and abiotic PCA datasets

Dissimilarity of community composition across stations in the dataset was examined using non-metric multidimensional scaling (NMDS) based on Bray-Curtis pairwise dissimilarities and Mantel tests. The latter were used to examine the amount of correlation between datasets, based on the resulting pairwise Bray-Curtis dissimilarities. Physicochemical parameters were standardized and compared using Euclidian distances, with the resulting distance matrix compared to biological community data using Mantel tests and subjected to Principal Coordinates Analysis (PCoA) Key findings (Fig. 11) include:

- Region IX (Barents Sea) stations hosted biological communities that were consistently different, i.e., formed a separate cluster in NMDS space. than other regions.
- The Mantel tests showed that 18S-based community data was the most consistent with community structure based on morphological data, implicating that 18S is the marker that best reveals the community response to the modelled parameters (depth, the hydrocarbon component of the PI, the Ba and Cu component, geographical distance, sand and gravel).

- The 18S dataset was also the most consistent with differences in abiotic parameters, more so than morphotaxonomy data, according to the Mantel tests.
- The PI and NSI correlated significantly with all NMDS components.
- Of the 25 most abundant taxa, eight were shared between COI and the morphological dataset. The lower resolution and database coverage of 18S did not allow a similar comparison.

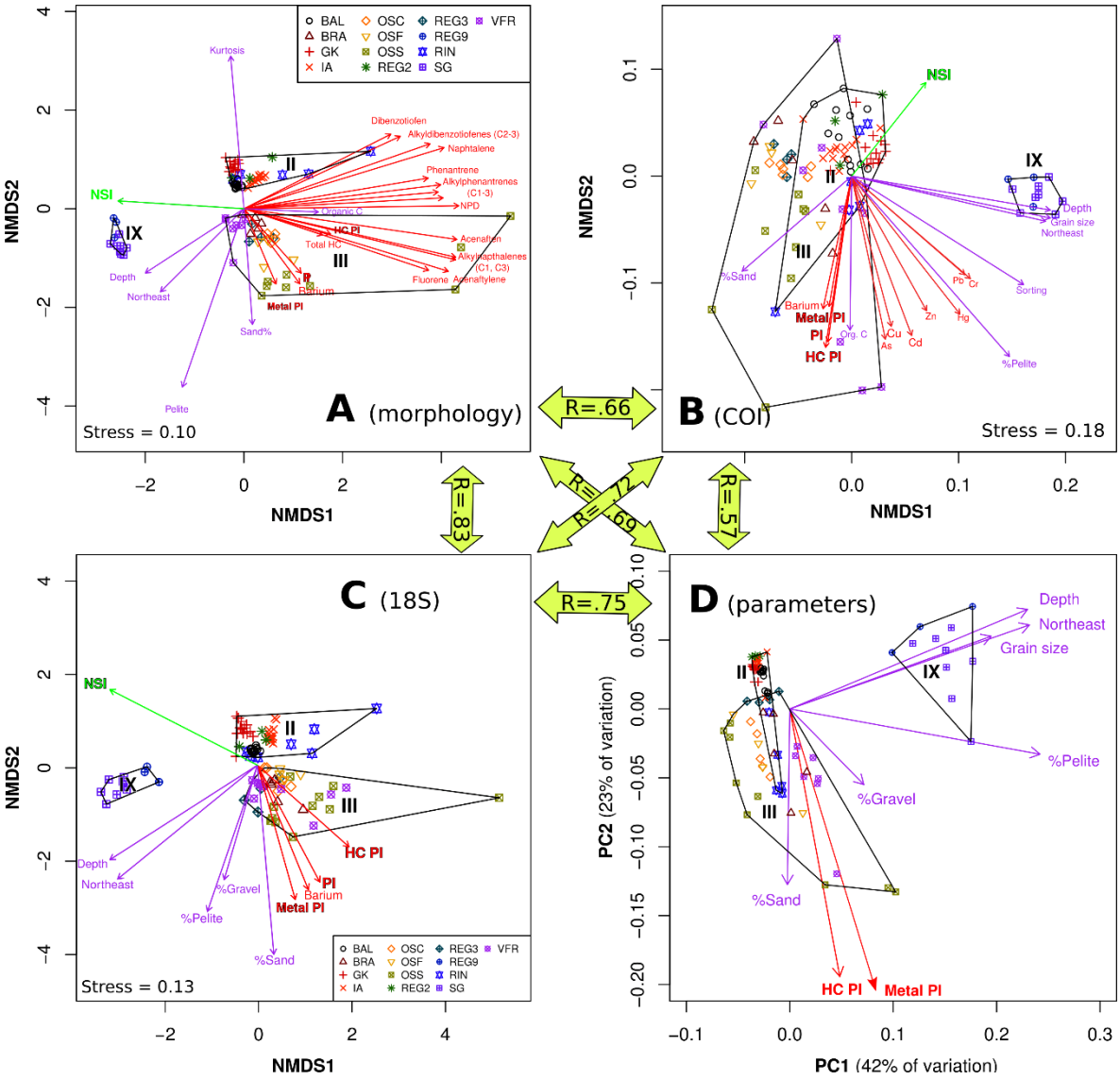


Figure 11. Correlation (Mantel tests, shown as arrows) between the beta diversity of the different datasets (NMDS plots) and to abiotic parameters (PCA plot).

3.1.4. NSI and *de novo* indices

The NSI index (Rygg & Norling 2013) was calculated for the morphological dataset and the parts of the COI dataset that could be identified to a sufficient level. The existing Norwegian NSI classification (Direktoratsguppen vanndirektivet 2018) was overlaid on scatterplots to investigate correlation in assigned classification between COI and morphological NSI values, PI and COI NSI values, and PI and morphological NSI values (Fig. 12).

- Around 52% of COI OTUs and 5% of 18S OTUs could be identified to genus level or lower.
- NSI values based on COI and morphology were correlated, both COI and morphology NSI values had moderate correlation to PI, but COI NSI values had additional outliers.

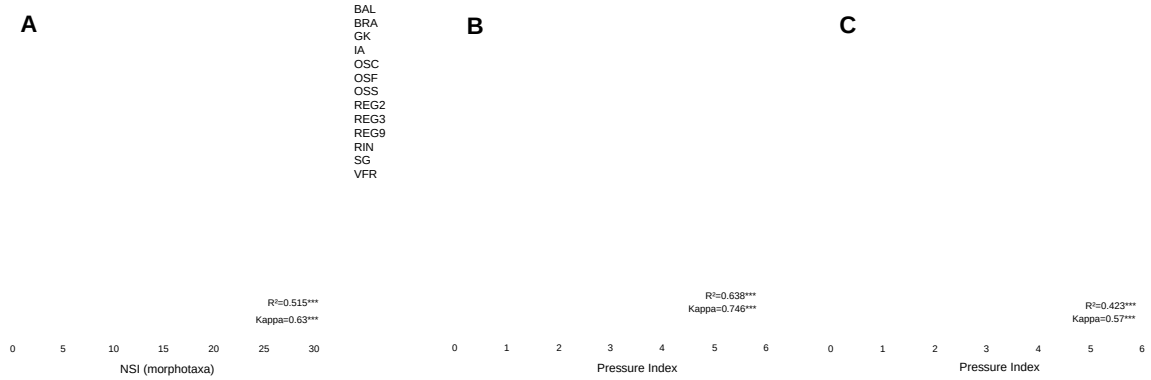


Figure 12. The correlation between (A) COI and morphology NSI values, (B) morphology NSI values and the pressure index (PI), and (C) COI NSI values and the PI.

The *de novo* indices were made with TITAN2 v2.1. This approach examines the abundance of each taxon (here OTU) along a gradient of an environmental parameter (here the PI). Significant positive correlation between OTU abundance and impacted sites suggests tolerance to impact, while negative correlation suggests sensitive species/OTUs. OTUs given a significant score using TITAN2 ($p<0.01$) were selected for additional analysis using quantile regression spines (QRS) and assigned an eco-group value (eco-group: classification of degree of sensitivity according to a value of 1-5, analogous to AMBI). This approach provides a list of bioindicator OTUs/taxa with defined eco-group values that forms the basis of the *de novo* index. Finally, by running iterations where different sets of the stations and installations were excluded, this *de novo* index could be cross validated to investigate the resilience of the index.

A separate *de novo* index was made for the 18S and COI data, containing 49 taxa for the new COI index and 118 taxa for the 18S index. The *de novo* index values were then correlated to PI values (Fig. 12):

- The COI *de novo* index performed slightly better than COI-based NSI values in its ability to predict PI values.
- The 18S *de novo* index, however, performed somewhat worse than COI and morphology, despite the better consistency of 18S data with both morphotaxonomy and physicochemical parameters, as revealed by Mantel tests.

3.1.5. Co-occurrence networks

Co-occurrence network analysis is a way of analyzing the correlations of specific taxa within the dataset and can thus be used to investigate ecological connections between the members of the communities within. Here, a subset of the full dataset, consisting of four installations with high impact (40-42% impacted stations, OSS, RIN, OSF, VFR), was used and split into a non-impacted and impacted dataset for comparison.

The majority of the 18S taxa identified as potential bioindicator using the above approach (TITAN2 followed by QRS), appeared in the 18S network as nodes, but only a third of COI taxa included in the *de novo* COI index appeared in the COI network as nodes. In the 18S network of the impacted stations, the taxa with highest connectivity tended to be taxa with low sensitivity scores (high tolerance), while the opposite was true in the non-impacted network. There was little overlap in potential keystone species between the impacted and non-impacted 18S networks. In terms of network topology, the number of positive correlations were higher than negative correlations for the 18S non-impacted network, but the opposite trend was apparent in COI. There was higher overlap in taxa composition between the impacted and non-impacted networks in the 18S data (80%) than in COI (54%) (Fig. 13).

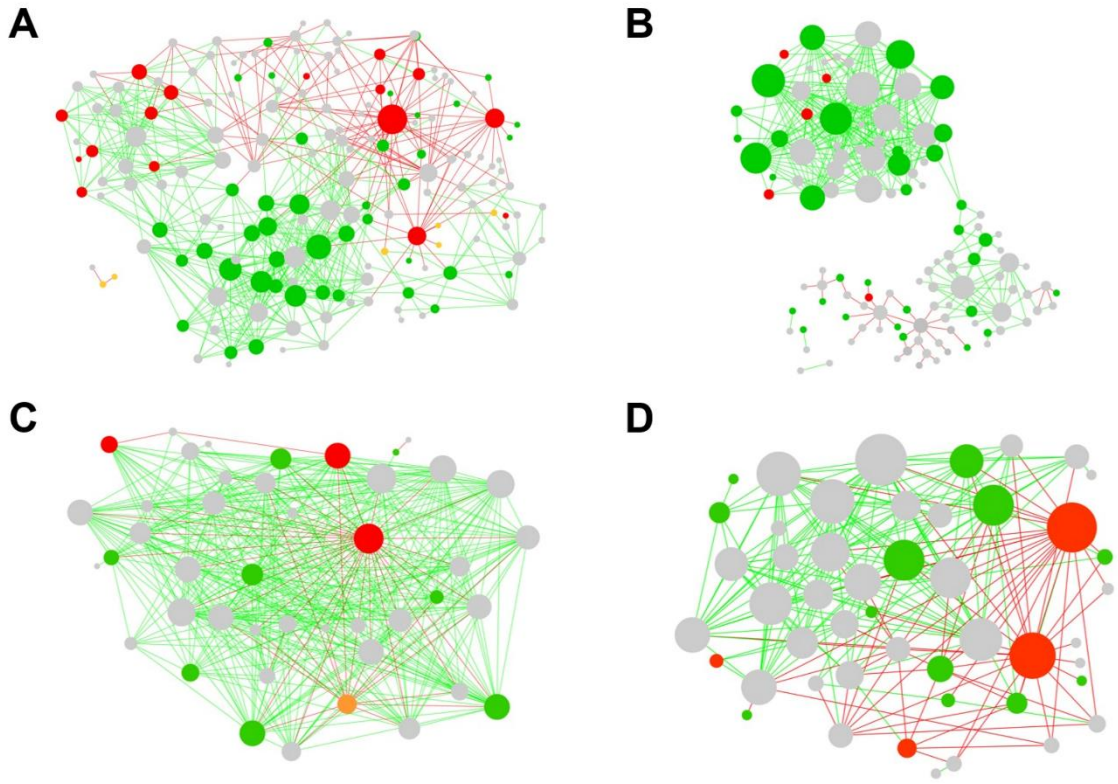


Figure 13. (A) Impacted 18S network, (B) non-impacted 18S network, (C) impacted COI network, (D) non-impacted COI network. The node colors show the eco group of OTUs in the de novo indices (I-II, green indicators of non-impact; III-V, red indicators of impact; grey, not part of the indices) based on the TITAN/QRS analysis above.

The networks showed clear differences between impacted and non-impacted sites, but results proved ambiguous, rendering data interpretation difficult.

3.1.6. Study conclusions

The main conclusions of the study are that metabarcoding data can indeed be used to detect environmental impact. Even for a relatively modest total dataset as seen here (97 stations) the patterns in the community composition as examined using 18S and COI data, could both be used to create new indices that perform at approximately the same level as the currently used NSI biotic index in terms of correlation to direct impact parameters (i.e., THC, PAH, Ba, Cu), as represented by the pressure index (PI) developed here.

In the case of the NSI, COI had a performance approaching the morphological dataset: COI and morphological NSI values correlated strongly with each other, and with the PI. On the other hand, the lower taxonomic resolution and lower sequence database coverage of the 18S marker meant that much fewer OTUs from the metazoan part of the 18S data could be identified to a genus or species level, and thus this marker did not support the calculation of NSI.

While this study provided a promising example of how *de novo* indices based on metabarcoding data are feasible for future environmental monitoring, the results here do not represent an improvement in impact assessment accuracy compared to existing methodology. An attempt was also made to use supervised machine learning (SML) on the metabarcoding data, but this did not produce significant results.

The quality of any new biotic index is dependent on the dataset that is used to create it. Thus, increasing the number of stations that are included in the dataset will help refine the inclusion and eco-group assignment of indicator species in any *de novo* index. This is especially relevant as the number of non-impacted sites is high, and many of the impacted stations are severely contaminated, skewing the dataset. An increased number of mid-level impacted stations is thus important to increase index quality. Other measures, such as a more sophisticated approach to remove pelagic sequences from the metabarcoding data, can also be expected to increase index accuracy.

4. WP1 – Quantitative indicator assays

While methods such as metabarcoding use markers with "universal" primers that are designed to include large groups of organisms, taxon-specific PCR primers make it possible to narrow down the range of organisms that are amplified to specific groups or species. Thus, it can be used to selectively identify specific taxa present in a given sample. In the context of eDNA, this approach is used together with methods that quantify the number of sequences that are present in the sample, including platforms such as quantitative PCR (qPCR), digital PCR (dPCR) or digital droplet PCR (ddPCR). While metabarcoding, due to amplification bias with regards to the variation of sequences that are present, does not produce quantitative data, such single organism assays can produce quantitative and sensitive results that enables both detection and quantification of organisms of interest from even extremely small amounts of trace DNA. Such organisms can for instance be sensitive or vulnerable species, such as cold-water corals (Kutti et al. 2020), invasive species (Crane et al. 2021), or tolerant indicator species for environmental impact.

4.1. Indicator taxon choice and assay design

As part of the MetaMon Work Package 1 metabarcoding study, a number of potential indicator taxa (118 18S OTUs and 47 COI OTUs) were identified based on their positive or negative correlation to a gradient of environmental impact. Based on these results, two indicator species (one for each of the markers 18S and COI) were chosen, based on high correlation to impact, for a ddPCR proof-of-concept study to demonstrate quantification of individual organisms using this method. From the 18S dataset, an OTU belonging to Haplosporida (SWARM 347) with strong negative correlation to impact (i.e., sensitive); from the COI dataset, the annelid *Capitella capitata* (SWARM 126) with a strong positive correlation to impact (i.e., tolerant). The name "*Capitella capitata"* used here refers to a group of sibling species that are not well defined morphologically (i.e., a species complex), but resolving this taxonomical issue is not within the scope of this project.

Like other PCR-based methods, a ddPCR assay consists of a primer pair to amplify the correct sequence. As such, the first step involves identifying new primers from the sequence of the chosen metabarcoding OTUs that selectively amplify only these OTUs and no other OTUs in the source sample. To increase the assay specificity a species-specific probe that sit within the amplified fragment can be used.

The sequences for the Haplosporida and *Capitella capitata* OTUs were used to conduct BLAST searches that identified similar sequences from the GenBank database. The 100 most similar sequences were downloaded and aligned using Geneious 6.14 (Fig 14).

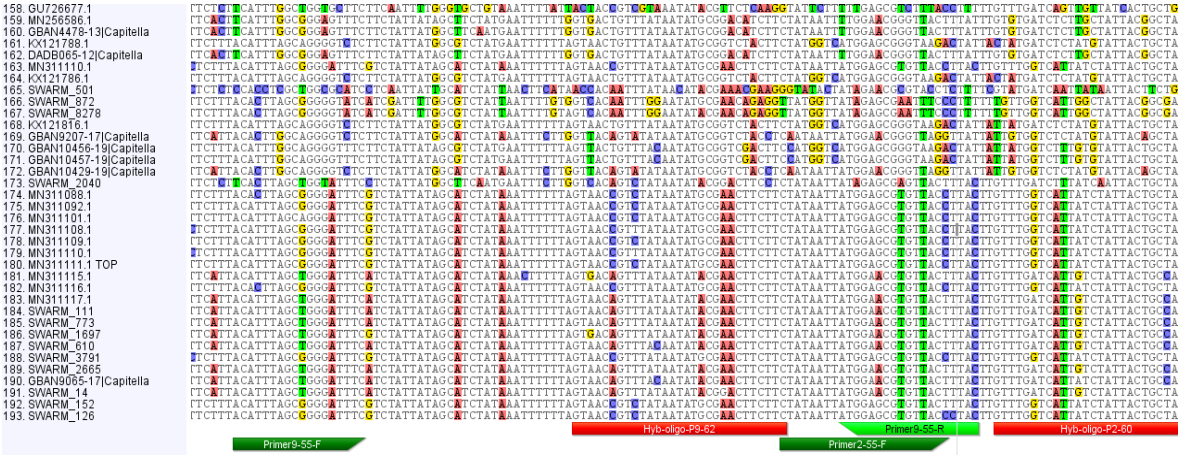


Figure 14. Alignment and manual examination of identified potential primer sites.

The web services primer-BLAST and Primer3 were used to find potential primer sites for the Haplosporida and *C. capitata* sequences, and potential primers were checked against the alignment to make sure they selectively only amplified these OTUs. For *C. capitata*, the decision was made to also design primers so that they would amplify sequences in the alignment that corresponded to a cluster of closely related *C. capitata* specimens collected from the Norwegian coast in a previous study. Two different primer pairs were identified for the haplosporidian and three for *C. capitata*, in addition to one specific probe for each. As the initial choice, we decided to test assays using primer pairs that by themselves had the required degree of specificity together with EvaGreen dye, with a list of alternatives if this approach failed (Table 2).

Table 2. A list of assays developed for ddPCR quantification of Haplosporida (left) and Capitella capitata *(right) and test strategies.*

Haplosporida assays			***Capitella capitata* assays**		
A: Specific SWARM 347 primers			**A: Specific SWARM 126 primers**		
	Name	**Sequence (5'->3')**		**Name**	**Sequence (5'->3')**
Forward	S347-1-18S-F	CGATTTGTACTATGAAACTGCA	Forward	S126-COI-F	GCGGGGATTTCGTCTATTAT
Reverse	S347-1-18S-R	CTTCCTTACAGTCGAGATTTTG	Reverse	S126-COI-R	CAGCAGCTAAAACAGGTAGA
Product length		129	Product length		157
Probe	S347-1-18S-hyb	TGATGGCTAACCTGTTACACGGAT	**B: General *Capitella* primers with 126-specific SWARM probe**		
B: SWARM 347 plus two closets GenBank matches				**Name**	**Sequence (5'->3')**
	Name	**Sequence (5'->3')**	Forward	Cap-1-COI-F	ACTTCTTCTATAATTATGGAGCGT
Forward	S347-2-18S-F	ATGGCTAACCTGTTACACG	Reverse	Cap-1-COI-R	TAATACCAGCAGCTAAAACAGG
Reverse	S347-2-18S-R	GAATCACCAACAAGAATGATG	Product length		81
Product length		81	Probe	S126-1-COI-hyb	CCTACTTGTTTGGTCATTATCTATTACTGCT
Test strategy:			**C: General *Capitella* primer**		
1. Test primer pair A				**Name**	**Sequence (5'->3')**
2. Test primer pair B			Forward	Cap-2-COI-F	CCTGTTTTAGCTGCTGGTATTA
3. Test primer pair A with probe			Reverse	Cap-2-COI-R	TAAGTGTTGATAAAGAACAGGATC
			Product length		105
			Test strategy:		
			1. Test primer pair B		
			2. Test primer pair C		
			3. Test primer pair B pair with probe		
			4. Test primer pair A		

4.2. Samples

To test the designed 18S haplosporidian and COI *C. capitata* assays, we chose a selection of installations with known contamination for certain stations: Veslefrikk (VFR, 10 stations, 30 grabs), Oseberg Sør (OSS, 9 stations, 26 grabs) and Ringhorne (RIN, 7 stations, 17 grabs) in addition to their associated regional baseline stations (REG3-13, 14, 15 and 19). Extracts were pooled at grab level, for a total of 84 North Sea samples.

As *Capitella capitata* is widely known to be a common indicator species for aquaculture eutrophication, we decided to also include some unrelated aquaculture samples for the *C. capitata* assays only: two stations from one Norwegian and five stations from two Scottish aquaculture localities; three replicates from each station.

Haplosporida sp. assay

Haplosporida is an order that includes many parasites of metazoans, but the exact affinity of the selected OTU, 18S SWARM 347, is unknown. The OTU is highly correlated to unimpacted sites but present in only low quantities in the metabarcoding dataset. Initial testing found that 2 µl template did not produce results for either primer pair (S347-1 and S347-2), so 6 µl template was used for the following assays, focusing on the first primer pair assay, S347-1. The assay produced a weak, but

clear signal for most stations, except impacted OSS and RIN stations (Fig. 15). While the results validated the approach, the low general abundance of this OTU makes the signal weaker than ideal. Thus, this approach should be repeated with a larger dataset, to identify more abundant indicator organisms.

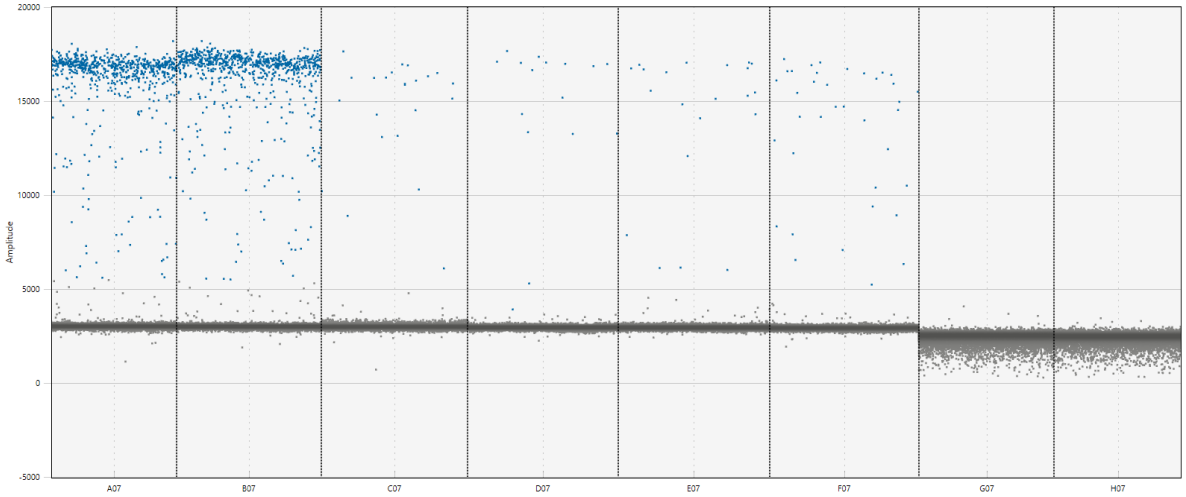


Figure 15. An example of eight samples showing moderate weak and no signal (blue dots) for the haplosporidian S347-1 assay.

***Capitella capitata* assay**

We started by testing the two identified assays that both targeted the *Capitella* OTU SWARM 126 as well as other closely related *Capitella* sequences in the metabarcoding data and on GenBank (assays Cap1 (assay B) and Cap2 (assay C)). Temperature gradient tests were done to find optimal annealing temperature, using an initial concentration of 2 µl template. For Cap1, 55.6°C was found to be an optimal annealing temperature, for Cap2, 59.3°C. The 2 µl template concentration worked well and was retained for subsequent ddPCR amplification runs.

The 84 North Sea samples were run with three ddPCR run replicates each for a total of 252 ddPCR reactions. Both assays worked well and identified stations where *Capitella* species had been identified in the metabarcoding dataset (Fig. 16).

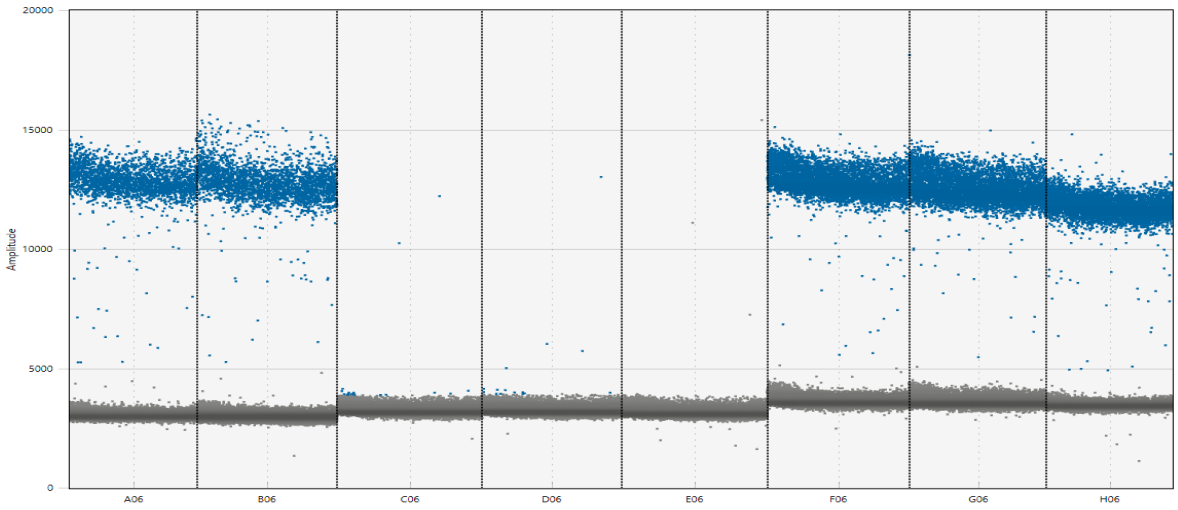


Figure 16. An example of eight samples showing strong signal (blue dots) for the Cap1 assay for impacted stations.

The assays were able to detect the presence of *Capitella capitata* sequence variants at the same stations where *Capitella* sequences were found in the metabarcoding data, with a few discrepancies (Fig. 17). These discrepancies in the results can be attributed to the non-quantitative nature of the metabarcoding process, especially for station OSS11B1: In this severely contaminated station, many sequence reads were recovered for *Capitella* in the metabarcoding data. However, a likely explanation for this result is that there were few other organisms available in the sample, meaning that the standard metabarcoding PCR amplification overestimate abundance of the little remaining DNA in the sample: a powerful demonstration of the limits of using metabarcoding abundance as a direct measure.

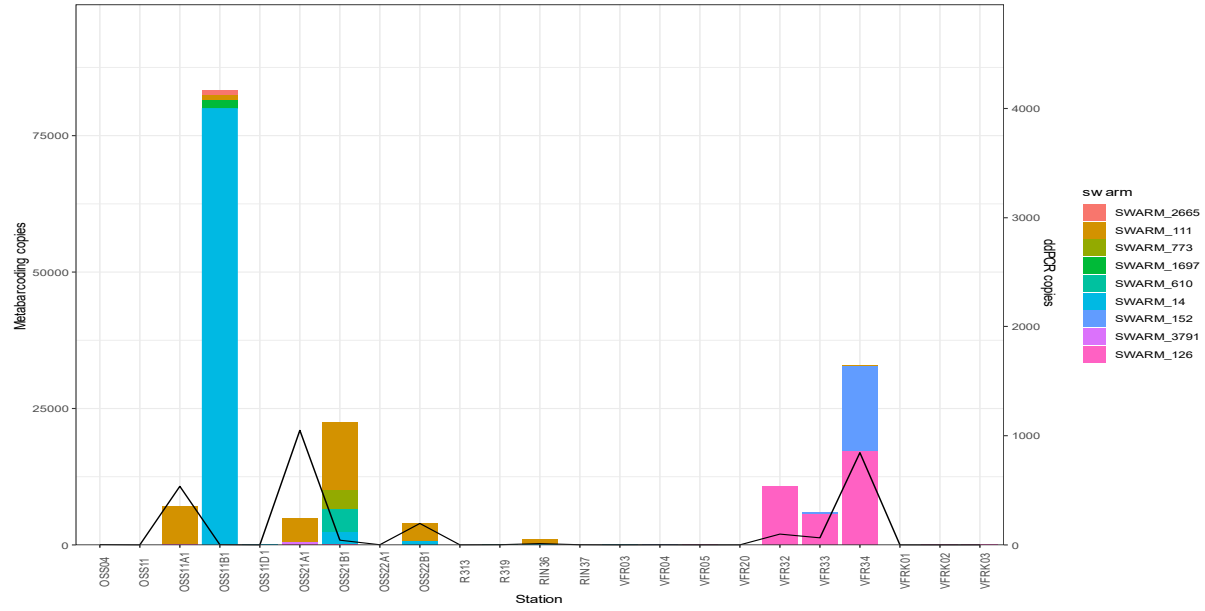


Figure 17. A comparison of signal strength for Capitella *OTUs in the metabarcoding data and number of copies detected through ddPCR for the Cap-1 primer pair. The number of sequence reads in the metabarcoding data is given as color coded bar charts, while the number of copies detected in the*

ddPCR assays is given as a solid line. In addition to SWARM126, the primer pair also detects other Capitella *in the dataset, given with SWARM number on the right.*

Capitella assay ddPCR results are in prep for submission to a scientific journal and will include a comparison to morphological data from the same offshore stations as for the metabarcoding and ddPCR results above, as well as inclusion of a smaller number of aquaculture samples to broaden the scope and applicability of the assay.

4.3. Main quantitative assay conclusions

Choosing which organisms were suitable as impact indicators required correlation of abundance for individual OTUs in the metabarcoding datasets with abiotic environmental parameters. For MetaMon, this was done as part of the approach to define the 18S and COI *de novo* indices, using TITAN2 and quantile regression splines. Using this method, a handful of potential bioindicator species were identified. However, this correlation was often noisy, and with relatively few datapoints (i.e., stations present) per OTU. While such noise is less important for eco group assignments in creating a *de novo* index, since noise in individual scores gets averaged over a larger number of OTUs, it provided a challenge when trying to identify optimal OTUs for individual indicator assays. For this reason, we recommend that the process is repeated for a larger dataset to more firmly establish OTU and impact correlation and include an abundance threshold as well. This last recommendation is since the haplosporidian, while correlating well with stations with no impact, was present also here in only minute quantities, making detection (i.e., rule out false negative results) more difficult than for an assay based on a more abundant organism.

5. WP3 – Metabarcoding for macrofaunal identification

5.1. Taxonomic database gap analysis

In order to discover the taxonomical identity of sequence OTUs from metabarcoding data, such OTUs can be compared to reference sequences coupled with species names found in one of a handful of online databases, including GenBank, the Barcode of Life Database (BOLD) or SILVA. GenBank contains multiple gene markers, the main part of BOLD comprises COI sequences from metazoans, and SILVA either the prokaryote (16S) or eukaryote (18S) ribosomal small subunit gene. Such comparison is typically referred to as taxonomical assignment. However, databases are far from complete: Depending on the similarity to closest database matches, metabarcoding OTUs can either only be identified all the way to species level, or just a higher-level group (i.e., more inclusive Linnean rank) such as family or even phylum. This partial lack of data (that gets smaller as more sequences are added to the databases) can be referred to as the database gap.

While species level identification is not feasible for microbial organisms, databases are more complete for multicellular animals due to the taxonomic and barcoding efforts over the last decades, especially for COI. Similarly, it can be expected that databases are more complete in areas where the marine fauna is better known, such as in the Northern Atlantic. Thus, an advantage of using metazoan data instead of microorganisms for monitoring is that more sequences can then be assigned to known animals living in the sediment, which allows a direct comparison to current morphological monitoring data. It also enables the use of existing indices, such as AMBI, ISI and NSI, on the parts of the metabarcoding data that can be identified to species or sometimes genus level.

For this reason, we conducted a study to estimate the percentage number of animals that could be recovered to lower taxonomical level for the COI and 18S marker genes, using GenBank for COI and 18S, BOLD for COI only, and SILVA 18S v1.28 for 18S data only. This was done through preparing a list of 1902 species (or in some cases higher level groups) reported from region IV through morphological identification, and separately for the 240 most common species reported from the same area. The databases were then queried for sequences belonging to these taxa, and results summarized in Table 3.

Worldwide, species level database coverage is poor (Kvist 2013). The North Sea stands out in a positive way: Roughly half of species reported in monitoring from this area were represented in at least one online database with COI data, with some variation between the GenBank and BOLD databases. The 18S coverage was lower, with between 36-21% coverage, and this result does not consider how large part of the whole 18S gene was present in the database. Still, the results show that additional resources need to be used to fill in existing gaps in database coverage, even for this comparatively well-studied area. For an objective that includes use of metazoan taxonomic data in metabarcoding monitoring, the most cost-efficient way of increasing database coverage is through targeted barcoding of retrieved organisms from monitoring cruises. Results from this work were published in Hestetun et al. (2020).

Table 3. Summary statistics for all species and according to phylum, showing the percentages that were present in the databases queried, for the COI and 18S marker genes respectively (from Hestetun et al. 2020).

	All groups		Annelida		Arthropoda		Mollusca		Echinodermata		Others	
	No	%	No	%	No	%	No	%	No	%	No	%
Total dataset												
Number of taxa	1802		620		483		451		88		157	
COI												
GenBank COI hits	1018	56.5%	303	48.9%	283	58.6%	247	54.8%	70	79.5%	115	73.2%
18S												
GenBank 18S hits	820	45.5%	299	48.2%	183	37.9%	178	39.5%	48	54.5%	112	71.3%
SILVA 18S hits	526	29.2%	240	38.7%	69	14.3%	115	25.5%	27	30.7%	33	21.0%
Species level												
Number at species level	1474		536		381		399		74		84	
COI												
GenBank COI hits	743	50.4%	241	45.0%	190	49.9%	204	51.1%	57	77.0%	51	60.7%
BOLD public COI hits	625	42.4%	180	33.6%	166	43.6%	200	50.1%	36	48.6%	43	51.2%
BOLD record hits	1034	70.1%	378	70.5%	279	73.2%	256	64.2%	65	87.8%	56	66.7%
COI GenBank only	165	11.2%	76	14.2%	34	8.9%	20	5.0%	22	29.7%	13	15.5%
COI BOLD public only	47	3.2%	15	2.8%	10	2.6%	16	4.0%	1	1.4%	5	6.0%
18S												
GenBank 18S hits	537	36.4%	233	43.5%	95	24.9%	132	33.1%	35	47.3%	42	50.0%
SILVA 18S hits	400	27.1%	207	38.6%	48	12.6%	96	24.1%	21	28.4%	28	33.3%
Genus level												
Number of genera	947		292		252		254		54		95	
COI												
GenBank COI hits	656	69.3%	222	76.0%	145	57.5%	168	66.1%	38	70.4%	83	87.4%
BOLD record hits	848	89.5%	265	90.8%	222	88.1%	220	86.6%	52	96.3%	89	93.7%
18S												
GenBank 18S hits	736	77.7%	216	74.0%	195	77.4%	191	75.2%	51	94.4%	83	87.4%
SILVA 18S hits	532	56.2%	206	70.5%	88	34.9%	138	54.3%	29	53.7%	71	74.7%

5.2. Targeted barcoding

As part of work package 3, macrofaunal animals from a subset of stations from the region III deep area ("Troll" area) were sieved in a regular fashion using 1 mm sieves as part of the regular monitoring cruise in 2019. Rather than the standard formalin fixation, these were fixed in 96% ethanol for further molecular analysis.

From these samples, macrofaunal species were retrieved from remaining sediment and identified to lowest possible taxonomic level by MERG in collaboration with the University of Bergen Natural History Museum. Species lacking barcodes or with only partial sequence coverage in the databases, confirmed using a similar method as in the previous section 5.1, were selected as candidates for a targeted barcode program, to improve the taxonomical performance of future metabarcoding studies from the Norwegian Shelf.

For the targeted barcoding, 96 specimens, representing 46 separate polychaete, mollusk and crustacean species, were sequenced separately using Sanger sequencing. The gene markers COI, 18S and 28S were selected for sequencing using the primers sets in Table 4. Note that for COI, several primer sets were tested, including degenerated universal Folmer primers (jgLCO and jgHCO) and group-specific primers for the polychaetes and mollusks.

Table 4. Primers used for the targeted barcoding study, comprising the markers 28S, 18S and COI.

Name	Marker		Sequence	Source
28S5F	28S	Forward	5'-CAAGTACCGTGAGGGAAAGTTG-3'	(Passamaneck et al. 2004)
PO28R4	28S	Reverse	5'-GTTCACCATCTTGGGGTCCCAAC-3'	(Struck et al. 2006)
HCO2198mollusk	COI	Reverse	5'-TATACTTCTGGATGACCAAAAAATCA-3'	(Jaksch et al. 2016)
jgLCO1490	COI	Forward	5'-TITCIACIAAYCAYAARGAYATTGG-3'	(Geller et al. 2013)
jgHCO2198	COI	Reverse	5'-TAIACYTCIGGRTGICCRAARAAYCA-3'	(Geller et al. 2013)
PolyLCO	COI	Forward	5'-GAYTATWTTCAACAAATCATAAAGATATTGG-3'	(Carr et al. 2011)
PolyHCO	COI	Reverse	5'-TAMACTTCWGGGTGACCAAARAATCA-3'	(Carr et al. 2011)
F-40	18S	Forward	5'-AAGATTAAGCCATGCATG-3'	(Hadziavdic et al. 2014)
R-1196	18S	Reverse	5'-TGTTGAGTCAAATTAAGC-3'	(Hadziavdic et al. 2014)

Table 5. Species where barcode sequences for the 28S, COI and 18S markers were successfully sequenced for at least one marker gene. Note that detailed analysis of sequence quality is not finished.

Species	18S	COI	28S	Species	18S	COI	28S
Antalis occidentalis	■	■	■	*Aponuphis bilineata*	■		■
Dacrydium ockelmanni			■	*Artacama proboscoidea*	■		■
Dacrydium vitreum	■		■	*Dasybranchus caducus*	■		■
Leptaxinus minutus	■	■	■	*Lanice conchylega*	■		■
Pulsellum lofotense	■		■	*Maldane koreni*	■		■
Limopsis cristata	■	■	■	*Marphysa belli*	■		■
Similipecten similis	■		■	*Octobranchus floriceps*	■	■	■
Cuspidaria lamellosa		■	■	*Octobranchus sikorskii*	■	■	■
Calathura norvegica		■		*Paradiopatra fjordica*	■		■
Haploops carinata	■	■		*Placostegus tridentatus*		■	
Haploops robusta	■	■		*Pseudopolydora pulchra*	■		■
Ilyarachna longicornis		■	■	*Myriochele heeri*		■	
Ilyarachna longicornis	■		■	*Macandrevia cranium*	■		
Laetmatophilus armatus	■	■		*Ilyarachna longicornis*	■	■	■
Leucon nasicoides	■	■		*Ischnomesus bispinosus*	■		■
Lysianassa costae	■	■	■	*Abra longicallus*	■		■
Philomedes globosus	■		■	*Similipecten greenlandicus*	■		■
Vargula norvegica	■	■	■	*Kelliella miliaris*	■		■
Nototropis vedlomensis	■	■		*Unciola planipes*	■	■	
Amage auricula	■			*Leptocheirus hirsutimanus*	■		

Preliminary results include 18S sequences for 34 of the 46 original species, 28S sequences for 28 species, and COI sequences for 19 species (Table 5). A final sequencing attempt currently for COI not yet analyzed may raise the number of COI sequences, and sequences will be uploaded to GenBank when these results are ready.

The success rate for the three markers is around average for a barcoding project of this type. COI in particular is tricky for a wide range of organisms due to its nature as a protein-coding gene, which is why several primer pairs were attempted for this marker in an iterative approach.

5.3. Community bulk sample analysis

Several studies (e.g. Aylagas et al. 2016b; Creer et al. 2010) have shown the promise of using metabarcoding extracts from bulk homogenate samples (i.e., collecting all animals in the sample, and blending them together into a paste) as an alternative metabarcoding approach to sediment samples for metazoans. This allows sampling a much larger volume from e.g., a grab or corer, either through sieving or elutriation, thus increasing the representativeness of the sample.

Table 6. The number of specimens and species identified from morphological taxonomy from the TRB, DUV and HUL installations. These specimens were homogenized into a paste representing each installation for subsequent DNA extraction and metabarcoding sequencing.

No. species (morphology)		**No. specimens**	
Total species	152	Total specimens	737
TRB species	78	TRB specimens	271
DUV species	61	DUV specimens	210
HUL species	63	HUL specimens	256

For the community bulk sample analysis, our aim was to compare the species composition of the sieved grab samples using morphology, COI and 18S metabarcoding data in terms of taxon composition and abundance, to see to which degree metabarcoding data reflected the underlying species composition as revealed through morphological identification.

Sieved >1 mm macrofauna from the Troll B, (TRB) Duva (DUV) and Huldra (HUL) installations was identified using morphological taxonomy to lowest possible level as part of the targeted barcoding approach in section 5.2 above. These animals were pooled to make homogenates with a known morphological species composition (Table 6): The three bulk samples were homogenized for 2 x 30 s using a Qiagen TissueRuptor. Five extraction replicate wet weight subsamples were removed and dried, with dry weight ranging from 6-8 mg (Troll B), 10-12 mg (Duva), and 3-5 mg (Huldra). ATL buffer was added to samples, and they were lysed with proteinase K overnight. Subsequent DNA extraction was done using the Qiagen Blood and Tissue Kit with the QIAsymphony extraction robot.

The extraction replicates from each homogenate sample were amplified and the 18S V1-V2 and COI marker genes sequenced using the same protocol as in WP1. The relative abundance of the major taxon groups is shown in Figure 18. In the morphological dataset, most specimens were polychaetes at all three areas, with almost all remaining abundance shared between mollusks and crustaceans. This phylum level abundance is mostly reflected in the metabarcoding data: Polychaetes retained the majority of reads in all cases except the Duva COI data. The relative abundance of lower taxa is

not representative of morphological abundance, however. COI data was typically dominated by individual taxa with low to moderate abundance in the morphological data, such as *Paradiopatra* for Troll B, an unidentified annelid at Huldra, and the isopod *Natatolana borealis* (consisting of only two morphological specimens) at Duva. But discounting these outliers, other abundances were more equally distributed. Relative abundances in the 18S data were less, but still significantly, dominated by certain taxa, most clearly visible for Huldra, where an unknown polychaeta represented 61% of all reads.

Apart from the *N. borealis* outlier, crustacean abundance was severely underrepresented and had low resolution in both the COI and 18S data. Most sequences belonged to either unidentified arthropods or amphipods. Finally, mollusks were comparably well represented in the COI data, while mollusk 18S resolution was low, however. A majority of 18S mollusk reads were assigned to unidentified Veneroida, a superseded name for the current bivalve order Venerida. While some of these reads could be *Kelliella miliaris*, it is likely that these reads also include a range of related taxa such as Cardiida and thyasirids.

While the major findings from this work are ready, the bioinformatic pipeline needs to be revised in light of recent research on optimal bioinformatic processing of metazoans (Antich et al. 2021; Brandt et al. 2021). Thus recommendations, together with final results from the targeted barcoding, are planned for publication using the pipeline currently in development for the follow-up project MetaBridge.

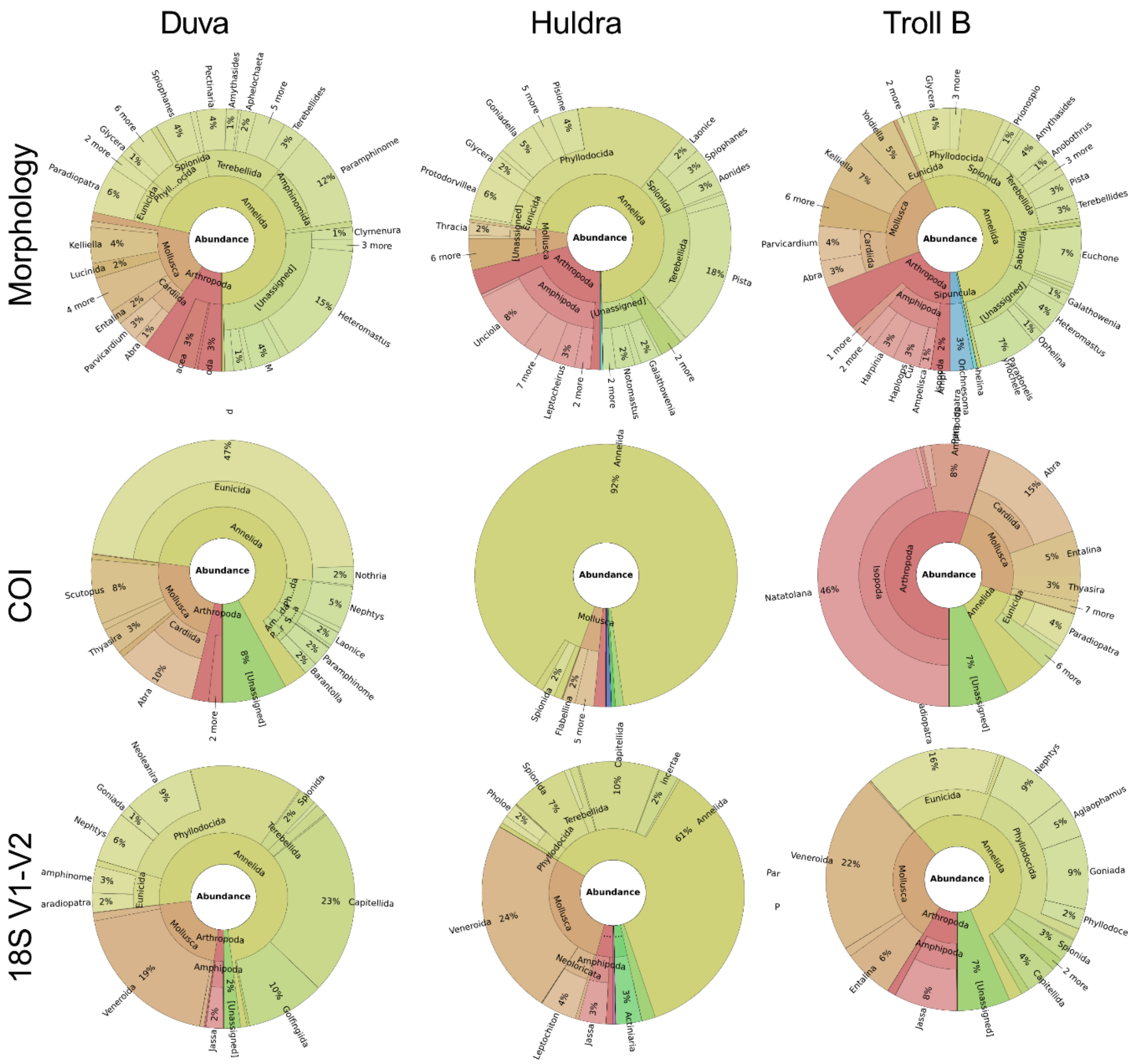


Figure 18. Preliminary Krona charts of the relative abundance of major taxa for Troll B, Huldra and Duva in the morphological, COI and 18S dataset respectively. Note that the analysis is scheduled to be redone with an updated bioinformatic pipeline.

6. Project findings and future directions

MetaMon was designed to address knowledge gaps towards using eDNA for marine benthic monitoring, including methodology, metabarcoding performance relative to morphological taxonomy, and the identification and analyses of macrofauna barcode data from online databases. As such, the project aimed to identify best practices for the use of eDNA in marine benthic monitoring, using both project results in themselves, but also drawing on evidence from the increased amount of other recent eDNA studies.

6.1. Methodology

The choices made for sampling design and processing of samples influence the final data. Thus, it is important to attest the influence of methodological factors to develop a protocol. This is especially true for monitoring purposes, as major changes to the protocol could make it difficult to compare time series of data from monitoring stations. Prior to MetaMon, Lanzén et al. (2017) and Lekang et al. (2015) already laid some of the groundwork towards an optimized protocol. MetaMon aimed to close additional knowledge gaps in the methodology that had not been previously investigated.

MetaMon aimed to investigate the optimal trade-off between cost-effectiveness and capturing as much of the present biodiversity as possible. Specifically, uneven distribution of organisms in the source sediment is a potential concern due to the comparatively low volumes of sediment analyzed in metabarcoding studies. While complete sampling of all organisms that are present at a sampling station is unfeasible (a reality for any biological sampling), sampling must be comprehensive enough to capture enough of the organisms present to give a consistent and representative view of the environmental conditions at the site.

Sampling comprehensiveness can be increased both by making additional DNA extracts per sediment sample, or by increasing the amount of source sediment for the extraction. Lanzén et al. (2017) showed that increasing the number of DNA replicates per sediment sample increased the number of captured organisms in the data. Smaller volume sediment extracts can also be incorporated into a semi-automatic processing protocol. Thus, we hypothesized that the most cost-effective approach was to increase the number of DNA replicates, rather than increasing sediment volume. As part of the experiments done to test this hypothesis, we also compared different intensity homogenization of samples during extraction, a previously unexplored aspect.

Another important consideration is the choice of molecular marker. Here, we chose to use two markers targeting the small subunit ribosomal RNA (SSU rRNA) gene and the mitochondrial cytochrome c oxidase subunit 1 (COI). The choice of markers was made after an extensive literature survey, in order to identify commonly used markers that would provide an insight into both a broad range of eukaryote organisms, including single-celled groups (18S), and animals, particularly macrofauna, specifically (COI). Since we expected that the distribution of the larger, multicellular, organisms would be more uneven than single-celled organisms, these two markers would also provide a contrasting view towards the amount of sampling needed for sufficient organism coverage.

The MetaMon sampling design was chosen in order to maximize the number of organisms in the source sediment from each station, even with a relatively modest amount of effort: In order to

increase the number of organisms present in the sediment samples, sediment samples from each grab were collected from different portions of the grab surface, pooled together and stirred before DNA extraction. Sediment was preserved by freezing at -20°C onboard the ship. Three grab replicates were made per station.

MetaMon findings showed that a DNA extract replicate strategy using semi-automated extraction, combined with a moderate intensity homogenizer program and pooling extract DNA prior to PCR amplification, seemed to provide an optimal strategy to minimize time and cost of these processing steps (Hestetun et al. 2021b). Nine extract replicates (three from each grab pooled sediment sample) were made from each station, which were again pooled into three PCR amplification reactions for each molecular marker. Additional tests were made examining the variability of the organism composition at different scales including from different areas of the same grab sample and between different grab samples from the same station (Hestetun et al. 2021a).

The MetaMon methodological results demonstrated the ability of the protocol to capture most organisms using a feasible number of DNA extract replicates using 18S and thus validated the protocol that was then used for the main MetaMon metabarcoding study. The results also showed that metazoans had a more uneven distribution of DNA in the sediment, which increased variability between samples for COI and the metazoan sequence fraction in the 18S data. While COI has been used in several metabarcoding studies using a similar approach as in MetaMon, e.g., extraction of DNA directly from the sediment, it is more commonly used on concentrated bulk tissue from sieved sediment fractions, a more time and work intensive approach.

- The methodological experiments provided additional data used to decide and evaluate the protocol developed for MetaMon.
- The currently developed protocol produces consistent results for single-celled organisms and provides a good trade-off between processing cost and time and the quality of the resultant data.
- For larger organisms, a DNA replicate strategy still provides the same or better increase in consistency compared to increasing the amount of sediment, and limits the ability of single, large animals to totally dominate read abundance numbers in the resultant data.
- Still, there is obviously more variability (“noise”) in metazoan organisms compared to single-celled organisms due to uneven distribution, which is difficult to mitigate using only sediment extracted DNA.
- The increased noise in the COI data does not preclude the use of this marker using a direct sediment extraction protocol such as the MetaMon protocol, as the COI data still seems to be able to predict environmental impact (see next section).
- The consistency of COI results increases when using indices such as NSI or AMBI, since these indices use mostly common organisms in the data, which are also more consistently detected in the COI data (as opposed to rare species).
- The possibility of developing an additional protocol for metazoans, that could be for instance considered as an alternative for a subset of monitoring stations, should be explored.

6.2. Comparative performance of metabarcoding and morphology

Using the protocol that was established as part of the MetaMon methodological experiments, we processed and sequenced 18S and COI data using sediment samples from 97 stations from regions II, III and IX for the main MetaMon metabarcoding study. The stations were chosen to include installations where environmental impact had been detected in previous monitoring surveys. The aim of this study was to compare the ability of the metabarcoding data to detect environmental impact with the existing methodology based on morphological taxonomy. As the stations in the metabarcoding study are also part of the regular monitoring surveys, the study was designed to allow direct comparison of metabarcoding data with both the morphological data, and abiotic parameters of environmental impact including hydrocarbon and metal concentrations.

The study design included A) creating an aggregated environmental pressure index (PI) comprising selected hydrocarbon and metal parameters, B) testing this PI through correlation to the currently used biotic NSI index, C) using the correlation between PI values and individual OTUs in the metabarcoding datasets to identify sensitive and tolerant OTUs, D) classifying the OTUs according to eco group to produce a new (*de novo*) biotic index for each of the two markers (18S and COI) and testing these indices, and E) examine correlations between individual OTUs using a co-occurrence network approach.

The main findings from this study were that the newly developed 18S and COI indices could predict environmental impact at roughly the same level as the existing NSI morphological taxonomy index, with some outliers, however. An alternative supervised machine learning approach did not yield significant results. The co-occurrence approach showed that the connections between individual OTUs were different in impacted vs non-impacted sites but proved difficult to interpret clearly without further context.

The *de novo* index approach is promising, but as the quality of any newly developed index is dependent on the source dataset, we recommend to further refine this approach using a larger dataset and additional markers, especially with regards to stations with medium severity impact as those were underrepresented in the MetaMon dataset.

6.3. Quantitative indicator species assays

The TITAN2/quantile regression splines strategy that examined correlation between individual OTUs and environmental impact was also used to identify potential species for quantitative indicator assays. However, we found that the MetaMon dataset was not large enough to provide unequivocal such correlation and decided to reduce the scope to two (from four) OTUs, to do a proof-of-concept study. One COI OTU, identified to be *Capitella capitata*, had clear correlation to high impact. One 18S OTU, an unidentified haplosporidian, had a clear negative correlation to high impact. Quantitative ddPCR assay alternatives were developed and tested for both. The results of the *Capitella* assay show clear promise and are currently being prepared for publication, while we found that the general low abundance of the haplosporidian makes this OTU less consistent. As with the metabarcoding study, the results clearly showed the viability of the approach used, but we would recommend increasing the amount of available data for more robust OTU impact correlation to identify indicator species.

6.4. Assessment of the archive and taxonomic gaps

Work package 3 (WP3) comprised three separate main tasks: A) an estimation of the percentage of reported region IV macrofaunal species present as COI or 18S sequences in GenBank and/or BOLD/SILVA, B) a targeted barcoding project involving specimens from region II and IX, and C) an evaluation of the use of COI for bulk samples with a known species composition from region III. We found that around 50% of species level taxa were represented with at least one COI barcode in one of the databases. For any part of 18S, corresponding number was 36.4%.

WP3 also included the targeted barcoding of specimens preserved on ethanol from the 2019 region III and IX monitoring cruises. Species from these samples were individually identified in collaboration with the Bergen university museum, and the resultant species lists were checked against the BOLD, SILVA and GenBank databases. Then, 96 specimens, representing 46 species lacking sequences, were chosen for individual Sanger sequencing so that they could be added for future metabarcoding studies. Using this targeted barcoding approach, we recovered 18S sequences for 34 of the 46 original species, 28S sequences for 28 species, and COI sequences for 19 species.

Finally, to get a better understanding of the bias introduced by the COI and 18S V1-V2 markers used in MetaMon, we performed a community experiment, extracted from a homogenized bulk tissue sample with a known morphological species composition. Preliminary comparison of the species composition of the morphological, COI and 18S datasets imply PCR biases as expected, but also a higher degree of affinity between bulk community samples and morphology compared to morphological samples and direct sediment metabarcoding samples. This is not surprising given that the community samples are sieved and hence, does not contain most of the biota that pass through a one-millimeter screen.

6.5. Visibility and outreach

MetaMon findings were communicated at a variety of venues during the project, including the International Workshops on Environmental Genomics (IWEG) annual meetings 2019-2021, the International Barcode of Life Conference (iBOL 2019), the World Conference of Marine Biodiversity (2020), the 1st DNAqua-Net International Conference, and at several smaller symposia and meetings with stakeholders and policymakers.

6.6. Conclusions and future recommendations

Studies of eDNA have massively increased in recent years. MetaMon represents a significant step forward to mature metabarcoding specifically, and eDNA generally, in Norwegian Shelf offshore monitoring. For the methodological work in MetaMon, we developed standardized sediment sampling and processing guidelines based on previous MERG project findings, scientific literature and MetaMon experiments for empirical validation of extraction and spatial replication choices. These guidelines were made to maximize the amount of the organism community recovered at a sampling location through sediment sampling design and processing. In addition, the protocol enables the use of an automated extraction robot during extraction. While MetaMon library prep was done in house, we would expect these steps to be outsourced to a core facility for future routine monitoring, further decreasing time and cost of lab processing.

We found that this design was able to provide consistent results for 18S total eukaryote metabarcoding data, but that the COI marker data were less consistent, due to more uneven distribution of macrofauna relative to microorganisms. Still, the increased noise in the COI dataset did not preclude robust analysis of this marker, in addition to 18S, for environmental impact analysis of the WP1 dataset.

- We would recommend that COI is retained in future metabarcoding monitoring, as it provides a connection to the existing macrofaunal data and can be related to known organisms at species level. We would, however, also recommend that additional testing be done for this marker on bulk samples, alternatively by pre-stirring the whole recovered sediment sample prior to extraction, to compare the resultant data consistency. For high consistency COI data, a more elaborate separate sieved bulk sample protocol could be considered as an alternative to sediment extraction and employed at a selected number of stations in future monitoring.
- As the total eukaryote dataset provided more consistent results with lower sediment volume, microorganisms are well placed for a central role in metabarcoding-based monitoring. As each marker has its own taxonomic bias, we would suggest that additional 18S markers, and prokaryote 16S, should be sequenced and their relative performance evaluated, in order to find an optimal selection of markers.
- In order to further validate the representativeness of the metabarcoding data from each station, we recommend revisiting MetaMon sampling stations in the future. Time series and comparing the changes in metabarcoding data with similar variation in the morphological data, would be an important validation of metabarcoding as a monitoring tool.
- Developing new biotic indices is a very promising approach to determine environmental impact from metabarcoding data and would need to be developed for each marker chosen in monitoring. While the MetaMon results were encouraging, more data is needed to more securely assign sensitivity values to taxa included in such indices.
- Similarly, while we have demonstrated two assays as a proof of concept in MetaMon, more robust sensitivity correlation is needed to accurately identify final indicator species for quantitative eDNA-methods.
- Supervised machine learning is a promising direction for monitoring, but the MetaMon data proved insufficient to significantly predict environmental impact using this approach.
- Input from policymakers is important in order to guide future directions in a manner compatible with regulatory requirements.

6.7. MetaBridge

The research questions examined by MetaMon have led to promising findings, but in most cases require a more substantial body of evidence in terms of available data, covering a range of environmental conditions and revisiting previous sites, to reach a maturity level that would allow regulators to consider routine implementation of eDNA in marine sediment monitoring. As the main findings from MetaMon as described above became clear, a proposal to a follow-up project was created with Equinor, Total and Lundin as industry partners and sent to the Norwegian Research Council in August 2020. This proposal, MetaBridge, was approved for funding in December 2020.

MetaBridge (2021-2024) builds directly on MetaMon findings by expanding the amount of data available to improve the foundation for developing biotic indices, individual species quantitative assays and a supervised machine learning algorithm. As in MetaMon, samples are collected as part of regular monitoring surveys, enabling comparison to currently used parameters. The project revisited MetaMon 2018 stations in 2021, and will revisit MetaMon 2019 stations in 2022, in order to validate data consistency in time. As additional deliverables, MetaBridge will also expand the number of molecular markers for both MetaMon and new MetaBridge samples, work to further refine macrofaunal methodology, work towards storage and curation solutions for physical samples and metabarcoding data and drive a closer dialogue with stakeholders and policymakers as the eDNA field matures.

7. References

Alberdi A, Aizpurua O, Gilbert MTP, and Bohmann K. 2018. Scrutinizing key steps for reliable metabarcoding of environmental samples. *Methods in Ecology and Evolution* 9:134-147. 10.1111/2041-210x.12849

Andújar C, Arribas P, Yu DW, Vogler AP, and Emerson BC. 2018. Why the COI barcode should be the community DNA metabarcode for the metazoa. *Molecular Ecology* 27:3968-3975. 10.1111/mec.14844

Antich A, Palacin C, Wangensteen OS, and Turon X. 2021. To denoise or to cluster, that is not the question: optimizing pipelines for COI metabarcoding and metaphylogeography. *BMC Bioinformatics* 22:177. 10.1186/s12859-021-04115-6

Atherton S, and Jondelius U. 2020. Biodiversity between sand grains: Meiofauna composition across southern and western Sweden assessed by metabarcoding. *Biodiversity Data Journal* 8. 10.3897/BDJ.8.e51813

Aylagas E, Borja Á, Irigoien X, and Rodríguez-Ezpeleta N. 2016a. Benchmarking DNA Metabarcoding for Biodiversity-Based Monitoring and Assessment. *Frontiers in Marine Science* 3. 10.3389/fmars.2016.00096

Aylagas E, Borja Á, Muxika I, and Rodríguez-Ezpeleta N. 2018. Adapting metabarcoding-based benthic biomonitoring into routine marine ecological status assessment networks. *Ecological Indicators* 95:194-202. https://doi.org/10.1016/j.ecolind.2018.07.044

Aylagas E, Borja Á, Tangherlini M, Dell'Anno A, Corinaldesi C, Michell CT, Irigoien X, Danovaro R, and Rodríguez-Ezpeleta N. 2017. A bacterial community-based index to assess the ecological status of estuarine and coastal environments. *Marine Pollution Bulletin* 114:679-688. https://doi.org/10.1016/j.marpolbul.2016.10.050

Aylagas E, Mendibil I, Borja Á, and Rodríguez-Ezpeleta N. 2016b. Marine Sediment Sample Pre-processing for Macroinvertebrates Metabarcoding: Mechanical Enrichment and Homogenization. *Frontiers in Marine Science* 3. 10.3389/fmars.2016.00203

Baird DJ, and Hajibabaei M. 2012. Biomonitoring 2.0: a new paradigm in ecosystem assessment made possible by next-generation DNA sequencing. *Molecular Ecology* 21:2039-2044. 10.1111/j.1365-294X.2012.05519.x

Bik HM, Porazinska DL, Creer S, Caporaso JG, Knight R, and Thomas WK. 2012a. Sequencing our way towards understanding global eukaryotic biodiversity. *Trends in Ecology & Evolution* 27:233-243. https://doi.org/10.1016/j.tree.2011.11.010

Bik HM, Sung WAY, De Ley P, Baldwin JG, Sharma J, Rocha-Olivares A, and Thomas WK. 2012b. Metagenetic community analysis of microbial eukaryotes illuminates biogeographic patterns in deep-sea and shallow water sediments. *Molecular Ecology* 21:1048-1059. 10.1111/j.1365-294X.2011.05297.x

Blaxter ML, De Ley P, Garey JR, Liu LX, Scheldeman P, Vierstraete A, Vanfleteren JR, Mackey LY, Dorris M, Frisse LM, Vida JT, and Thomas WK. 1998. A molecular evolutionary framework for the phylum Nematoda. *Nature* 392:71-75. 10.1038/32160

Borja A, Franco J, and Pérez V. 2000. A Marine Biotic Index to Establish the Ecological Quality of Soft-Bottom Benthos Within European Estuarine and Coastal Environments. *Marine Pollution Bulletin* 40:1100-1114. https://doi.org/10.1016/S0025-326X(00)00061-8

Bourlat SJ, Borja A, Gilbert J, Taylor MI, Davies N, Weisberg SB, Griffith JF, Lettieri T, Field D, Benzie J, Glöckner FO, Rodríguez-Ezpeleta N, Faith DP, Bean TP, and Obst M. 2013. Genomics in marine monitoring: New opportunities for assessing marine health status. *Marine Pollution Bulletin* 74:19-31. https://doi.org/10.1016/j.marpolbul.2013.05.042

Brandt MI, Trouche B, Quintric L, Günther B, Wincker P, Poulain J, and Arnaud-Haond S. 2021. Bioinformatic pipelines combining denoising and clustering tools allow for more comprehensive prokaryotic and eukaryotic metabarcoding. *Molecular Ecology Resources* 21:1904-1921. https://doi.org/10.1111/1755-0998.13398

Brannock PM, and Halanych KM. 2015. Meiofaunal community analysis by high-throughput sequencing: Comparison of extraction, quality filtering, and clustering methods. *Marine Genomics* 23:67-75. https://doi.org/10.1016/j.margen.2015.05.007

Carr CM, Hardy SM, Brown TM, Macdonald TA, and Hebert PDN. 2011. A Tri-Oceanic Perspective: DNA Barcoding Reveals Geographic Structure and Cryptic Diversity in Canadian Polychaetes. *PLOS ONE* 6:e22232. 10.1371/journal.pone.0022232

Chariton AA, Ho KT, Proestou D, Bik H, Simpson SL, Portis LM, Cantwell MG, Baguley JG, Burgess RM, Pelletier MM, Perron M, Gunsch C, and Matthews RA. 2014. A molecular-based approach for examining responses of eukaryotes in microcosms to contaminant-spiked estuarine sediments. *Environmental Toxicology and Chemistry* 33:359-369. 10.1002/etc.2450

Collins RA, Bakker J, Wangensteen OS, Soto AZ, Corrigan L, Sims DW, Genner MJ, and Mariani S. 2019. Non-specific amplification compromises environmental DNA metabarcoding with COI. *Methods in Ecology and Evolution* 10:1985-2001. 10.1111/2041-210x.13276

Cordier T, Esling P, Lejzerowicz F, Visco J, Ouadahi A, Martins C, Cedhagen T, and Pawlowski J. 2017. Predicting the Ecological Quality Status of Marine Environments from eDNA Metabarcoding Data Using Supervised Machine Learning. *Environmental Science & Technology* 51:9118-9126. 10.1021/acs.est.7b01518

Cordier T, Forster D, Dufresne Y, Martins CIM, Stoeck T, and Pawlowski J. 2018. Supervised machine learning outperforms taxonomy-based environmental DNA metabarcoding applied to biomonitoring. *Molecular Ecology Resources* 18:1381-1391. 10.1111/1755-0998.12926

Cordier T, Lanzén A, Apothéloz-Perret-Gentil L, Stoeck T, and Pawlowski J. 2019. Embracing Environmental Genomics and Machine Learning for Routine Biomonitoring. *Trends in Microbiology* 27:387-397. https://doi.org/10.1016/j.tim.2018.10.012

Cordier T, and Pawlowski J. 2018. BBI: an R package for the computation of Benthic Biotic Indices from composition data. *Metabarcoding and Metagenomics* 2:e25649.

Crane LC, Goldstein JS, Thomas DW, Rexroth KS, and Watts AW. 2021. Effects of life stage on eDNA detection of the invasive European green crab (Carcinus maenas) in estuarine systems. *Ecological Indicators* 124:107412. https://doi.org/10.1016/j.ecolind.2021.107412

Creer S, Fonseca VG, Porazinska DL, Giblin-Davis RM, Sung W, Power DM, Packer M, Carvalho GR, Blaxter ML, Lambshead PJD, and Thomas WK. 2010. Ultrasequencing of the meiofaunal biosphere: practice, pitfalls and promises. *Molecular Ecology* 19:4-20. https://doi.org/10.1111/j.1365-294X.2009.04473.x

Daan R, Mulder M, and Van Leeuwen A. 1994. Differential sensitivity of macrozoobenthic species to discharges of oil-contaminated drill cuttings in the North Sea. *Netherlands Journal of Sea Research* 33:113-127. https://doi.org/10.1016/0077-7579(94)90056-6

Davis NM, Proctor DM, Holmes SP, Relman DA, and Callahan BJ. 2018. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome* 6:1-14. DOI 10.1186/s40168-018-0605-2

Deagle BE, Jarman BE, Coissac E, Pompanon F, and Taberlet P. 2014. DNA metabarcoding and the cytochrome *c* oxidase subunit I marker: not a perfect match. *Biology Letters* 10:20140562. doi:10.1098/rsbl.2014.0562

Direktoratsguppen vanndirektivet. 2018. Veileder 02:2018. Klassifisering av miljøtilstand i vann. Oslo: Direktoratsgruppen for gjennomføringen av vannforskriften. p 222.

DNV GL. 2021. The Environmental Monitoring Database MOD. https://projects.dnv.com/MOD/MODKart/.

Edgar RC. 2016. UNCROSS: Filtering of high-frequency cross-talk in 16S amplicon reads. *bioRxiv*:088666. 10.1101/088666

Elbrecht V, and Leese F. 2015. Can DNA-Based Ecosystem Assessments Quantify Species Abundance? Testing Primer Bias and Biomass--Sequence Relationships with an Innovative Metabarcoding Protocol. *PLOS ONE* 10:e0130324-e0130324. 10.1371/journal.pone.0130324

Elbrecht V, and Leese F. 2017. Validation and Development of COI Metabarcoding Primers for Freshwater Macroinvertebrate Bioassessment. *Frontiers in Environmental Science* 5. 10.3389/fenvs.2017.00011

Farell EM, and Alexandre G. 2012. Bovine serum albumin further enhances the effects of organic solvents on increased yield of polymerase chain reaction of GC-rich templates. *BMC Research Notes* 5:257. 10.1186/1756-0500-5-257

Faria LCd, Di Domenico M, Andrade SCS, Santos MCd, Fonseca G, Zanol J, and Amaral ACZ. 2018. The use of metabarcoding for meiofauna ecological patterns assessment. *Marine Environmental Research* 140:160-168. https://doi.org/10.1016/j.marenvres.2018.06.013

Folmer O, Black M, Hoeh W, Lutz R, and Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3:294-299.

Frøslev TG, Kjøller R, Bruun HH, Ejrnæs R, Brunbjerg AK, Pietroni C, and Hansen AJ. 2017. Algorithm for post-clustering curation of DNA amplicon data yields reliable biodiversity estimates. *Nature Communications* 8:1-11. 10.1038/s41467-017-01312-x

Geller J, Meyer C, Parker M, and Hawk H. 2013. Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. *Molecular Ecology Resources* 13:851-861. 10.1111/1755-0998.12138

Gray JS, and Pearson TH. 1982. Objective Selection of Sensitive Species Indicative of Pollution-Induced Change in Benthic Communities. I. Comparative Methodology. *Marine Ecology Progress Series* 9:111-119.

Guardiola M, Uriz MJ, Taberlet P, Coissac E, Wangensteen OS, and Turon X. 2015. Deep-Sea, Deep-Sequencing: Metabarcoding Extracellular DNA from Sediments of Marine Canyons. *PLOS ONE* 10:1-26. 10.1371/journal.pone.0139633

Guardiola M, Wangensteen OS, Taberlet P, Coissac E, Uriz MJ, and Turon X. 2016. Spatio-temporal monitoring of deep-sea communities using metabarcoding of sediment DNA and RNA. *PeerJ* 4:e2807. 10.7717/peerj.2807

Hadziavdic K, Lekang K, Lanzen A, Jonassen I, Thompson EM, and Troedsson C. 2014. Characterization of the 18S rRNA gene for designing universal eukaryote specific primers. *PLOS ONE* 9:e87624-e87624. 10.1371/journal.pone.0087624

Hestetun JT, Bye-Ingebrigtsen E, Nilsson RH, Glover AG, Johansen P-O, and Dahlgren TG. 2020. Significant taxon sampling gaps in DNA databases limit the operational use of marine macrofauna metabarcoding. *Marine Biodiversity* 50:70. 10.1007/s12526-020-01093-5

Hestetun JT, Lanzén A, and Dahlgren TG. 2021a. Grab what you can – an evaluation of spatial replication to decrease heterogeneity in sediment eDNA metabarcoding. *PeerJ*.

Hestetun JT, Lanzén A, Skaar KS, and Dahlgren TG. 2021b. The impact of DNA extract homogenization and replication on marine sediment metabarcoding diversity and heterogeneity. *Environmental DNA* n/a. https://doi.org/10.1002/edn3.223

ISO. 2014. ISO 16665:2014. Water quality — Guidelines for quantitative sampling and sample processing of marine soft-bottom macrofauna. International Organization for Standardization. p 33.

Jaksch K, Eschner A, Rintelen TV, and Haring E. 2016. DNA analysis of molluscs from a museum wet collection: a comparison of different extraction methods. *BMC Research Notes* 9:348. 10.1186/s13104-016-2147-7

Kapa Biosystems. 2016. KAPA3G Plant PCR Kit – Technical Data Sheet. p 4.

Keeley N, Wood SA, and Pochon X. 2018. Development and preliminary validation of a multi-trophic metabarcoding biotic index for monitoring benthic organic enrichment. *Ecological Indicators* 85:1044-1057. https://doi.org/10.1016/j.ecolind.2017.11.014

Kelly RP. 2016. Making environmental DNA count. *Molecular Ecology Resources* 16:10-12. https://doi.org/10.1111/1755-0998.12455

Krolicka A, Gomiero A, and Baussant T. 2020. qPCR-based assessment of microfaunal indicators of oil for monitoring benthos around oil and gas platforms. *Science of The Total Environment* 736:139527. https://doi.org/10.1016/j.scitotenv.2020.139527

Kutti T, Johnsen IA, Skaar KS, Ray JL, Husa V, and Dahlgren TG. 2020. Quantification of eDNA to Map the Distribution of Cold-Water Coral Reefs. *Frontiers in Marine Science* 7. 10.3389/fmars.2020.00446

Lanzén A, Lekang K, Jonassen I, Thompson EM, and Troedsson C. 2016. High-throughput metabarcoding of eukaryotic diversity for environmental monitoring of offshore oil-drilling activities. *Molecular Ecology* 25:4392-4406. 10.1111/mec.13761

Lanzén A, Lekang K, Jonassen I, Thompson EM, and Troedsson C. 2017. DNA extraction replicates improve diversity and compositional dissimilarity in metabarcoding of eukaryotes in marine sediments. *PLOS ONE* 12:e0179443-e0179443. 10.1371/journal.pone.0179443

Lanzén A, Mendibil I, Borja Á, and Alonso-Sáez L. 2020. A microbial mandala for environmental monitoring: Predicting multiple impacts on estuarine prokaryote communities of the Bay of Biscay. *Molecular Ecology* 00:1-19. 10.1111/mec.15489

Laroche O, Kersten O, Smith CR, and Goetze E. 2020. Environmental DNA surveys detect distinct metazoan communities across abyssal plains and seamounts in the western Clarion Clipperton Zone. *Molecular Ecology* n/a. 10.1111/mec.15484

Le JT, Levin LA, Lejzerowicz F, Cordier T, Gooday AJ, and Pawlowski J. 2021. Scientific and budgetary tradeoffs between morphological and molecular methods for deep-sea biodiversity assessment. *Integrated Environmental Assessment and Management* n/a. https://doi.org/10.1002/ieam.4466

Lejzerowicz F, Esling P, Pillet L, Wilding TA, Black KD, and Pawlowski J. 2015. High-throughput sequencing and morphology perform equally well for benthic monitoring of marine ecosystems. *Scientific Reports* 5:13932. 10.1038/srep13932

Lekang K, Thompson EM, and Troedsson C. 2015. A comparison of DNA extraction methods for biodiversity studies of eukaryotes in marine sediments. *Aquatic Microbial Ecology* 75:15-25.

Leray M, and Knowlton N. 2016. Censusing marine eukaryotic diversity in the twenty-first century. *Philosophical Transactions of the Royal Society B: Biological Sciences* 371:20150331. doi:10.1098/rstb.2015.0331

Leray M, and Knowlton N. 2017. Random sampling causes the low reproducibility of rare eukaryotic OTUs in Illumina COI metabarcoding. *PeerJ* 5:e3006. 10.7717/peerj.3006

Leray M, Yang JY, Meyer CP, Mills SC, Agudelo N, Ranwez V, Boehm JT, and Machida RJ. 2013. A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. *Frontiers in Zoology* 10:34. 10.1186/1742-9994-10-34

Lobo J, Shokralla S, Costa MH, Hajibabaei M, and Costa FO. 2017. DNA metabarcoding for high-throughput monitoring of estuarine macrobenthic communities. *Scientific Reports* 7:15618. 10.1038/s41598-017-15823-6

Macher J-N, Vivancos A, Piggott JJ, Centeno FC, Matthaei CD, and Leese F. 2018. Comparison of environmental DNA and bulk-sample metabarcoding using highly degenerate cytochrome c oxidase I primers. *Molecular Ecology Resources* 18:1456-1468. 10.1111/1755-0998.12940

Mahé F, Rognes T, Quince C, de Vargas C, and Dunthorn M. 2015. Swarm v2: highly-scalable and high-resolution amplicon clustering. *PeerJ* 3:e1420-e1420. 10.7717/peerj.1420

Makiola A, Compson ZG, Baird DJ, Barnes MA, Boerlijst SP, Bouchez A, Brennan G, Bush A, Canard E, Cordier T, Creer S, Curry RA, David P, Dumbrell AJ, Gravel D, Hajibabaei M, Hayden B, van der Hoorn B, Jarne P, Jones JI, Karimi B, Keck F, Kelly M, Knot IE, Krol L, Massol F, Monk WA, Murphy J, Pawlowski J, Poisot T, Porter TM, Randall KC, Ransome E, Ravigné V, Raybould A, Robin S, Schrama M, Schatz B, Tamaddoni-Nezhad A, Trimbos KB, Vacher C, Vasselon V, Wood S, Woodward G, and Bohan DA. 2020. Key Questions for Next-Generation Biomonitoring. *Frontiers in Environmental Science* 7:1-14. 10.3389/fenvs.2019.00197

Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *2011* 17:3. 10.14806/ej.17.1.200

Martínez A, Eckert EM, Artois T, Careddu G, Casu M, Curini-Galletti M, Gazale V, Gobert S, Ivanenko VN, Jondelius U, Marzano M, Pesole G, Zanello A, Todaro MA, and Fontaneto D. 2020. Human access impacts biodiversity of microscopic animals in sandy beaches. *Communications Biology* 3:175. 10.1038/s42003-020-0912-6

Mauffrey F, Cordier T, Apothéloz-Perret-Gentil L, Cermakova K, Merzi T, Delefosse M, Blanc P, and Pawlowski J. 2020. Benthic monitoring of oil and gas offshore platforms in the North Sea using environmental DNA metabarcoding. *Molecular Ecology* 00:1-16. 10.1111/mec.15698

Medlin LK, and Orozco J. 2017. Molecular Techniques for the Detection of Organisms in Aquatic Environments, with Emphasis on Harmful Algal Bloom Species. *Sensors* 17:1184. 10.3390/s17051184

Nascimento FJA, Lallias D, Bik HM, and Creer S. 2018. Sample size effects on the assessment of eukaryotic diversity and community structure in aquatic sediments using high-throughput sequencing. *Scientific Reports* 8:11737. 10.1038/s41598-018-30179-1

Norwegian Environment Agency. 2020. M-408. Guidelines for environmental monitoring of petroleum activities on the Norwegian continental shelf, 2020 revision.: Norwegian Environment Agency. p 68.

Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Szoecs E, and Wagner H. 2019. vegan: Community Ecology Package. Version 2.5-6 [R package / software]. 2.5-6 ed. Available from: http://CRAN.R-project.org/package=vegan.

Pansu J, Chapman MB, Hose GC, and Chariton AA. 2021. Comparison of an extracellular *v*. total DNA extraction approach for environmental DNA-based monitoring of sediment biota. *Marine and Freshwater Research*:-. https://doi.org/10.1071/MF20269

Passamaneck YJ, Schander C, and Halanych KM. 2004. Investigation of molluscan phylogeny using large-subunit and small-subunit nuclear rRNA sequences. *Molecular Phylogenetics and Evolution* 32:25-38. https://doi.org/10.1016/j.ympev.2003.12.016

Pawlowski J, Kelly-Quinn M, Altermatt F, Apothéloz-Perret-Gentil L, Beja P, Boggero A, Borja A, Bouchez A, Cordier T, Domaizon I, Feio MJ, Filipe AF, Fornaroli R, Graf W, Herder J, van der Hoorn B, Iwan Jones J, Sagova-Mareckova M, Moritz C, Barquín J, Piggott JJ, Pinna M, Rimet F, Rinkevich B, Sousa-Santos C, Specchia V, Trobajo R, Vasselon V, Vitecek S, Zimmerman J, Weigand A, Leese F, and Kahlert M. 2018. The future of biotic indices in the ecogenomic era: Integrating (e)DNA metabarcoding in biological assessment of aquatic ecosystems. *Science of The Total Environment* 637-638:1295-1310. https://doi.org/10.1016/j.scitotenv.2018.05.002

Pearson T, and Rosenberg R. 1978. Macrobenthic succession in relation to organic enrichment and pollution of the marine environment. *Oceanogr Mar Biol Ann Rev* 16:229-311.

Ramirez-Llodra E, Brandt A, Danovaro R, De Mol B, Escobar E, German CR, Levin LA, Martinez Arbizu P, Menot L, Buhl-Mortensen P, Narayanaswamy BE, Smith CR, Tittensor DP, Tyler PA, Vanreusel A, and Vecchione M. 2010. Deep, diverse and definitely different: unique attributes of the world's largest ecosystem. *Biogeosciences* 7:2851-2899. 10.5194/bg-7-2851-2010

Ratnasingham S, and Hebert PDN. 2007. bold: The Barcode of Life Data System (http://www.barcodinglife.org). *Molecular ecology notes* 7:355-364. 10.1111/j.1471-8286.2007.01678.x

Rognes T, Flouri T, Nichols B, Quince C, and Mahé F. 2016. VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 4:e2584. 10.7717/peerj.2584

Rygg B. 1985. Distribution of species along pollution-induced diversity gradients in benthic communities in Norwegian fjords. *Marine Pollution Bulletin* 16:469-474. https://doi.org/10.1016/0025-326X(85)90378-9

Rygg B. 2006. NIVA 5208-2006. Developing indices for quality-status classification of marine soft-bottom fauna in Norway. NIVA Report: Norwegian Institute for Water Research. p 38.

Rygg B, and Norling K. 2013. NIVA 6475-2013. Norwegian Sensitivity Index (NSI) for marine macroinvertebrates, and an update of Indicator Species Index (ISI). NIVA Report: Norwegian Institute for Water Research. p 47.

Schander C, and Willassen E. 2005. What can biological barcoding do for marine biology? *Marine Biology Research* 1:79-83. 10.1080/17451000510018962

Schirmer M, Ijaz UZ, D'Amore R, Hall N, Sloan WT, and Quince C. 2015. Insight into biases and sequencing errors for amplicon sequencing with the Illumina MiSeq platform. *Nucleic Acids Research* 43:e37-e37. 10.1093/nar/gku1341

Sinniger F, Pawlowski J, Harii S, Gooday AJ, Yamamoto H, Chevaldonné P, Cedhagen T, Carvalho G, and Creer S. 2016. Worldwide Analysis of Sedimentary DNA Reveals Major Gaps in Taxonomic Knowledge of Deep-Sea Benthos. *Frontiers in Marine Science* 3:1-14. 10.3389/fmars.2016.00092

Struck TH, Purschke G, and Halanych KM. 2006. Phylogeny of Eunicida (Annelida) and Exploring Data Congruence Using a Partition Addition Bootstrap Alteration (PABA) Approach. *Systematic Biology* 55:1-20. 10.1080/10635150500354910

Taberlet P, Coissac E, Pompanon F, Brochmann C, and Willerslev E. 2012. Towards next-generation biodiversity assessment using DNA metabarcoding. *Molecular Ecology* 21:2045-2050. 10.1111/j.1365-294X.2012.05470.x

Tang CQ, Leasi F, Obertegger U, Kieneke A, Barraclough TG, and Fontaneto D. 2012. The widely used small subunit 18S rDNA molecule greatly underestimates true diversity in biodiversity surveys of the meiofauna. *Proceedings of the National Academy of Sciences* 109:16208-16212. 10.1073/pnas.1209160109

Uthicke S, Lamare M, and Doyle JR. 2018. eDNA detection of corallivorous seastar (Acanthaster cf. solaris) outbreaks on the Great Barrier Reef using digital droplet PCR. *Coral Reefs* 37:1229-1239. 10.1007/s00338-018-1734-6

Wangensteen OS, Palacín C, Guardiola M, and Turon X. 2018. DNA metabarcoding of littoral hard-bottom communities: high diversity and database gaps revealed by two molecular markers. *PeerJ* 6:e4705. 10.7717/peerj.4705

Zhan A, and MacIsaac HJ. 2015. Rare biosphere exploration using high-throughput sequencing: research progress and perspectives. *Conservation Genetics* 16:513-522. 10.1007/s10592-014-0678-9

Zinger L, Bonin A, Alsos IG, Bálint M, Bik H, Boyer F, Chariton AA, Creer S, Coissac E, Deagle BE, De Barba M, Dickie IA, Dumbrell AJ, Ficetola GF, Fierer N, Fumagalli L, Gilbert MTP, Jarman S, Jumpponen A, Kauserud H, Orlando L, Pansu J, Pawlowski J, Tedersoo L, Thomsen PF, Willerslev E, and Taberlet P. 2019. DNA metabarcoding—Need for robust experimental designs to draw sound ecological conclusions. *Molecular Ecology* 28:1857-1862. 10.1111/mec.15060