eDNA IN ENVIRONMENTAL MONITORING

Technical Report from DCE – Danish Centre for Environment and Energy No. 133 2019

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Data sheet

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Abstract: The use of environmental DNA (eDNA) for environmental monitoring has been presented as a technique to replace existing traditional monitoring techniques; being faster, easier and more accurate. In both aquatic and terrestrial environments detection of specific species or group of organisms as well as biodiversity assessment have shown promising results with eDNA. However, several technological and data assessment issues have to be resolved prior to full employment in environmental monitoring. Sharing, harmonizing and consolidating the available knowledge is therefore of prime importance in order to develop standardized procedures through all the steps of the process (sampling, DNA extraction, amplification primers and conditions, bioinformatic analysis). eDNA-based results generally show different aspects of the environmental state with increasing detailed knowledge on a different scale than traditional monitoring methodology. Future techniques will probably allow metabarcoding based on longer reads thanks to advances in sequencing technologies. Another branch of eDNA analysis is expected to focus on direct sequencing of eDNA omitting PCR and the biases associated with this. In addition, the bioinformatics analyses are anticipated to be standardized and detailed in standard operating procedures.

Keywords: eDNA, aquatic, terrestrial, pathogens, biodiversity, metagenome, sequencing, qPCR

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Introduction

Anne Winding

In microbial ecology, the extraction of DNA from environmental samples and analysis of the amount and diversity of bacteria and fungi hidden in the extracted DNA have been studied for decades. However, the interest in studying the DNA from multicellular and larger organisms (plants and animals) has increased within the last decade. The focus on DNA extracted directly from environmental samples has arisen along with a great development in the possibilities of DNA amplification and sequencing and hence gaining insight into the information hidden in the DNA. Especially, knowledge has been gained about the species present and their diversity, but data also provide indications of their abundance and activity. Together, this has led to speculations and ambitions of using molecular tools in monitoring of the environmental state as well as in screenings for the presence of specific species such as threatened or non-indigenous species. Several initiatives of investigating these possibilities have been reported from around the world and for different environments, such as marine- and freshwater, agricultural soils and for organisms such as pollinating insects.

This report is the outcome of a collaboration in the Danish eDNA Center at Aarhus University between several disciplines within natural science. The objective is to evaluate the possibilities of using eDNA for environmental monitoring of both ecological status, presence of specific species including plant pathogens, non-indigenous species and endangered species.

Objectives

Anne Winding

The objectives of the report are to provide:

- An overview of eDNA techniques, including the need of development and adjustment of techniques.
  - qPCR versus sequence-based methods.
  - pros and cons of eDNA for environmental monitoring.
- Examples of eDNA used for environmental monitoring and of scientific areas with potentials and developments.

For the design of an environmental monitoring program, Deiner et al. (2017) supplied guiding questions and a workflow presenting challenges and necessary considerations on applying eDNA in environmental monitoring regarding most levels of sampling design, primer design, bioinformatics biases etc. We adapt this workflow as a framework for the current report:

1. ***Study design*** including considerations of presence/absence, diversity assessment or absolute quantification; targeted taxa; comparison of traditional *vs.* eDNA monitoring; statistical power of sample design.

2. ***Field sampling*** including considerations of: sample type; number of replicates; contamination including positive and negative controls; other known biases as inhibitors and sample volume.

3. ***Laboratory analyses***

   I. Sample handling phase including considerations of: extraction methods; primers; reference sequence data; technical replicates; library preparation method; samples to be pooled or indexed; sequence depth; read length.
   II. DNA processing phase including considerations of: sequence platform; appropriate quality assurance (e.g. mock communities); positive and negative controls; other known biases as primer bias, coverage, taxonomic resolution.

4. ***Data processing*** including considerations of: quality of database; sequencing coverage; software tools; appropriate quality; filtering biological conclusions compared to eDNA conclusions.

The area of eDNA has great attention in these years and several reviews and reports covering various aspects of the technique have been published within the last approximately five years (Coble et al. 2019, Deiner et al. 2017, Taberlet et al. 2018). Hence, this report will focus on experience and possibilities in the Danish environment.

Sammenfatning

Anvendelse af miljø-DNA (environmental DNA, eDNA) til miljøovervågning præsenteres som en hurtigere, nemmere og mere præcis metode til erstatning af traditionel overvågning. Både den teknologiske og økonomiske udvikling af eDNA-teknikker går i øjeblikket meget hurtigt, og ambitionerne for anvendelse af eDNA-teknikker i overvågningssammenhænge er store. I både det akvatiske og terrestriske miljø har eDNA-teknikker vist lovende resultater ved både detektion af specifikke arter og grupper af organisme samt ved bestemmelse af biodiversitet. Inden for det teknologiske område og m.h.t. databehandling og –fortolkning er der dog udfordringer, der skal løses og standardiseres, før udbredt anvendelse af eDNA til overvågning kan implementeres. Deling, harmonisering og konsolidering af den tilgængelige viden er af afgørende betydning for standardisering og videreudvikling af procedurer for alle trin i processen (såsom prøvetagning, DNA-ekstraktion, primer design, bioinformatisk analyse) samt håndtering af falsk positive og falsk negative prøver. Videnskabelig litteratur viser tydeligt at på trods af nemmere prøveindsamling, er dannelse af sekventeringsbiblioteker stadig udfordrende, mens standardiserede bioinformatiske procedurer (pipelines) medfører nemmere analyser.

Undersøgelser baseret på eDNA viser generelt andre aspekter af miljøets tilstand; især en mere detaljeret viden på en anden skala end traditionel overvågning. Dette gælder både for biodiversitet og artsspecifik tilstedeværelse og forekomst. De konventionelle lange tidsserier af moniteringsdata er af høj værdi, og det bør sikres, at disse ikke kompromitteres ved overgang til eDNA-baserede teknikker. Hvis vi umiddelbart skifter til eDNA-baserede teknikker, mister vi muligheden for undersøgelser, der kræver lange tidsserier. Derfor anbefales det, at udføre parallel overvågning med eDNA teknikker og traditionelle metoder indtil tilstrækkelig erfaring og data er indsamlet til, at analyser af lange tidsserier kan gennemføres med sikkerhed for tilstrækkelig kvalitet.

Fremtidige eDNA-teknikker vil sandsynligvis omfatte muligheder for metabarcoding, med længere læste DNA strenge, takket være teknologiske fremskridt. Direkte sekventering af DNA uden forudgående PCR-amplificering er en anden mulig teknologisk udvikling, hvorved usikkerhed og fejlrisici ved PCR undgås. De bioinformatiske analyser forventes også at blive standardiseret og udførligt beskrevet. eDNA-baseret *in situ* overvågning vil sandsynligvis blive mulig, enten gennem automatisk indsamling koblet med PCR eller vha. bærbar sekvenatorudstyr.

Summary

The use of environmental DNA (eDNA) for environmental monitoring has been presented as a technique to replace existing traditional monitoring techniques, being faster, easier and more accurate. Both the technical and economic development in the area is currently very fast and ambitions for the use of eDNA for environmental monitoring are high. In both aquatic and terrestrial environments detection of specific species or group of organisms as well as biodiversity assessment have shown promising results with eDNA. However, several technological and data assessment issues have to be resolved prior to full implementation in environmental monitoring. Sharing, harmonizing and consolidating the available knowledge are therefore of prime importance in order to develop standardized procedures throughout all the steps of the process (e.g. sampling, DNA extraction, amplification primers and conditions, bioinformatic analysis) including handling of false positives and negatives. Databased scientific literature clearly demonstrates that even though sampling might be easier and faster, sequencing library preparation is still costly, although continuously decreasing, while standardized bioinformatics procedures (pipelines) make bioinformatic analyses more efficient.

eDNA-based results generally show different aspects of the environmental state with increasing detailed knowledge on a different scale than traditional monitoring. This applies both to biodiversity and species-specific occurrence and abundance. The conventional long time series of environmental monitoring is of high value and great care should be taken not to compromise these without careful evaluation of the benefits of moving to eDNA approaches. If we convert all environmental monitoring to eDNA and stop collecting specimens for collections, we lose the possibility of certain studies requiring e.g. long time series. Therefore, it is recommended to perform parallel monitoring with eDNA-based techniques and the traditional monitoring until sufficient experience and data have been obtained to be able to follow the environmental state back to the time before eDNA monitoring.

Future techniques will probably allow metabarcoding based on longer reads thanks to advances in sequencing technologies. Another branch of eDNA analysis is expected to focus on direct sequencing of eDNA omitting PCR and the biases associated with this. In addition, the bioinformatics analyses are anticipated to be standardized and detailed in standard operating procedures. *In situ* monitoring will most likely be possible, either through autonomous samplers coupled with qPCR assays or portable sequencers.

1. Techniques

Anne Winding, Toke Bang-Andreasen, Lars H. Hansen, Frank Panitz

Environmental DNA (eDNA) is defined as DNA extracted from an environmental sample (e.g. soil, water, sediment, air) without first isolating any target organisms (Taberlet et al. 2012). Ancient DNA (aDNA) is broadly defined as DNA sequences from museum specimens, archaeological finds, fossil remains etc. (Pääbo et al. 2004) and have been used for environmental studies on past ecosystems (e.g. Zobel et al. 2018). Relic DNA (rDNA) is a term used for extracellular persistent DNA from dead organisms in the environment (Carini et al. 2016). The longevity of rDNA is dependent on the environmental conditions like pH and cation exchange capacity. Extracted soil microbial DNA can be composed of up to 40% extracellular DNA. The differences between eDNA, rDNA, and aDNA are mainly related to time, and recently Ushio et al. (2018) has added to the definition of eDNA that it is persistent DNA in the environment, which makes the difference between eDNA, rDNA and aDNA smaller.

Community DNA is described as DNA from a bulk-extracted mixture of organisms separated from an environmental sample, e.g. all invertebrates in a soil sample (Deiner et al. 2017). The use of eDNA and community DNA has proven useful in various settings targeting organisms from all three domains of life across most of Earth's biosphere (e.g. Jørgensen and Boetius, 2007; Thomsen and Willerslev, 2015; Valentini et al., 2016; Willerslev et al., 1999; Temkiv et al., 2012; Michaud et al., 2014; Inskeep et al., 2013; Drummond et al., 2015).

Recently, more subgroups of DNA have been termed, like invertebrate-derived DNA (iDNA). It should also be mentioned that within the area of molecular microbial ecology, eDNA used to be the abbreviation of extracellular DNA and iDNA the abbreviation of intercellular DNA (Alawi et al., 2014). However, this use of the abbreviations have generally been replaced by environmental DNA (eDNA) and invertebrate-derived DNA (iDNA).

In the present report, we focus on recent environmental DNA (not aDNA or rDNA) with occasional use of community DNA.

1.1 DNA extraction

Anne Winding

Extracted DNA includes extracellular DNA, which has been actively or passively extruded by organisms or from cell lysis (Pietramellara et al., 2009) and also includes relic DNA from dead organisms due to the relatively slow degradation of DNA (Levy-Booth et al., 2007; Nielsen et al., 2007). The efficiency of extracting DNA is obviously a very important parameter for the further downstream DNA analysis. In microbiology, much effort and considerations have been undertaken to distinguish DNA originating from living or dead organisms versus extracellular DNA (Kowalchuk et al. 2004). Today a consensus seems to be that extracted microbial DNA originates from intact cells as well as extracellular DNA.

In soil and sediment, the physical and chemical conditions lead to strong binding of DNA to especially clay particles. This binding increases the longevity of extractable DNA from soil and sediment considerably compared to aquatic habitats where DNA is likely to be degraded much faster (Deiner et al. 2017).

At present, DNA is commonly extracted using commercial kits, such as Qiagen (earlier MoBio), KingFisher or GenLute. The amount of environmental sample used for DNA extraction affects the sensitivity and detection limit. Hence, aquatic samples are often filtered in order to extract DNA from several liters of water, while soil and sediment samples usually contain higher amounts of DNA per unit and extraction is done on decimals of grams up to a few grams. Both national and international technical guidelines of DNA extraction are available (Carim et al. 2015; Laramie et al., 2015; Danish EPA 2017).

Along with the DNA, RNA is also extracted. RNA is less stable in the environment and represents the gene expression of the active organisms present, while DNA gives information on presence of organisms. Parallel to DNA analyses, a rapid development of RNA analyses is occurring in the scientific community. Techniques based on RNA are, however, still at a developmental stage where it is too early to include in environmental monitoring.

1.2 PCR Primers

Toke Bang-Andreasen, Anne Winding

PCR primers are typically used in sets of two, a forward and a reverse primer (~15-30 bp in length), that flanks the region of interest. During PCR amplification, these primers, after an initial denaturation step that splits DNA into two single-stranded templates, bind to the template strands (annealing step) thereby allowing DNA polymerases to produce the complementary strand (extension step). The marker gene region of interest is thereby doubled for every PCR amplification cycle and eventually resulting in an amplicon of the marker gene region.

The choice of the marker and the design of the primers to target the specific region is the very basis of amplicon-based methods and a major concern in eDNA assessments. Unspecific primers result in misleading and skewed community composition estimates (Schirmer et al., 2015; Lanzén et al., 2011; Hugerth and Andersson, 2017; Eloe-Fadrosh et al., 2016; Taberlet et al., 2012b, 2012a). The optimal marker gene region must fulfill multiple requirements (e.g. Epp et al., 2012; Valentini et al., 2009), including:

- Close to identical nucleotide sequence among members of target organisms, whether that be a single species or a group of organisms (e.g. bacteria, fish, earthworms) but different nucleotide sequences between groups resulting in high taxonomic resolution,
- Sufficient phylogenetic information to assign higher taxonomic rank to undescribed species (genus, family, order, etc.),
- Variable region flanked by highly conserved regions allowing binding of amplification primers,
- Primer sites sufficiently conserved to include all members of the target organism group with minimal bias.

For different taxonomic groups, different regions are targeted (Freeland, 2017 and references therein): for bacteria, typically, the 16S rRNA gene, for fungi the ITS1 or ITS2 regions, for eukaryotes the 18S rRNA gene, for specific eukaryotes the mitochondrial gene cytochrome oxidase I (COI) and for plants regions of chloroplast DNA (cpDNA) including matK and rbcL (see further details in Freeland, 2017). COI has been widely used for eukaryotes and can provide primers for specific detection. It is, however, no longer recommended for biodiversity assessment, as it will address fewer than 30% of the species (Stat et al., 2017). Instead, primers based on the 18S rRNA gene and the use of multiple primer sets is recommended (Stat et al., 2017).

Several software tools are available for design of primers, and OBItools is presently promising (Taberlet et al., 2018). During *in silico* testing, primers are aligned to sequences of reference databases to test complementarity, and thus coverage and specificity, of the primer set against the taxonomic groups available in the reference database. Freely available tools allows for rapid *in silico* tests of primer sets against reference databases, e.g. OBITools (Boyer et al., 2016), TestPrime 1.0 (Klindworth et al., 2013), DegePrime (Hugerth et al., 2014), PrimerProspector (Walters et al., 2011). Primer biases can also be evaluated by aligning primers to sequences obtained by primer independent metagenomics (Eloe-Fadrosh et al., 2016). Newly designed primers should always be tested *in silico* prior to in vivo application, and using mock communities, which is a DNA mixture from known organisms.

For analysis of aDNA and detection of rare taxa, the targeted marker gene region should be short enough to include partly degraded DNA. However, the short length of the targeted marker gene region is not important in studies assessing the living fraction in soil. Instead, the length is restricted by limitations of current sequencing platforms in sequence read length and by e.g. available DNA polymerases (and the proofreading ability of these) to produce long nucleotide amplicons of high quality. However, rapid development of sequencing technologies together with improvement of reagents used in molecular biology will allow amplification and sequencing of longer marker gene regions in the near future.

Degenerate primers having base-level variations (wobble nucleotides) can increase the number of targeted taxa and thus increase the coverage of various taxa. However, this increases the risk of amplifying unwanted DNA regions thereby decreasing the specificity of the primer towards the target taxonomic group. Additionally, preferential binding of sequences containing C/G rather than A/T at a degenerate position also causes biases in the microbiome profile (Lanzén et al., 2011).

Marker gene regions for metabarcoding that fulfill all requirements listed above are not yet described and might not exist (Nielsen and Matz, 2006; Baker and Cowan, 2004; Martinez-Porchas et al., 2017). Typical biases associated with the choice of marker gene region and thus the primer choice includes inadequate complementarity of all members within the target group resulting in false negatives. This leads to lower coverage of specific taxonomic groups within the target group. Another typical bias is low specificity of the selected marker gene region towards the target taxonomic group. This can lead to false positives through amplification of sequences belonging to organisms outside the target group. These unwanted sequences have to be filtered during bioinformatic processing and can result in removal of a substantial fraction of the obtained operational taxonomic units (OTU). Other important

biases associated with PCR amplification involve differential amplification efficiencies among the target gene that together with gene copy number variation between species of the target groups can result in skewed relative abundance estimates. As a consequence of primer biases, studies using different marker gene regions are generally incomparable (Soergel et al., 2012; Nossa, 2010; Yang et al., 2016).

1.3 Analysis of eDNA

Anne Winding, Toke Bang-Andreasen

The extracted eDNA can be analyzed using different molecular techniques of which the most common are:

1. Quantitative PCR (qPCR), sometimes called real-time PCR (RT-qPCR), is used to quantify the concentration of specific DNA fragments in environmental samples. The DNA fragments are amplified with primers targeting specific small sequences of the target DNA. During the PCR amplification the product is quantified. While for higher eukaryotes, qPCR is typically used to detect and quantify specific species, for bacteria and fungi qPCR it is also used for quantification of broader taxonomic groups.

2. High throughput amplicon sequencing in which a specific DNA region from all genomes present in the environmental sample is amplified and sequenced to yield a multispecies community profile (Kelly 2016). This technique is also called metabarcoding as a "barcode", i.e. a specific DNA sequence, is targeted and amplified with the primers used.

3. Direct sequencing of all the DNA in the environmental sample, also yielding a multispecies community profile and circumventing the difficulties and constraints of identifying and designing the primers. This technique called metagenomics or shotgun sequencing has become possible with the drastically decreasing price of sequencing and the development of databases with DNA sequences from a huge and ever increasing number of species.

1.3.1 Quantitative PCR

In qPCR the production of the amplicon itself provides a quantitative measure. This happens as fluorescent reporter molecules binds to double stranded DNA and activate fluorescence. The fluorescence is measured after each amplification cycle and converted to a concentration of the amplicon by comparing to a DNA standard series (Heid et al., 1996).

qPCR is mostly used either when investigating groups at low taxonomic rank e.g. in assessment of specific species such as a specific mollusk (e.g. Andersen and Wiberg, 2017), or used to quantify overall pools of eDNA from more general taxonomic ranks such as the Bacterial domain or the Fungal kingdom. However, large variability in the number of copies of the target marker gene per genome from different taxa and variable numbers of nuclei of different eukaryotic taxa make the absolute number of target organisms in an entire community difficult to address using this approach (Gong et al., 2013; Geisen et al., 2015; Klappenbach et al., 2000).

Recently, high resolution melting curve analysis of qPCR output has provided qualitative insight into microbial community changes and has been proposed

as a rapid screening tool to investigate microbial community changes between samples (Hjelmsø et al., 2014; Everman and Wang, 2017; Vestergård et al., 2017). This is based on the different melting temperature of DNA based on difference in G:C ratios.

qPCR has the clear advantage that the limit of detection is very low and hence qPCR can be used for detection of rare organisms. In Denmark this technique is being used for detection of Non-Indigenous Species (NIS) in the marine environments.

1.3.2 Metabarcoding (High throughput amplicon sequencing)

In high throughput amplicon sequencing, DNA is PCR amplified using primers targeting more or less specific genetic traits, and after the following sequencing of the PCR products (amplicons), the sequences are processed bioinformatically leading to taxonomic assignment of the sequences against a reference database. The output is typically a taxonomic table presenting species or higher taxonomic identity based on the sequences (in some cases to species level). Amplicon sequencing also provides semi-quantitative measures as the number of sequences assigned to different taxonomic ranks is provided (resulting in relative abundances). This has proven useful when investigating microbial community differences between samples (Pilloni et al., 2012). Furthermore, richness and diversity measures of organisms can be calculated, including organisms that remain undescribed, by clustering sequences into OTU, sometimes called molecular operational taxonomic units (MOTU), based on sequence nucleotide similarity thresholds or by amplicon sequence variants based on exact sequence variants (Nguyen et al., 2016; Callahan et al., 2016, 2017; Mahé et al., 2014; Hugerth and Andersson, 2017).

Metabarcoding is presently the most widely used eDNA analysis. However, amplicon based approaches can introduce biases in various procedural steps including polymerase chain reaction (PCR) amplification, sequencing and bioinformatic processing. This is in addition to the general biases in sample collection and nucleic acid extraction. Many of these biases have been reviewed previously (Taberlet et al., 2012; Zinger et al., 2012; Hugerth and Andersson, 2017; Schirmer et al., 2015; Alberdi et al., 2018).

1.3.3 Metagenomics (shotgun sequencing)

Reported primer biases described above highlights the hampering effect amplicon based approaches typically introduce during the PCR. Metagenomic approaches avoid the amplification of marker genes by sequencing the total pool of extracted DNA from a sample. Instead of PCR amplification, a fragmentation step is included to adjust nucleotide sequences to a sequencing-able length, followed by sequencing of essentially all DNA. The sequencing of total DNA has the advantages of including genomic material from all three domains of life in one go (Coble et al. 2019). This does not only allow for taxonomic assignment of DNA sequences using marker genes, but can also reveal functional potential of the assessed organisms. Moreover, full genomes can be assembled from metagenome data (Albertsen et al., 2013; Sharon and Banfield, 2013) and likewise can full-length marker genes be assembled resulting in improved taxonomic assignment of sequences (Miller et al., 2011). The rapid development of sequencing techniques, with increasing sequence output at a lower cost, makes metagenomic approaches increasingly available for most eDNA laboratories.

With metagenomic investigation, rare taxa and taxa that occupy smaller fractions of the total nucleic acid pool are included, but the detection limit is rather high, which is in contrast to qPCR. For instance, any metazoan group will occupy a small fraction of the total DNA in a typical soil in comparison to unicellular organisms. Hence, DNA has to be extracted from a larger sample and very deep sequencing has to be conducted to get a comprehensive taxonomical insight on the rarer taxonomic group, making the approach less cost effective. Different pre-DNA-extraction enrichment procedures can be used to circumvent this. This involves community DNA methods and traps or other approaches that concentrate the taxa of interest (Yu et al., 2012; Schnell et al., 2015). Also, hybridization capture methods allows for the capture of specific genes, e.g. mitochondrial DNA, prior to sequencing and thus allows for sequencing of full marker genes without the need for PCR amplification (Jacobsen, 1995; Liu et al., 2016; Shokralla et al., 2016; Gasc et al., 2016). In this way, specific taxonomic groups can be targeted without introducing most of the biases associated with the use of universal primers. However, Stat et al. (2017) found higher eukaryotic diversity by metabarcoding and concluded that metagenomics analyses presently lacks resolution.

1.4 Databases

Liselotte W. Andersen, Frank Panitz, Paul H. Krogh

A main limitation of next generation sequencing approaches lies in the vastly incomplete sequence reference databases (Hugerth and Andersson, 2017). This is true for both amplicon based and metagenomics approaches. Many DNA sequences are often assigned as unknown organisms or at a very high taxonomic rank due to the limited taxonomic coverage of the reference databases. The ability to assign taxonomy at a low taxonomic rank is important in e.g. diversity assessment. Continuous expansion and curation of reference databases is strongly needed to increase usefulness and reliability of eDNA results in diversity assessment. To assign species information to the generated reads, these have to be compared to a sequence database e.g. the 'nr' (nonredundant) database for searches in the Basic Local Alignment Search Tool (BLAST). BLAST finds regions of local similarity between sequences and compares nucleotide to sequence databases and calculates the statistical significance of matches (https://blast.ncbi.nlm.nih.gov/Blast.cgi). For characterizing an unknown sample composition it is, however, critical to obtain up-to-date versions of sequence databases that are as 'complete' as possible, since only matches to annotated sequences in the database will provide the information in question. Depending on the type of database used (genome, expressed genes, translated protein) and how the scoring is performed (local or global) the thresholds for scoring matches have to be tested and adjusted. In addition, the user has to decide for each experiment on the thresholds to be applied to filter 'successful' hits from potential false positives. Another approach is to create a dedicated database with all species with a high probability of being present in the target environmental area. When interpreting the results from the eDNA metabarcoding to identify the species, a high cut-off between 99-100% match to the sequences in the database should be considered. Furthermore, different species might have different error rates regarding eDNA-based detection. This suggest that there is a tradeoff between the number of species to be detected, the number of different primers (markers) to be applied, and the cost of the monitoring program. The lack of complete and trustworthy databases leads to the suggestion of avoiding the species assignment and instead use MOTU as the measure of diversity.

1.5 Data handling and bioinformatic analysis

Frank Panitz, Liselotte W. Andersen

A wide range of so-called next generation sequencing (NGS) technologies is currently used to investigate environmental DNA (e.g. Roche 454, IonTorrent, Illumina MiniSeq, MiSeq, HiSeq and NextSeq, and Oxford Nanopore) (Besser et al., 2018; Buermans and den Dunnen, 2014) indicating the rapid development of high throughput platforms during the last decade. A major challenge in applying these 'next generation' technologies is to make sure the users understand the potential limitations of the individual methods and protocols applied, e.g. sequencing library preparation, error rates or base composition biases, all of which can influence the interpretation of eDNA detection results.

One essential point of handling the data from NGS or other large-scale assays is to work towards standardization of the basic processing steps to ensure quality and reproducibility within and across eDNA studies. Sharing of analysis workflows and pipelines will increase reproducibility and the recording of retained and removed data provides transparency. The primary bioinformatics processing step should include removal of adapter sequences (introduced during sequence library preparation), quality filtering and length trimming of the reads to reduce unspecific and false-positive matches in database searches. Some authors (Chambert et al., 2015; Ficetola al., 2016; Lahoz-Monfort et al., 2016; Guillera-Arroita et al., 2017; Hunter et al., 2017; Brost et al., 2018) have suggested different approaches of how to monitor and control for false positives and false negatives, going from excluding or including them in the interpretation of the presence/absence in different modeling approaches. With this setup, it would be able to assign a probability to the occurrence of a species in a certain area. Still, there is a lack of common guidelines on how to include uncertainties associated with the methods in a Bayesian modeling setup.

Further, removing PCR-duplicates, chimeric reads, singleton sequences as well as filtering for sequence abundance are valid steps to reduce the complexity (and search space) of the sample prior to subsequent analyses. Ideally, all treatments and manipulation of the data, from the instrument raw sequencing reads to the final analysis, should be recorded. By sharing the scripts and analysis pipelines together with the quality cleaned raw data other researchers can not only redo the analysis but also reproduce the results. To this end, the (raw) data should be submitted to public sequence archives like the European Nucleotide Archive (ENA; https://www.ebi.ac.uk/ena), the Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra) or the Dryad Digital Repository (https://datadryad.org). In addition, users are to be encouraged to actively test different algorithm parameters or software settings in order to refine the analysis pipeline as standard options of bioinformatics tools are not necessarily tuned to be specific or selective enough for eDNA analysis.

Apart from the bioinformatics standard procedures to establish high-quality data sets (Thomsen and Willerslev, 2015; Creer et al., 2016), the complete workflow from sampling to laboratory procedures has an impact on the analysis results, e.g. the selection of the type of sequencing as well as the experimental design (Deiner et al., 2017). Among many other aspects, the following questions have to be considered:

- How many samples should or can be collected?

- Are technical replicates necessary for analysis?
- What meta data should be recorded?
- What sequencing platform will be applied?
- What read length is needed, and is paired-end sequencing required?
- What library preparation method should be applied?
- What sequencing depth or coverage is needed?
- How many samples are to be indexed, barcoded, multiplexed or pooled?
- What quality controls are to be included, e.g. qPCR, bioanalyzer measurements, spike-in of (unrelated) control samples?
- What potential biases have been identified during sampling and laboratory procedures, e.g. sampling volumes, reaction inhibitors, PCR primer biases, sequence coverage?
- What measures have been undertaken to minimize potential contamination, e.g. positive or negative controls?
- Is the reference database completeness representative for the taxa or species to be identified – or should the reference catalogue be improved before analysis of the experimental data?

The answers will depend on the specific research question and the specific conditions, including access to sample, statistical design of experiment etc.

2. Examples of eDNA used for environmental monitoring in aquatic environments

2.1 Aquatic plants and algae

Dorte Krause-Jensen, Peter Stæhr

Marine angiosperms and macroalgae are key elements for assessing ecosystem health in the framework of the European Water Framework Directive and the Marine Strategy Framework Directive. Traditional monitoring of aquatic plants and algae is usually conducted by visual field observations by divers of cover and species, or based on interpretation of underwater videos. This involves information such as:

- Biodiversity/species lists
- Site/depth-specific occurrence of key species, non-indigenous species, endangered species
- (Relative) abundance of species/functional groups

A recent review concluded that among the various biological quality elements (phytoplankton, benthic flora, benthic invertebrates and fish) the potentials for eDNA-based identification in ecological status assessment under the European Water Framework (WFD) is least appropriate for benthic flora (Hering et al., 2018). This group is typically surveyed in the field rather than being sampled and brought to the lab as a standard sample and indices typically rely on cover values as proxies for abundance. When species are easily detected with non-destructive traditional sampling, eDNA methods of species detection may, hence, not be more efficient than trained observers (Goldberg et al., 2016).

However, eDNA may supplement existing monitoring of marine angiosperms and macroalgae in several ways:

- eDNA techniques have a clear potential with regard to early screening for non-indigenous species or rare species that may be difficult to realize through traditional monitoring due to limited occurrence (Goldberg et al., 2016; Trivedi et al., 2016).
- eDNA may support species level identification of e.g. macroalgae and seagrass species for which traditional identification is challenging and barcodes are available (Trivedi et al., 2016).
- For assessing the diversity of species-rich macroalgal communities, eDNA techniques may also prove cost-efficient. The use of eDNA to monitor biodiversity on boulder reefs in Danish waters was recently explored (Stæhr et al., 2016). Diver-determined hard bottom flora and fauna along with pelagic taxonomic survey of phyto- and zooplankton were compared with eDNA diversity indices derived using metabarcoding. The results showed an overall good agreement between different measures of diversity. However, the traditional and eDNA-based species lists were not easily comparable, partly because several of the local taxa did not appear in the available DNA reference library. Hence, eDNA techniques are not yet mature for delivering full species lists as complete barcode libraries are not yet available for macroalgae and seagrasses.

- eDNA techniques are highlighted as being advantageous when conventional survey methods are logistically difficult to apply, have negative impacts on individuals or populations, have low probabilities of detection or are very costly (Goldberg et al., 2016). Hence, in remote areas, such as along Arctic coastlines where traditional monitoring is logistically challenging and costly, eDNA may prove useful once reference libraries are sufficient well developed.
- eDNA- analysis of water and sediment samples may provide information on the connectivity between benthic vegetated habitats and their near and far surroundings of marine surface sediments.
- eDNA analysis of marine surface sediments may also deliver important information on the contribution of benthic vegetated communities to sequestration of organic carbon. A recent study has used eDNA to identify the provenance of organic carbon in sediments of blue carbon habitats, i.e. coastal vegetated habitats (Reef et al., 2017). Such knowledge of the sources and fate of organic carbon stored in marine sediments is important for both managing coastal blue carbon stocks and understanding carbon cycling.
- Another complementary use of eDNA in the monitoring of aquatic vegetation is in relation to habitat-associated biodiversity. For this, both eDNA in water samples and, more integrative, eDNA in surface sediments could be relevant.

In the near future when more complete barcode libraries are likely to be available for aquatic vegetation, DNA techniques could, in principle, also be developed for use in alternative vegetation indices based on the relative abundance of species along the lines recently suggested for benthic invertebrate indices (Lobo et al., 2017).

2.2 Benthic invertebrates

Peter Stæhr

2.2.1 Why use molecular techniques for benthic monitoring and assessment?

Several directives and guidelines require sustainable management of marine resources and conservation of marine ecosystem health. To assess this, macrobenthic invertebrates are one of the key compulsory components in the framework of the European Marine Strategy Framework Directive and the Water Framework Directive (European Commission 2000, 2008). These programs are based on almost exclusively morphology-based approaches for species identification. This process is time-consuming, labor intensive and skills reliant, resulting in an expensive process. The use of DNA-based tools can circumvent many of these shortcomings, complementing traditional ecological sampling and providing a fast method to support monitoring and management strategies.

2.2.2 Experiences with benthic monitoring and assessment

For the moment, several marine institutions are experimenting with different molecular approaches, but this is still in its infancy, and a concerted routine application and implementation in biological monitoring and management does not exist. Molecular methods in benthic invertebrate monitoring is a relatively new area in environmental sciences, and knowledge and experience

have increased enormously over the recent years. National science foundations have supported various national projects like the Metabenthomics project in Norway at the NORD University, and a project on Molecular Taxonomy of Marine Organisms in Germany under the SENCKENBERG center. Both projects have improved the knowledge on potential best practice of introducing DNA-based methods in routine monitoring and increased the number of species sequenced in open source libraries.

A recent study compared morphological and eDNA/eRNA-based inventories of metazoans from samples collected around a fish farm in Scotland. It was found that molecular data faithfully reflected the morphology-based indices and provided an equivalent assessment of the impact associated with fish farms activities (Lejzerowicz et al. 2015). From this study, it was advocated that future benthic monitoring should integrate metabarcoding as a rapid and accurate tool for the evaluation of the quality of marine benthic ecosystems. Similar results were reported by Aylagas et al. (2016) who recommended that metabarcoding is valid for environmental status assessment and will contribute to accelerating the implementation of this technique to traditional monitoring programs.

The use of eDNA for monitoring of marine systems, including benthic invertebrates, is an area of great interest. One example concerns monitoring of non-indigenous species (NIS), where samples recently have begun to be taken for comparison of eDNA with traditional taxonomical identification (Fossing and Stæhr, 2017). Samples have been taken and initial steps have been made to prepare comparison with traditional monitoring data. As part of this, a connectivity modeling study is conducted to evaluate the spread of DNA from a few key Non-Indigenous Species (NIS). The use of eDNA to monitor biodiversity on boulder reefs in Danish waters was recently published (Stæhr et al., 2016) as described earlier (page 17).

2.2.3 Challenges in using eDNA as a tool for benthic invertebrate monitoring

A reliable reference library is important for the use of DNA-based approaches for monitoring of benthic invertebrates, especially since the genetic monitoring tool has to be widely applicable and transferable into specific environmental impact assessments throughout the monitoring area. Therefore, it is important that the DNA reference database comprises a wide range of bottom fauna species. It is not only important to include as many species as possible, but it is also key to include the regional variability within species, because a good knowledge of intra- and interspecific variability is crucial to accurately link taxonomic and functional information to sequence data.

There is some evidence that genetic-based indices of benthic marine fauna status (gAMBI) can be used in a similar way as the "traditional" AMBI (Aylagas et al., 2016). However, eDNA techniques are not expected to fully substitute traditional monitoring, but rather supplement. Overlapping periods with same samples analyzed with both technologies is found essential. It should be acknowledged that eDNA techniques provide different insights into diversity issues compared to traditional methods. It must be acknowledged that biotic indices rely on community analyses and metabarcoding or metagenomics are therefore needed. However, if a given species or a few species such as NIS or endangered rare species are targeted, then the qPCR approach is a good alternative as this method allows quantification and determination of thresholds.

2.3 Larger animals in aquatic environments

Liselotte W. Andersen, Frank Panitz

eDNA has been used to detect several larger animal species in the aquatic environment. The studies have focused on different species both pure aquatic (all life cycles in water) - as well as semi-aquatic animals. Amongst pure aquatic species studied are several fish species (Valentini et al., 2016; Atkinson et al., 2017; Clusa et al., 2017 (salmon); Balasingham et al., 2018 (non-indigenous species); Sigsgaard et al., 2015 (weather loach)), mussels (Stoeckle et al., 2016; Andersen and Wiberg, 2017 (freshwater pearl mussel); Deiner and Altermatt, 2014 (Unio tumidus); De Ventura et al., 2017 (zebra and quagga mussel)) and amphibians (Valentini et al., 2016; Harper et al., 2018) although not entirely aquatic. The truly aquatic species can be divided into sessile species like mussels and mobile species such as fish. They can be further grouped into whether they mainly occur in streams (Stoeckle et al., 2016) or lakes/ponds (Agersnap et al., 2017 (signal and narrow-clawed crayfish)). These environmental factors are important for the probability of detecting the species using eDNA. It is expected that there is a higher probability of detecting eDNA from species in stagnant water as in a pond compared to a stream with higher rate of water exchange. Common for the studies is that the majority use species-specific eDNA detection (mainly qPCR), reporting presence/absence of species and attempting to quantify their presence relatively (see e.g. Lacoursière-Roussel et al., 2016; De Ventura et al., 2017; Lopes et al., 2017). It is still difficult to quantify presence of species as numbers of individuals with eDNA. Semi-aquatic animals like otter are more difficult to detect using eDNA as the DNA is shed sporadically when the animal is in the water and especially in streams (Thomsen et al., 2012 (mainly lakes/ponds); Andersen et al., 2018 (stream)). Thomsen et al. (2012) searched for otters in streams, ponds and lakes with known otter populations by using qPCR with species-specific primers. The low detection rate of 27% was attributed to their semi-aquatic lifestyle and the large size of territories. Later, Andersen et al. (2018) searched for otters in streams categorized as inhabited by no, semi-high or high density of otters by sampling water at one time point. Positive otter detection was only possible in one sample. Increasing the sampling events, e.g. sampling every hour for 24 hours and/or filtering larger water volumes, are expected to increase the detection rate. Hence, to monitor larger animals the sampling designs have to be extremely well planned.

Primer-based eDNA metabarcoding is used mainly for monitoring biodiversity (biomonitoring), i.e. detecting the presence of several species and here, as well as for the species-specific detection method, quantification of the species is also attempted (Valentini et al., 2016; Pont et al., 2018). To use primer-based eDNA metabarcoding for biomonitoring in Denmark it is necessary to analyze several reference localities to obtain the same bio-indices for both, conventional and eDNA monitoring. This has been shown to work e.g. for diatoms that are now used as bioindicators of water quality in Switzerland (Visco et al., 2015).

For larger animals with a limited species range, it may be recommendable to create a dedicated database containing all the species in the area that are expected to have a high probability of being present. When interpreting the results from the eDNA metabarcoding a high cut-off between 99-100% match to the sequences in the database should be set for taxonomy, discarding all other matching results.

3. Examples of eDNA used for environmental monitoring in terrestrial environments

3.1 Plant pathogens and pests (vira, bacteria, fungi, and nematodes)

Mogens Nicolaisen, Niels B. Hendriksen, Rumakanta Sapkota

Up to now, numerous eDNA studies related to plant pathogens are dealing with basic questions regarding e.g. the ecology of pathogens, their interaction with the plant, other microbes including beneficials and the effect of the environment on the plant microbiomes (e.g. Agler et al., 2016; Walder et al., 2017 and references therein). Relatively few studies have dealt with eDNA as a tool for monitoring or diagnosing plant pathogens. These studies (Maree et al., 2018 and references therein) indicate that eDNA techniques do have the potential to be used for diagnostics and monitoring of plant pathogens. When the causal agent of symptomatic plants is unknown and no targeted analysis is possible, eDNA may be relevant for diagnosis (Rott et al., 2017). eDNA technology may also be relevant for monitoring the environment for infectious propagules in irrigation water, soils and air. This could be used in early warning systems to enable timely and targeted treatment of crops before symptoms are visible or even before spores have landed on the plant. For example, fungal pathogens were detected using air samplers placed in urban settings or on agricultural fields to clarify the potential for early warning systems (Nicolaisen et al., 2017). Likewise, eDNA methods could be used for monitoring air or irrigation water for harmful organisms in greenhouses.

In relation to conventional diagnostics (determination of the causal agent of a disease), next generation sequencing technology has until now mostly been used for plant-virus diagnostics. The genetic material of most viruses is RNA and there are no consensus sequences that can be used for genetic amplification. Instead, total plant RNA or virus-enriched RNA is used to generate reads of total RNA in the plant (Maree et al., 2018). This genetic technology made it possible to obtain a much more complete picture of the viral content of plant tissues compared to targeted analysis. This includes the actual causal agent of a given disease but also leads to identification of many other viruses that are apparently not causing any symptoms in the plant. Identification of this high number of hitherto unknown plant viruses has led to discussions of the legislation and regulation of viruses that are not causing any apparent yield decrease or other problems.

One of the challenges in using eDNA methods for identification of pathogens and pests of fungal, nematode or bacterial origin is that the genetic resolution of marker genes in many cases has to be very high to be able to discriminate between pathogenic and non-pathogenic species or even strains. This is currently not possible with the most commonly used markers for fungi (the internal transcribed spacer (ITS)), bacteria (16S rDNA) or nematodes (18S rDNA). Within many species of bacteria and fungi there are many pathovars, formae speciales etc. that are very host specific meaning that identification to species level may not imply that the organism is pathogenic on a given host. The 18S region used for nematode identification is only capable of discriminating at genus level. In a few cases markers with a higher resolution are being

introduced, for instance in Fusarium where the use of an elongation factor-based marker increases resolution (Karlsson et al., 2016).

For a specific disease or symptom, identification of the primary causal agent is often problematic due to secondary infection by saprotrophs or necrotrophs. The weakened or dead plant tissue, which is more susceptible, and hence, an infection site will typically be enriched with several bacterial or fungal species.

3.2 Soil fungi, protists and invertebrates

Paul H. Krogh, Toke Bang-Andreasen, Susana Santos, Anne Winding

3.2.1 Fungi

Fungal community composition is mainly assessed using primers targeting the eukaryotic internal transcribed spacer (ITS) regions (Peay et al., 2008). These regions are situated between the chromosomal small and large subunit rRNA genes. The full ITS region has an average length of 600 bp across all fungal lineages (Porter and Golding, 2011). In eukaryotes, two ITS regions are present. ITS1 is located between 18S and 5.8S rRNA genes while ITS2 is located between 5.8S and 28S (in animals and fungi). These regions contain well-conserved fungal specific priming sites, which flank multiple highly variable regions thereby allowing for taxonomic discrimination of fungi by targeting these. Other marker genes have been used for fungal diversity assessment (e.g. CO1, 18S rRNA and 28S rRNA) but the ITS2 regions has been shown to provide the most successful identification for the broadest range of fungi and has been recognized as the formal fungal DNA barcoding region (Schoch et al., 2012; Seifert, 2009).

3.2.2 Protists

Protists are the most diverse and the least known group of soil eukaryotes, and their functional importance expands well beyond being bacterivorous and fungal feeders (Geisen and Bonkowski, 2018). Universal primer sets for the assessment of soil protists are challenged by the polyphyletic nature of the protist group (Pawlowski et al., 2012; Baldauf et al., 2000) and the high genetic divergence between and within major protistan groups (Fiore-Donno et al., 2016). Even closely related protist species fundamentally differ in copy numbers of targeted barcode genes, which makes it inapplicable to relate qPCR copy number to abundance for a wider range of protists (Geisen and Bonkowski, 2018). Marker gene regions of protists targeted by universal primers (18SrRNA gene) typically result in biases towards a few lineages. For instance, the phylum *Amoebozoa* is often highly underrepresented in amplicon-based assessment of protists, while primer-independent metagenomics approaches identifies *Amoebozoa* as a main protist group in soils (Fiore-Donno et al., 2016). This underrepresentation is likely caused by amoebozoans having longer sequence reads of the targeted marker-gene region resulting in reduced success in PCR amplification in comparison to shorter sequences from the same marker-gene region of other protists (Geisen et al., 2015). In contrast, cilliates are most often highly overrepresented when universal primers are used due to their shorter SSU rRNA sequences that ease amplification, their multiple nuclei and their high SSU rRNA gene copy number (Gong et al., 2013; Geisen et al., 2015). Large fractions of obtained sequences will originate from organisms outside the target group (plants, fungi, metazoan) and filtering out of

relatively large fractions of the obtained sequences must be expected. Nevertheless, in focused studies where the same methodology is being used, amplicon-based studies provide invaluable tools that allow untargeted analyses of nearly the entire diversity of, so far, unknown protists in soils. Due to the polyphyletic nature and the challenges associated with this, recent biodiversity studies of protists generally focus on a specific group, e.g. amoebae (Le Calvez et al., 2012), Cercozoans (Harder et al. 2016; Fiore-Donno et al., 2017), or ciliates Lara et al. (2007). Using metabarcoding with universal primers in addition to ciliate specific primers, Santos et al. (in prep.) found protist as well as ciliate diversity in agricultural soils to vary across Europe depending on moisture, precipitation and also land-use. Moreover, primer biases can nowadays be avoided by applying new molecular methods such as metagenomics and metatranscriptomics to reveal soil protist communities (Geisen et al., 2015; Jacquiod et al., 2016). Using a metatranscriptomic approach Geisen et al. (2015) concluded that *Rhizaria* and *Amoebozoa* protist communities were dominating soils from different forests and grasslands soils, while *Alveolata* were most abundant in peat soils.

3.2.3 Collembola

Often collembolans are proposed as indicators of environmental conditions so the state-of-the-art of collembolan reference databases is considered here. Currently, the number of collembolans on The Barcode of Life Data System (BOLD) (Ratnasingham and Hebert, 2007), which is assigned an unequivocal species name, is 292 and when including species lines the number is 347. However, the number of BOLD collembolan BINs (Barcode Index Number) is 4989, which is in the order of the number of described species of 8854 (Bellinger et al., 2018). These BINs will include many as, yet, undescribed species, but will deliver verified operational taxonomic units (OUT) information for monitoring studies. The most recent inventory of Danish collembolans (https://allearter.dk/english/) arrives at 248 species of which 97 have a perfect species name match with BOLD. Expectedly, if all 4989 public barcodes of collembolans had been assigned a species name, most of the Danish collembolans would find a match among those. In other words, current BINs will cover the majority of Danish species and reference databases are therefore sufficiently populated with COI barcodes to support eDNAbased environmental monitoring. Enchytraeids are also relatively well covered with COI and the H3 gene barcodes (Ratnasingham and Hebert, 2007; Clark et al., 2016). Earthworms are very well covered and references are at an adequate level (Ratnasingham and Hebert, 2007; Clark et al., 2016).

3.2.4 Monitoring

In soil ecosystems, the use of eDNA includes:

- monitoring of biodiversity which is a cornerstone in conservation biology (Frøslev et al., 2017; Thomsen and Willerslev, 2015),
- assessing soil quality and fertility of production soils having agronomic potentials (Chaparro et al., 2012; Bender et al., 2016),
- retrieving species knowledge of palaeoecological importance (Willerslev et al., 2003; Haile et al., 2009; Willerslev et al., 2014),

- assessing microbial responses and their impact on global climate change (Hultman et al., 2015; Zhou et al., 2011)

- investigation and improval of bioremediation and bioaugmentation of contaminated soils (Techtmann and Hazen, 2016; Liu et al., 2017; De Vrieze et al., 2017).

A necessary prerequisite for eDNA biodiversity monitoring programs is extensive DNA-reference databases. Such databases are continuously growing, so this issue will be gradually solved in the future. However, until then MOTU will be used to assess biodiversity (Pawlowski et al., 2018). For a proper environmental monitoring program to operate efficiently it needs baseline data and thresholds to guide the management decisions of land and mitigation measures. Baseline data for soil invertebrates are also in its beginning where the German edaphobase (https://portal.edaphobase.org/) holds data mostly from Germany, but are in the process of being supplemented with European data presently scattered in local national databases and by individual researchers. At the European Joint Research Centre (JRC) soil physicochemical data are available in databases, however, the spatial resolution of these is still too coarse to serve this purpose. JRC are now describing soil biodiversity employing metabarcoding eDNA techniques across Europe in the LUCAS18 (Land Use and Coverage Area frame Survey, https://esdac.jrc.ec.europa.eu/projects/lucas).

No public environmental monitoring program has yet employed eDNA techniques for soil invertebrates and protists. However, the research and development underpinning this endeavor is well under way. Currently, the German Environment Agency UBA, overseeing the permanent soil monitoring sites of the German federal states, so-called BDF (Bodendauerbeobachtungsflächen), is launching an investigation of the eDNA-tools for monitoring of earthworms, enchytraeids and collembolans. Although reference databases and metabarcoding protocols exist for these taxons at the Danish eDNA Center at Aarhus University, there are still questions concerning sampling designs and possible habitat differences that may require particular methodological consideration.

3.3 Endangered species

Liselotte W. Andersen

The few studies that have traced animals using eDNA metabarcoding based on eDNA from soil are conducted under controlled conditions, where the animals were known to occur, like safari parks or zoos (Andersen et al., 2011), or from natural areas where the species are known to occur.

Drummund et al. (2015) studied eukaryote species variation above and below ground using eDNA from soil but were not able to detect endangered species. Other studies used iDNA (invertebrate derived DNA) from insects like carrion flies (Rodgers et al., 2017) or leeches (Schnell et al., 2012; Schnell et al., 2015) to monitor terrestrial mammal biodiversity e.g. to successfully trace the Saola antelope (*Pseudoryx nghetinhensis*) in Vietnam. Schnell et al. (2012) succeeded to discover two species only recently described, the Truong Son muntjac (*Muntiacus truongsonensis*) and Annamite striped rabbit (*Nesolagus timminsi*).

Another approach to detect mammals and potentially endangered species is to sample eDNA from natural saltlicks and trace mammal biodiversity by eDNA metabarcoding (Ishige et al., 2017). The authors targeted the sampling towards areas where the rare and endangered Bornean orangutans occur and they succeeded to detect eDNA from the orangutans. Ushio et al. (2017) used water samples from forest ponds to detect terrestrial mammal diversity and showed the feasibility to detect some of the sequences from the mammals in the area, but observed no endangered species. Using water from waterers, wallows or artificial wallows as eDNA source, Williams et al. (2018) traced feral pigs suggesting the possibility to use these eDNA sources to trace terrestrial mammals. These studies illustrate that there is a fundamental difference between tracing eDNA from e.g. freshwater compared to soil. Using eDNA from soil to trace mammals is context dependent but also dependent on abundance and volume and can be used when mammals are large and common; e.g. Andersen et al. (2011) traced elephant eDNA at all localities in the Zoo.

Generally, the knowledge of the ecological behavior of the mammal, e.g. whether it returns to the same spot frequently, is essential for the sampling design. Based on the studies reported, there are three kinds of eDNA that might be useful to trace terrestrial and/or endangered mammals:

- targeted traces like feces, hair, urine, or footprints sampled in areas where the mammals are expected to occur,
- from carrion flies/insects and leeches containing DNA from mammals/endangered species,
- where the DNA ends - i.e. ponds/drinking water, saltlicks etc.

3.4 Rewilding

Liselotte W. Andersen

The use of eDNA in rewilding is an emerging field. One direct approach is tracing regeneration and rewilding of an ecosystem using primer-based eDNA metabarcoding to monitor biodiversity after anthropogenic effects like deforestation in the rainforest or in connection with nature restoration of biodiversity to evaluate the effect of the initiatives (Bohmann et al., 2014; Clare, 2014; Cristescu and Hebert, 2018). An indirect approach is through the analysis of diet composition, tracing foraging effects on the ecosystem of herbivore species (Nichols et al., 2015; Fløjgaard et al., 2017), either naturally occurring or introduced. Another indirect approach to track rewilding is using species-specific detection answering questions of species recolonization in an area, like the wolf in Denmark and Sweden (Åkesson et al., 2016) or the unintentional rewilding of bighorn sheep on Tiburón Island (Wilder et al., 2014). In these cases, the eDNA sampling is targeted in animal remains like feces or other physical remains left by the species in question. These colonization processes are reflecting passive rewilding, tracking nature restoration with no intervention.

4. Conclusion

Anne Winding, Paul H. Krogh, Liselotte W. Andersen

Environmental monitoring using eDNA is receiving great attendance, while actual practical use of the technique is less frequent. The technique is promising but several issues have to be well documented before eDNA can be fully included in national environmental monitoring programs. In this report the use of eDNA for monitoring in both aquatic and terrestrial environments, with detection of specific species or group of organisms as well as biodiversity assessment, is discussed. Several issues and considerations have been discussed and examples given of environmental monitoring using eDNA. Generally, eDNA techniques have shown promising results. In most areas of environmental monitoring eDNA is being considered and incorporated alongside, supplementing or replacing traditional monitoring. However, some concerns and areas of development still exist:

Among the technical issues that needs attention are:

- Longevity of eDNA and the occurrence of partially degraded DNA in the environment and across different environmental conditions needs further clarification.
- Detection of specific organisms by eDNA is feasible using qPCR.
- Robust Standard Operating Procedures should be developed including design of PCR primers and bioinformatics analysis.
- Quantifying abundance (either as biomass or numbers of individuals) of organisms using eDNA is still in the developmental phase.
- Reference databases for eukaryotes are generally less fully developed compared to prokaryotes, and significant improvements are ongoing.
- Handling and modelling of uncertainties associated with the different steps (field sampling, laboratory procedures, bioinformatics etc.) of eDNA-based techniques need further investigation and standardization.

The use of eDNA for environmental monitoring has been presented as a technique of replacing existing traditional monitoring techniques, being faster, easier and more accurate. At the present level of the methodology this has not been achieved, although the potentials of eDNA approaches are increasingly unfolding. Currently, no routine genetic monitoring is taking place from a management perspective, but different trials using eDNA approaches are ongoing in several countries. Sharing, harmonizing and consolidating the available knowledge is therefore of prime importance in order to develop standardized procedures throughout all the steps of the process (sampling, DNA extraction, amplification primers and conditions, bioinformatic analysis), including handling of false positives and negatives. Databased scientific literature clearly demonstrates that even though sampling might be easier and faster, sequencing library preparation is still costly despite continuously decreasing, while standardized bioinformatics procedures (pipelines) make analyses more efficient. eDNA-based results generally show different aspects of the environmental state with increasing detailed knowledge on a different scale than traditional monitoring. This applies to biodiversity and species-specific occurrence and abundance.

The eDNA methods will often reflect the state of different elements of the ecosystem compared to traditional methods. This has raised the question of whether we actually need the parameters measured with traditional methods or whether the paradigm should be changed, including how well the environmental status is defined in time and space. This is beyond the current report, but raises the question if we need to know the species name for evaluating the environmental quality or if it can be evaluated by sequencing data in terms of molecular operational taxonomic units (MOTU).

The conventional long time series of environmental monitoring is of high value and great care should be taken not to compromise these without careful evaluation of the benefits of moving to eDNA approaches. If we convert all environmental monitoring to eDNA and stop collecting specimens for collections, we lose the possibility of certain studies requiring e.g. long time series. Therefore, it is recommended to perform parallel monitoring with eDNA-based techniques and the traditional monitoring until sufficient experience and data have been secured to be able to follow the environmental state back to the time before eDNA monitoring.

The standardized sample collection and treatment and the following data analysis are among the many benefits of eDNA-based monitoring. The easy storage of the eDNA samples for later analysis opens new possibilities of adopting future advanced analytical tools addressing hitherto unknown parameters. Sampling across the year and seasons is recommended as this will pave the way for generating a fuller genetic picture.

Future techniques will probably allow metabarcoding based on longer reads thanks to advances in sequencing technologies. Another branch of eDNA analysis is expected to focus on direct sequencing of eDNA omitting PCR amplification and the biases associated with this. In addition, the bioinformatics analyses are anticipated to be standardized and detailed in standard operating procedures. *In situ* monitoring will most likely be possible, either through autonomous samplers coupled with qPCR assays or portable sequencers.

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eDNA IN ENVIRONMENTAL MONITORING

The use of environmental DNA (eDNA) for environmental monitoring has been pre-sented as a technique of replacing existing traditional monitoring techniques, being faster, easier and more accurate. In both aquatic and terrestrial environments detection of specific species or group of organisms as well as biodiversity assessment have shown promising results with eDNA. However, several technological and data assessment issues have to be resolved prior to full employment in environmental monitoring. Sharing, harmonizing and consolidating the available knowledge is therefore of prime importance in order to develop standardized procedures throughout all the steps of the process (sampling, DNA extraction, amplification primers and conditions, bioinformatic analysis). eDNA based results generally show different aspects of the environmental state with increasing detailed knowledge on a different scale than traditional monitoring. Future techniques will probably allow metabarcoding based on longer reads thanks to advances in sequencing technologies. Another branch of eDNA analysis is expected to focus on direct sequencing of eDNA omitting PCR and the biases associated with this. Also, the bioinformatics analyses are anticipated to be standardized and detailed in standard operating procedures.

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