ENVIRONMENTAL GENOMICS RESEARCH PROGRAMME

An IOGP programme

International Association of Oil & Gas Producers

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eDNA Sampling Standards and Guidelines

This Guidance is publicly available for readers to review and use. Any comments that improve the content or workability of the guidance are welcomed by the JIP. We will endeavor to address comments in future updates to the guidance. Updates are planned every 1 to 2 years to capture the rapid advances in this field. Please send comments to info@iogp-edna.org.

Prepared by Stantec and NatureMetrics for the International Oil and Gas Producers' Association Joint Industry Program on Environmental Genomics.

Stantec

NATURE METRICS

Disclaimer

Revision history

REVISION	NAME	COMPANY	DATE OF ISSUE	COMMENTS
R1	Marc Skinner	Stantec	2024-Jan-25	First release.
R2	Marc Skinner	Stantec	2024-Mar-22	Revision of initial release following feedback from JIP members
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R6	Michael Wright	eDNAtec	2025-Jun-18	Cross-referencing and alignment in terminology between chapters
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Executive Summary

Energy companies collect ecological samples to characterise environments in which they operate to pursue environmental stewardship, sustainability goals, and regulatory compliance. Conventional ecological measurement approaches are challenging to implement and due to inherent imprecision may not reflect the biodiversity that is present as many species are difficult to detect. Further, these surveys are often expensive and risk human safety due to reliance on specialised and heavy equipment in potentially harsh monitoring environments.

Environmental genomics is an emerging technology that offers the potential to mitigate these risks, specifically in the areas of probability of detection, specimen identification, data quality, survey and processing time, safety, and overall project cost. While environmental genomics has advanced considerably, bringing these tools into mainstream use for biodiversity surveys requires standardisation of protocols. Our goal is to provide oil and gas business line staff with a guidance document for the industry-specific application and integration of environmental genomics as a routine approach for biological monitoring to support environmental management activities.

Specifically, this guidance document advises on minimum sampling design requirements to permit practitioners to deploy environmental genomics technology with confidence in results and identify critical knowledge gaps to be filled to design a robust sampling program. This guidance focuses on the "First Principles" of sampling and monitoring programs to address environmental requirements, nuanced with specific considerations related to environmental genomics. We leverage existing international guidance and standards for the application of environmental genomics for biological survey and monitoring. We consider current limitations and knowledge gaps to provide high-level recommendations towards the goal of fostering regulatory uptake and confidence to minimise constraints on obtaining environmental permitting approvals for energy industry activities. Evaluation, prioritisation and developing means of addressing these knowledge, understanding and methodology gaps are key to countering regulator uncertainty and reluctance to employ environmental genomics in decision-making processes.

Many regulatory jurisdictions have relatively little or no previous experience with industrial permitting requests proposing genomic tools but are generally receptive to these approaches, particularly for geographies or ecological groups which are data-poor or previously unstudied. Our work indicates that most regulators do not generally rely on this technology for the assessment of industrial proposals yet will readily utilise environmental genomics to satisfy internal mandates related to biodiversity surveys, invasive species detection, or effects assessments. To maximise regulatory uptake and confidence, industry must address five intersecting knowledge gaps related to: understanding environmental eDNA persistence and dispersal; large-scale integration of eDNA data with different data types; improvement of reference library databases; molecular refinement of taxonomic indices; and standardisation. Additionally, emerging techniques and technologies must be incorporated into this constantly evolving field of science. We present recommendations related to employment of environmental RNA (eRNA), in-field analyses, automated eDNA samplers, airborne and passive eDNA collection, and collecting eDNA by swabbing surfaces of various substrates previously contacted by target species.

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Abbreviations

AMBI	AZTI Marine Biotic Index
ASV	Amplicon Sequence Variant
BOEM	Bureau of Ocean Energy Management
BOLD	Barcode of Life Data Systems
bp	Base Pair
BSA	Bovine Serum Albumin
COI	Cytochrome Oxidase I
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSA	Canadian Standards Association
ddPCR	Droplet Digital PCR
DEFRA	Department for Environment, Food and Rural Affairs
DFO	Fisheries and Oceans Canada
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DoC	Department of Conservation
eDNA	Environmental DNA
EIA	Environmental Impact Assessment
EPA	Environmental Protection Agency (United States)
eRNA	Environmental RNA
GRDI	Genomics Research and Development Initiative
HTS	High-Throughput Sequencing
IEC	International Electrotechnical Commission
IOGP	International Association of Oil and Gas Producers
IPC	Internal Positive Control
ISO	International Organization for Standardization
JIP	Joint Industry Program
MBARI	Monterey Bay Aquarium Research Institute
MOTUs	Molecular Operational Taxonomic Units
MSFD	Marine Strategy Framework Directive
NEA	Norwegian Environment Agency
NEPA	United States *National Environmental Policy Act*

NGS	Next-Generation High Throughput Sequencing
NOPSEMA	National Offshore Petroleum Safety and Environmental Management Authority
O&G	Oil and Gas
OPRED	Offshore Petroleum Regulator for Environment and Decommissioning
OSPAR	The Convention for the Protection of the Marine Environment of the North-East Atlantic
OTUs	Operational Taxonomic Units
qPCR	Quantitative Real Time PCR
rbcl	Ribulose-Bisphosphate Carboxylase Gene
RNA	Ribonucleic Acid
ROV	Remotely Operated Vehicle
rRNA	Ribosomal RNA
RT-PCR	Reverse Transcription- Polymerase Chain Reaction
SOP	Standard Operating Procedures
UK	United Kingdom
UV	Ultraviolet

1 Introduction

To pursue environmental stewardship, sustainability goals, and to comply with regulatory mandates, energy companies undertake a variety of ecological measurements to characterise and/or monitor the environments in which they operate. These measurements apply to all stages in the life cycle of energy projects, including exploration and appraisal; development (site selection and construction); operations; and decommissioning/post-decommissioning.

Although broadly used, these conventional approaches are challenging to implement and may not accurately capture the biodiversity that is present. For example, many species are difficult to detect using visual methods and acoustic devices observations. These conventional approaches can be inaccurate and/or imprecise as: many species are difficult to visually record/identify using morphology-based techniques or audibly with passive acoustic monitoring devices (PAM); microscopic taxa or ambiguous/cryptic species are difficult to capture and/or identify; and rare species are difficult to detect. Further, surveys performed using conventional methodology often incur high expenses and risk to human safety due to reliance on specialised and heavy equipment, time spent in the field and waiting for results from the surveys, and the cost of hiring specialists for specimen identification or visual field observations. For example, the identification of biotic specimens relies on expert taxonomists who are often unavailable or require a long lead time for specimen identification, sometimes on the order of months to years. Additionally, in-situ monitoring of megafauna (e.g., marine mammals) pose challenges because they are often difficult to detect due to low density, low visibility for observation, harsh monitoring environments, and the need for customised detection techniques (e.g., acoustic detection for vocalizing fauna).

Environmental genomics is an emerging technology that offers advantages and improvements over conventional ecological approaches, specifically in the areas of probability of detection, specimen identification, data quality, survey and processing time, safety, and overall project cost. Environmental genomics (also known as ecological genomics) broadly refers to the study of genetic material recovered directly from environmental samples. Environmental genomics has the potential to be less costly, faster, and provide more complete biodiversity inventories than conventional methods. The approach relies on sampling the habitat where target organisms live (i.e., water, soil, sediment) to examine the genetic material that is present, whether this be direct detection of microbes or indirect detection of other fauna based on DNA that has been shed into the environment.

In recent years, the application of environmental genomics has gained momentum as an alternative, non-invasive, scalable, time- and cost-effective approach for biological surveys. Although these methods have already advanced considerably, bringing environmental genomics into mainstream use for biodiversity surveys requires standardised approaches and requires standardisation of protocols. The goal of this document is to provide Business line staff with a guidance document for the industry-specific application of environmental genomics methods to support environmental management activities in the energy industry and thereby facilitate the

integration of environmental genomics as a routine approach for biological monitoring across the energy industry.

1.1 Objectives

Environmental genomics methods have advanced considerably within the research realm; guidance is needed to bring these methods into routine use for biological monitoring. Business line staff of resource extraction companies may not possess specific technical experience in genomics yet are required to solicit and evaluate the proposals or deliverables of contractors, consultants, or subsidiary companies. The goal of this guidance document is to translate the potentially dense technical details of environmental genomics into a business line user-friendly tool to assist in the guidance on sampling standards and guidelines. The guidance presented in this document:

- stipulates sampling design considerations that allow environmental practitioners within industry to deploy environmental genomics technology or have technical oversight of deployed technology and confidently rely on results
- is relevant to a range of environments, taxa and questions, so that it is broadly applicable across the energy industry
- allows identification of critical knowledge gaps to be filled to design a robust sampling program

1.2 Approach

The aim is for the main document to provide background information and guidance for specialists and non-specialists with technical details provided in appendices that can be kept updated as new information becomes available on applications of environmental genomics tools. The front matter of this guidance focuses on the “First Principles” of sampling and monitoring programs to address environmental requirements while applying genomic-specific considerations, particularly in the context of energy sector operations. As outlined in Figure 1-1, we begin with background information on environmental genomics and an overview of approaches used for biological surveys and monitoring. Next, we provide a summary of guidance and standards that have been developed for the application of EG. Then we identify the first principles that lay the foundation for the design and implementation of environmental genomics for biological survey and monitoring. This portion of the guidance provides foundational elements of environmental monitoring for specialists and non-specialists to aid non-specialist and specialist technical staff alike.

Specific technical details are provided in supporting appendices that can be easily updated as new information becomes available. This approach will allow the main document deliverable to remain accessible to non-specialists and provide a depth of technical resources as attachments for the benefit of more experienced genomic practitioners.

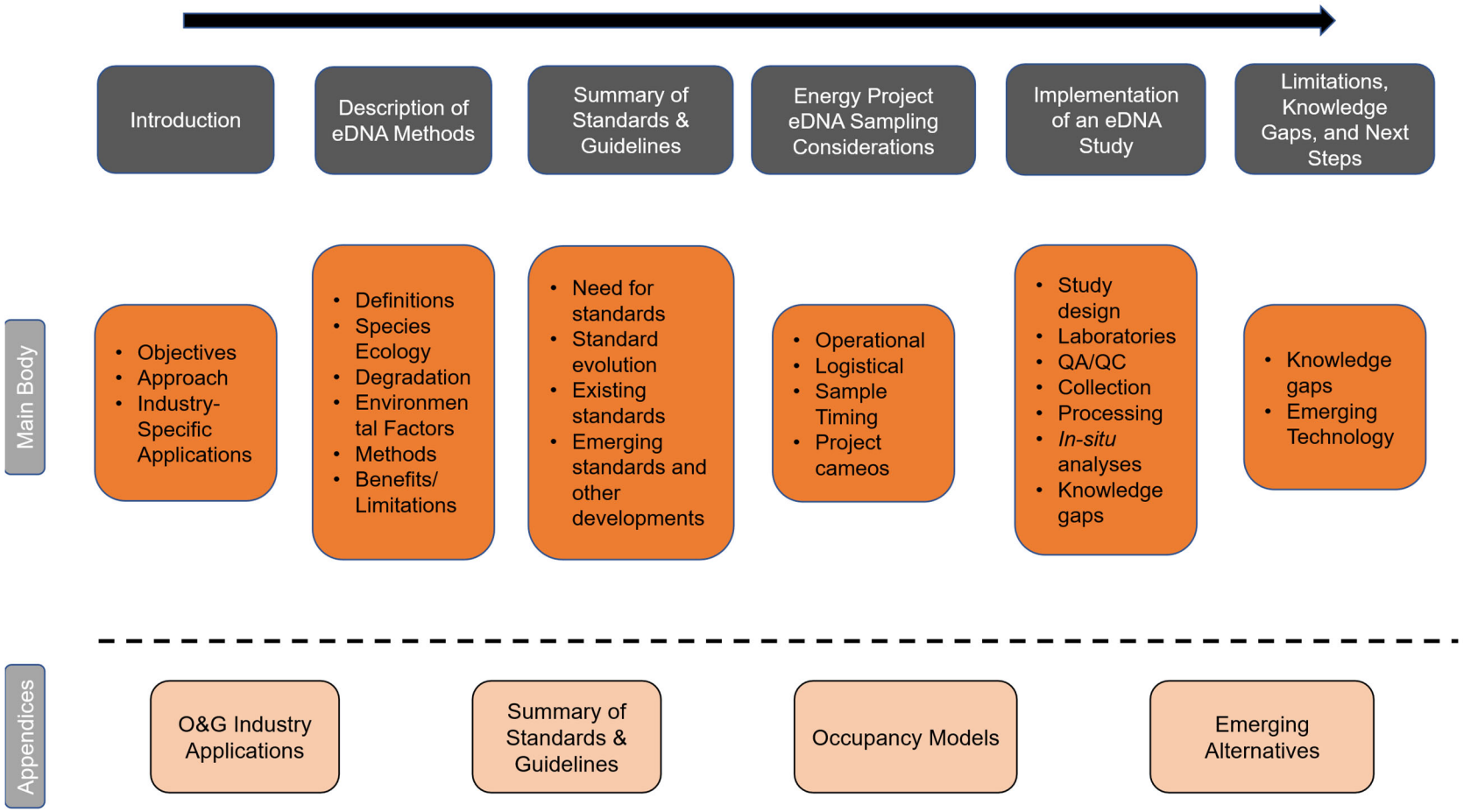

Figure 1-1 Guidance Document Structure

1.3 Industry-Specific Applications

The priority areas for development of this guidance were previously set by the IOGP-JIP. The IOGP-JIP developed a matrix identifying the Potential Industry-Specific Applications of greatest interest to energy companies (Appendix A). The matrix, as well as the current guidance are intended to be "evergreen" such that they can be updated by the IOGP-JIP as the technology area and applications progress. Both are intended to reflect the current understanding of the industry regarding potential applications of environmental genomics. The Potential Industry-Specific Applications currently identified within the matrix are:

- rapid assessment of potential invasive species
- early detection of newly introduced invasive or competitive species
- detection of key species
- population status & dynamics
- habitat delineation
- baseline assessments
- monitoring of environmental effects of oil & gas activities
- remediation / restoration
- bio-corrosion
- early warning and micro-leakage detection.

Within the matrix, the IOGP-JIP prioritised the Potential Industry-Specific Applications from lowest (1) to highest (3) according to each member company's level of interest in the individual Industry Focus Areas. Potential Industry-Specific Applications with an average priority score greater than 2 (Appendix A, Table A.1) were considered in this report while those with scores less than 2 generally were not unless they were considered immediately relevant (Appendix A, Table A.2). For further information on these applications, please refer to Environmental Genomics Applications for Environmental Management Activities in the Oil and Gas Industry: State of the Art Review and Future Research Needs (Stantec, 2020).

While considerations relevant to the application of eDNA technology to compliance and monitoring of environmental effects of energy project activities are of high priority for industry, these will be addressed in subsequent IOGP guidance.

2 Description of eDNA Methods

2.1 Introduction to eDNA

DNA-based taxonomic and functional profiling is widely used for the characterisation of organismal communities across an array of practical applications and research areas e.g., the role of microbiomes in health and disease, biological monitoring, and estimation of both microbial and metazoan species richness. Prior to delving into the finer details of this topic, we define and contextualise some fundamental terms and concepts below as there is an overlap and contradiction between the existing medical and ecological literature.

2.1.1 Definition of eDNA

Environmental DNA (eDNA) is defined as genetic material that has detached or sloughed off from an organism (in either intra- or extracellular form) into non-living components of an ecosystem such as air, water, or sediment (Díaz-Ferguson & Moyer, 2014; Pilliod et al., 2013a; Thomsen & Willerslev, 2015). Organisms will shed eDNA into the environment through death or from bodily functions such as renewal of skin cells, regurgitation and discharge of mucus, urine, feces, and gamete release or via the process of cell death and release of DNA (Díaz-Ferguson & Moyer, 2014; Pedersen et al., 2015; Pilliod et al., 2013a).

As noted previously, environmental genomics broadly encompasses examination of genetic material that is present within the environment of interest and includes a spectrum from direct collection of microbes and microfauna to indirect collection of macrofauna based on DNA that has been shed into the environment. For the purposes of this guidance document, the terms environmental genomics and eDNA are both applied to discussions of microbial communities.

2.1.2 Detection of eDNA

The field of eDNA technology has rapidly developed and eDNA methods have been applied to a wide range of research and monitoring projects globally. Non-invasive, rapid, sensitive, and scalable detection of practically any species in any environment, including cryptic, rare, elusive, and microscopic taxa, makes eDNA a transformative approach to biomonitoring. Several works have provided detailed eDNA reviews that outline important considerations for specific topics associated with eDNA sampling and analysis, and these can be found in Appendix B (Table B-2).

The success of an eDNA program relies on characteristics relating to the state of eDNA when released to the environment, which includes factors that influence shedding and degradation rates, which will affect the probability of collection and ultimately the detection of an eDNA molecule (Figure 2-1). We briefly introduce these concepts below, considering the type of media (water, soil, or sediment) and the environment (freshwater, marine, or terrestrial) being sampled. Further practical details are provided in Sections 4 and 5.

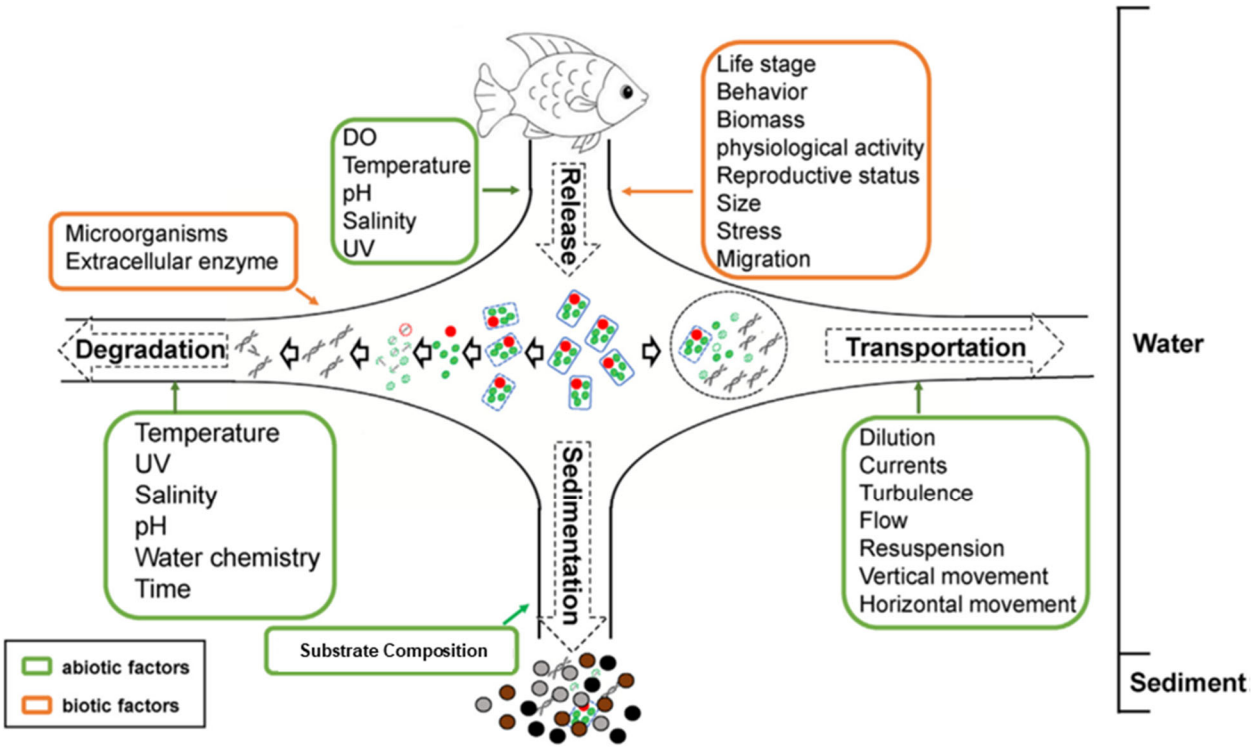

Figure 2-1 Schematic diagram illustrating the known associations of various influencing factors (biotic and abiotic) and the basic processes and fates of fish eDNA (from Wang et al. 2021).

2.1.2.1 Ecology of the Species of Interest

Understanding the ecology of the target species or taxonomic group is a crucial consideration for planning an eDNA sampling design. The ability to detect a species will depend on the concentration of its eDNA within the environment. Therefore, detection of a target species through eDNA is influenced by seasonal patterns of its ecology and spatial and temporal movements. In addition, eDNA shedding rates are heavily linked to metabolic activity, as has been demonstrated for seastars and fish in relation to food availability (Klymus et al., 2015; Kwong et al., 2021). Seasonal patterns and fluctuations in eDNA detection have been found for crayfish, where the probability of detection greatly increased immediately following egg hatching when juveniles displayed high growth rates and high metabolic activity, while probability of detection was lowest during cold months when the crayfish burrowed and displayed little to no movement (Troth et al., 2021). Similarly, eDNA detection probability of a salamander and a turtle in Alabama, USA, were dependent upon the sampling season that corresponded to highest biological activity and metabolic rates for each species (de Souza et al., 2016). Freshwater mussels have likewise been found to release higher eDNA concentrations during warmer months associated with high filtration and metabolic rates (Schill & Galbraith 2019; Wacker et al., 2019). Furthermore, many aquatic organisms release free-floating gametes directly into the water column during spawning events, which will provide high concentrations of eDNA if water sampling occurs during spawning season (Tilliotson et al., 2018). Therefore, an optimal sampling design will involve an understanding of the biology of the target species.

2.1.2.2 Degradation Rates

Degradation rates of eDNA will affect the ability to collect and detect eDNA within the environment (Mauvisseau et al., 2022). Nagler et al. (2022) provides a review detailing the process of eDNA degradation breaking down from pieces of whole tissue (e.g., organelle DNA) to small fragments of DNA (free extracellular DNA), and how choices in the sampling design may impact the accumulation of eDNA from an environmental sample. Within the environment, larger size-fractions (e.g., intact cells or organelles) will quickly degrade into small fractions (e.g., extracellular DNA), which may then be detectable for a few hours to days (Jo et al., 2022). Degradation of eDNA is heavily dependent upon environmental parameters, such as temperature, pH, and ultraviolet (UV) radiation, which can directly destabilise the structure of DNA fragments, or indirectly break down eDNA by influencing microbial and enzyme activity. Salinity also plays a role in degradation rates, with marine environments typically displaying faster degradation rates compared to that of freshwater environments (Jo et al., 2022; Lamb et al., 2022). Therefore, it is important to collect environmental variables alongside eDNA to aid in the interpretation of site-specific eDNA detection (Nicholson et al.,2020).

For eDNA in sediment, the decay rate is typically lower than that of aqueous eDNA (Turner et al., 2015; Sakata et al., 2020). It has been shown that sediments and other particulate matter will reduce the rate of biologically and chemically driven DNA decay by adsorbing both DNases and DNA molecules (Pietramellara et al., 2009; Shogren et al., 2017; Corinaldesi et al., 2011). This means that the detectable period of sedimentary eDNA is typically longer compared to aqueous eDNA (Turner et al., 2015). Although it is known that DNA molecules remain for a long time in low-oxygen environments, such as deeper sediments, there is less information on the decay rate of sedimentary eDNA on the surface before deposition occurs into the deep anoxic layers. However, Sakata et al. (2020) demonstrated that the decay rate of surface sedimentary eDNA can be very slow (detected up to 537 days), with sedimentary eDNA concentration often greater than that of aqueous eDNA for the same sample weight.

2.1.2.3 Environmental Factors

In aquatic environments, the persistence and detection of eDNA is affected by many factors including water movement, temperature, and sunlight, which may show seasonal patterns. For example, the probability of detection of eDNA for the invasive Corbicula Asian clam was found to be substantially higher during summer months compared to autumn months when streams displayed faster flows that likely led to high dilution of eDNA (Curtis et al., 2021). For water flow, the probability of eDNA detection for a freshwater turtle decreased when sample collection occurred following a recent rain event (Akre et al., 2019). eDNA has been shown to degrade faster in relation to high temperature, low pH, ultraviolet light, and elevated microbial and enzyme activity. The probability of eDNA detection is also affected by factors that inhibit eDNA analysis, such as the collection of sediments and humic acids within a water sample (Stoeckle et al., 2017; Lance & Guan, 2021).

Within the water column, most studies indicate that the community detected may often be localised due to rapid degradation and deposition of eDNA material. For example, in marine

habitats community differences have been found in samples collected only 60-100 m apart (Port et al., 2016) while species detections ceased when sampling only 30 m away from the source (Murakami et al., 2019). Distinct communities can be even more localised when looking at changes through depth, with different communities detected from <20 m apart (Monuki et al., 2021; Jeunen et al., 2020). Detection through time is also relatively short from a couple of hours to a couple of days (Collins et al., 2019). Detection can last 2-8 hours (Ely et al., 2021; Murakami et al., 2019) to 48 hours (Collins et al., 2019) and there are clear differences between day and night communities at the same location (Jensen et al., 2022). It is also worth noting that although tides have minimal influence on the detected community composition (Larson et al., 2022; Kelly at al., 2018; Lafferty et al., 2021), tidal stage has been shown to have an effect on eDNA dispersion from the source organism (Baetscher et al., 2024). In summary, eDNA remains detectable on the scale of hours and over tens of meters. While many laboratory studies have found eDNA persistence to last for many days to weeks (Marshall et al., 2021; Lamb et al., 2022), eDNA degradation appears to be more rapid within the natural environment, lasting less than 48 hours (Collins et al., 2019; Ely et al., 2021; Murakami et al., 2019).

2.2 Methods of eDNA Analysis

Individual species and entire community assemblages can be detected from an environmental sample using a wide range of molecular genetic tools (Taberlet et al. 2018). The genetic laboratory workflow typically involves the use of a molecular assay and PCR to generate millions of copies of DNA for the target taxa of interest. Different methodologies can be implemented to detect and quantify the resultant copies of DNA following PCR. A PCR assay or primer set is defined as two short, single-stranded nucleic acid molecules (typically 20 bp or longer) consisting of a sequence of DNA bases that are designed to match the target DNA at a particular point in the genome. Regardless of the molecular tools employed, eDNA approaches typically target short fragments of DNA (~100 – 400 base pairs [bp]) to allow for the successful detection of potentially heavily degraded pieces of DNA. Additional environmental genomic methods, and guidelines for laboratory processes can be found in IOGP-JIP Chapter 3.

2.2.1 Single-Species Applications

DNA barcoding is defined as the use of a short segment of DNA from a specific gene to identify a unique, individual species or taxon. The most commonly-used segment for DNA barcoding is a ~600 base pair (bp) sequence of the mitochondrial gene Cytochrome Oxidase I (COI) (Stein et al., 2014) (Figure 2-2 Blue Box). This gene region has been useful in phylogenetic studies because it provides high genetic variation between species, yet comparatively minor variation within species (Kress et al., 2015). Other barcode regions can be used for species identification, with other common gene regions including ribosomal DNA such as 12S, 16S, 18S, and 28S, and other mitochondrial regions such as Cytochrome b. Each gene region has its advantages and disadvantages and as such gene regions may be used for different purposes and taxonomic targets (Haarsma et al., 2016). DNA barcoding typically refers to single species identification applications, with the analysis of samples that originate from a single species (such as hair, skin, blubber, fish fin clip, or feces) (Figure 2-2A).

Molecular methods for the analysis of eDNA often involve targeted species-specific approaches using conventional PCR (Ardura et al., 2015; Sakai et al., 2019), quantitative PCR (qPCR) (Gargan et al., 2017; Wineland et al. 2019), or digital PCR (dPCR) methods which encompass droplet digital PCR (ddPCR) (Lafferty at al., 2018; Lehman et al., 2020). All three of these molecular approaches involve the use of a species-specific assay that is designed to target a particular species of interest. Quantitative PCR is currently the most widely used technique for the detection of a single taxon eDNA from environmental samples, as it tends to be more sensitive than either conventional PCR (Xia et al., 2018; Fediajevaite et al., 2021) or the broad community metabarcoding approaches described below (Blackman et al., 2020; Marshall et al., 2022).

2.2.2 Metabarcoding

When interested in monitoring more than a few, or the taxa of interest are not specifically defined, such as in community-level descriptions, the use of single-species qPCR analysis is not appropriate. High-throughput sequencing (HTS) (also referred to as next-generation sequencing [NGS]) can now simultaneously sequence billions of DNA molecules within the same sample, allowing for the detection of multiple concurrent species from eDNA samples (Harper et al., 2018; Bakker et al., 2017; Pearman et al., 2020). Two principal HTS approaches are currently used to assign taxonomy to DNA sequences: metabarcoding and metagenomics, though other approaches exist and are discussed in IOGP-JIP Chapter 3. Metabarcoding (Figure 2-2B) involves implementing PCR and HTS technology to amplify and sequence a particular gene region of interest for all target taxa within a sample.

Similar to DNA barcoding, in metabarcoding a gene region is chosen based on its ability to differentiate species using a library of DNA sequences derived from specimens of known identity called a reference database. However, unlike DNA barcoding, the source of the DNA sample does not need to originate from a single individual. Thus, metabarcoding is an ideal molecular tool for eDNA, where genetic material from individuals of many species may be present. Metabarcoding assays can target broad taxonomic groups, such as all eukaryotes (Bakker et al., 2019; Salonen et al., 2019) or all bacteria (Zhang et al., 2020), or they can target specific taxonomic groups, such as fish (Miya et al., 2020; DiBattista et al., 2017) or freshwater mussels (Marshall et al., 2022). Targeted assays are more sensitive to the DNA from the chosen taxonomic group and may identify species that otherwise would be missed with a broad taxonomic assay. For example, a broad-spectrum vertebrate assay would identify many fish species, but a fish targeted assay would likely get better resolution of the entire fish community (Collins et al. 2019). In many cases, multiple metabarcoding assays can be performed on the same eDNA sample, allowing for the simultaneous analysis of multiple taxonomic groups.

As previously mentioned, a reference database is a library of DNA sequences derived from specimens of known identity. Sequence data obtained from environmental samples (e.g., via metabarcoding) can be matched against a reference database to assign taxonomic names to the sequences. The two most common reference libraries employed for DNA-based taxonomic identification are the Barcode of Life Data System (BOLD) and the National Center for Biotechnology Information's (NCBI) GenBank. BOLD is specifically developed for DNA barcoding and is highly curated but contains a limited selection of barcode genes, while NCBI GenBank is

far more extensive but is not curated and contains a high level of error that must be accounted for in taxonomic assignment pipelines. Custom reference databases can also be made for specific projects, to allow for confident identification of important species. Table 2-1 below provides a list of some of the more commonly used reference databases. IOGP-JIP Chapter 4 discusses guidelines for the generation of reference database at length.

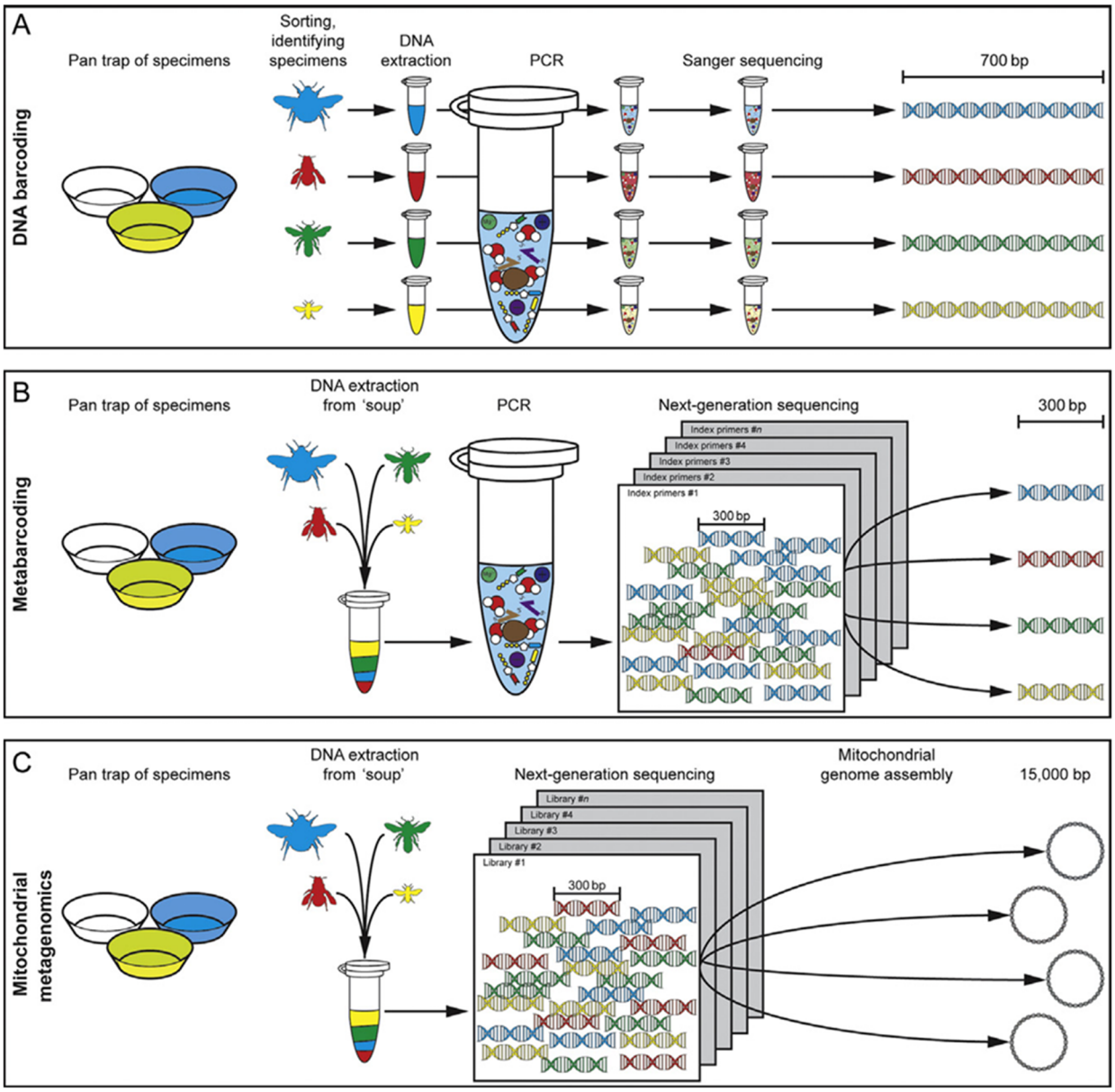

Figure 2-2 Barcoding, metabarcoding, and metagenomics pipeline (from Gill et al., 2016)

The passive detection of a metabarcoding approach allows for the monitoring of key and/or invasive species without prior knowledge of community composition before the sampling event. Thus, metabarcoding presents advantages when little is known about the species assemblage within a project site. Studies have demonstrated that, when compared to conventional sampling techniques, there are multiple benefits of eDNA metabarcoding for characterising community composition. Some of these benefits, as presented in a review by Pawloski et al., (2018), include its scalability and resource effectiveness, and its ability to improve taxonomic resolution and comparability across regions, detection of early life stages and/or fragments of specimens, and the detection of a wider range of taxonomic groups that are difficult to identify by conventional means. IOGP-JIP Chapter 1 provides a comprehensive comparison of environmental genomics approaches to conventional approaches, in industry-relevant contexts. See Bruce et al., (2021) and Deiner et al., (2017) for comprehensive lists of developed metabarcoding assays.

2.2.3 Metagenomics

While metabarcoding describes species assemblages based on analysis of a targeted gene, metagenomics, for which shotgun sequencing is the NGS technique most often used (Figure 2-2C), broadly characterises the entire genomic composition within a sample (Gilbert & Dupont, 2011; Zepeda Mendoza et al., 2015; Ruppert et al., 2019). For this approach, the sequence data output is reassembled into scaffolded genomes by overlapping reads to build longer sequences, enabling better classification, and then comparing the scaffolds to reference databases. Known associations between the scaffolded genomes and environmental conditions can then be used to consider the stresses experienced by the biological community. Data from metagenomic analysis (Figure 2-2C) can therefore provide information used for functional characterisation of the environment as well as taxonomic identification (Zepeda Mendoza et al., 2015).

Table 2-1 List of the most common eDNA assay and genetic reference databases.

Database	Description	Environments			Taxonomic groups	Reference
		Freshwater	Marine	Terrestrial		
eDNA validation	Compiled list of targeted qPCR assays used for eDNA analysis	✓	✓	✓	Macro-organisms	https://edna-validation.com
GAPeDNA	Extractable sequences for RTE species available per habitat, region and target region	✓	✓		Fish	GAPeDNA v1.1.1 (cnrs.fr)
MetaZooGene Barcode Atlas and Database	Reference database of marine zooplankton COI barcodes		✓		Zooplankton	MetaZooGene Atlas & Database
NCBI Genbank	An annotated collection of publicly available DNA sequences	✓	✓	✓	All	GenBank Overview (nih.gov)
BOLD	The Barcode of Life Data System (BOLD) is an online workbench and database that supports the assembly and use of COI sequence data	✓	✓	✓	Macro-organisms	BOLD Systems v4
EMBL's European Bioinformatics Institute	Data at EMBL-EBI spans genomics, proteins, expression, small molecules, protein structures, systems, ontologies and scientific literature.	✓	✓	✓	All	EMBL-EBI: EMBL's European Bioinformatics Institute | EMBL's European Bionformatics Institute
MitoFish	Comprehensive and standardised fish mitochondrial genome database	✓	✓		Fish	MitoFish: Mitochondrial Genome Database of Fish (u-tokyo.ac.jp)
UNITE	Reference database of eukaryotic fungal nuclear ribosomal ITS sequences	✓	✓	✓	Fungi	https://unite.ut.ee/
SILVA	A comprehensive on-line resource for quality checked and aligned ribosomal RNA sequence data.	✓	✓	✓	All	SILVA (arb-silva.de)

2.3 Benefits and Current Limitations of eDNA

Analysis of eDNA presents an opportunity for rapid species detections, quantification, and assessment of biodiversity. Because eDNA is generally sampled from non-living ecosystem components (abiotic media such as water, sediment, or soils), it provides a non-invasive means of conducting large-scale ecological surveys without physically capturing, handling, or harming organisms (Tréguier et al., 2014), which is particularly important for species-at-risk and other species of interest (e.g., species of commercial and/or cultural importance).

When compared with conventional methods, eDNA methods provide a safer sampling environment, with lower sampling effort and cost (Evans et al., 2017), permitting the collection of fewer samples with increased confidence. The cost for laboratory analysis of eDNA samples can be offset by lower logistics costs (e.g., conventional taxonomy and/or field mobilization, and permit collection). By analysing eDNA from a sample of environmental media (i.e., water, air, soil/sediment), it is possible to determine if a species of interest is present, regardless of life stage (e.g., gametes, larvae, adults) or whether specimens are intact or fragmented.

When assessing the performance and limitations of eDNA relative to conventional biomonitoring approaches, it is critical to highlight that conventional approaches and techniques are similarly bound by practical and theoretical limitations to their effectiveness and interpretations of the data collected. For example, the use of conventional capture methods, such as grabs, trawls, nets, or physical/digital trapping devices implicitly assume certain encounter probabilities, collection efficiency, attraction/avoidance biases, and other considerations (Eleftheriou & Moore, 2005). Environmental DNA methodologies are considered to be less prone to morphological identification bias (Buxton et al., 2022; Li et al., 2019) and spatial autocorrelation (Deiner et al., 2016) than conventional monitoring methods. Across a wide range of taxa, several studies have demonstrated comparable or higher rates of probability of detection via eDNA sampling compared to conventional methods (Pukk et al., 2021; Moss et al., 2022).

As noted in Section 2.1.2, detection probability is dependent on the life history of target species, population density, environmental conditions, PCR inhibitors, distance from shedding source, and primer/PCR performance (Fediajevaite et al., 2021). Aspects of eDNA methods that may contribute to false positive detections include equipment contamination, eDNA transport, and poor reference database completion. To date, eDNA does not provide complete information regarding population status and stability, sex, size, or health condition (Fediajevaite et al., 2021), although there is considerable research being undertaken to address this information. Amongst regulators and stakeholders, a desire exists for environmental genomics to replicate, or otherwise approximate classic ecological endpoints grounded in estimates of abundance. Although several laboratory studies clearly demonstrate the potential utility of eDNA as a tool to estimate species-specific abundance, attempts to duplicate these efforts in natural environments have met with limited success (Yates et al., 2019).

While the utility of eDNA has advanced greatly in a short period of time with demonstrated potential of its benefits, other aspects of best management practices are still lacking. Most

notably, there is a lack of standardised approaches to study design, sampling, reporting results and levels of uncertainty and acknowledgement of limitations to interpretation, which has hampered uptake with regulators and stakeholders.

A limitation specific to metabarcoding is that disagreement may occur between morphotaxonomic inventories and molecular datasets. This disagreement is largely due to incomplete or inaccurate DNA reference libraries (Cowart et al., 2015; Pawlowski et al., 2018; Taberlet et al., 2012a). Without a robust reference library, eDNA sequences can remain unassigned to a taxon or result in incorrect identifications (Lejzerowicz et al., 2015; Pawlowski et al., 2018). Further complicating matters, some of the major limitations of eDNA analysis occur during the data interpretation step because the bioinformatic programs and packages used for analysis are not standardised which makes it difficult to compare results across studies. However, one opportunity to address the limitations from database completion is that reference libraries are constantly updating with new sequences, which allows for the sequence data from metabarcoding studies to be reanalysed periodically to update taxonomic assignments without the need of additional sequencing. This is another benefit of the eDNA approach, whereby extracted DNA that has been appropriately stored, or digitally stored results, can be revisited over time as methodologies or reference libraries are improved.

Environmental DNA sampling for the early detection of targeted species, including newly introduced, invasive and competitive species has fewer limitations than the metabarcoding analysis of complex environmental samples. In fact, the limitations associated with morphological identification in the context of early detection of newly introduced or invasive species makes targeted eDNA-based methods more widely recognised by resource managers (Darling & Mahon, 2011). However, species detection using eDNA limits the information obtained about a target species in comparison to conventional monitoring, which can collect additional information (e.g., life stage, viability, reproductive status). In addition, eDNA monitoring for targeted species requires extensive and often costly laboratory assay development and validation prior to operational use (Bruce et al., 2021), - guidance on which can be found in IOGP-JIP Chapter 3.

As noted above, limitations are implicit in any environmental sampling methodology. Considering the current benefits and limitations of eDNA outlined above and throughout this document, there is general support amongst the international scientific community and regulatory agencies for the theoretical and practical potential of eDNA to characterise the environments in which the energy industry operates.

3 Summary of Standards & Guidelines

The field of environmental genomics has rapidly developed, and methods have been applied to a wide range of research and monitoring projects globally, resulting in a high level of methodological variation at all stages of an eDNA workflow. As this field continues to evolve, the successful mainstreaming of environmental genomics will require some level of standardisation of protocols across industry, academic, and regulatory bodies. This shift is needed to both improve communication with regulators and stakeholders, and to permit greater confidence in, and reliability on, the results of environmental genomics data. There is a clear desire for development of scientific best-practices in many areas, and yet there are no definitive global standards or regulations governing the use of DNA-based applications for environmental biomonitoring.

Accordingly, various national and international efforts have been undertaken to standardise methods and integrate them into monitoring frameworks (Pilliod et al., 2019; Loeza-Quintana et al., 2020; Minamoto et al., 2021; Pawlowski et al., 2020). In many countries, there is an increasing body of eDNA guidelines based on consultation between government, industry, and academia in the form of working groups or initiatives. While there is no consensus on standards yet, the dialogue between these different groups at an international scale has resulted in a shared understanding of what is important and what minimum requirements are needed to constitute a standard (Bruce et al., 2021). Additionally, there are efforts underway by several global organizations, such as the International Organization for Standardisation (ISO), to harmonise eDNA sampling methods as well as the many biodiversity metrics and indicators currently in use to streamline reporting standards for biodiversity assessments. Appendix B provides a detailed analysis of existing and emerging standards and guidelines and discusses how the standardisation of eDNA methods is currently evolving.

4 Energy Project eDNA Sampling Considerations

Several important considerations should be assessed through a hierarchy of tiers (Figure 4-1) to determine whether an eDNA study will be beneficial to an energy project. Failure to account for the potential influence of any of these tiers can significantly impair the ability to produce rigorous, interpretable and defensible conclusions from an eDNA survey at later study phases. The hierarchy of tiers for implementation of an eDNA study is as follows.

- Energy Project Phase Considerations
- Logistical Considerations (Health and Safety, Site Access, Field Conditions, Crew Availability and Experience)
- Ecological Considerations (Biota, Habitat Type[s], Species Phenology, DNA shedding rates, dispersion rates, degradation rates, representativeness)

This report section details the energy project phase and logistical tiers, providing the appropriate information required to frame the study goals, constraints, and opportunities. This hierarchical implementation is necessary to prepare the reader to consider the specifics of the

Ecological tier when implementing an eDNA study, further described in detail in Section 5. As eDNA sampling can be expanded to all industry personnel and contractors, it is useful for this broad demographic to be aware of the different sampling considerations for eDNA.

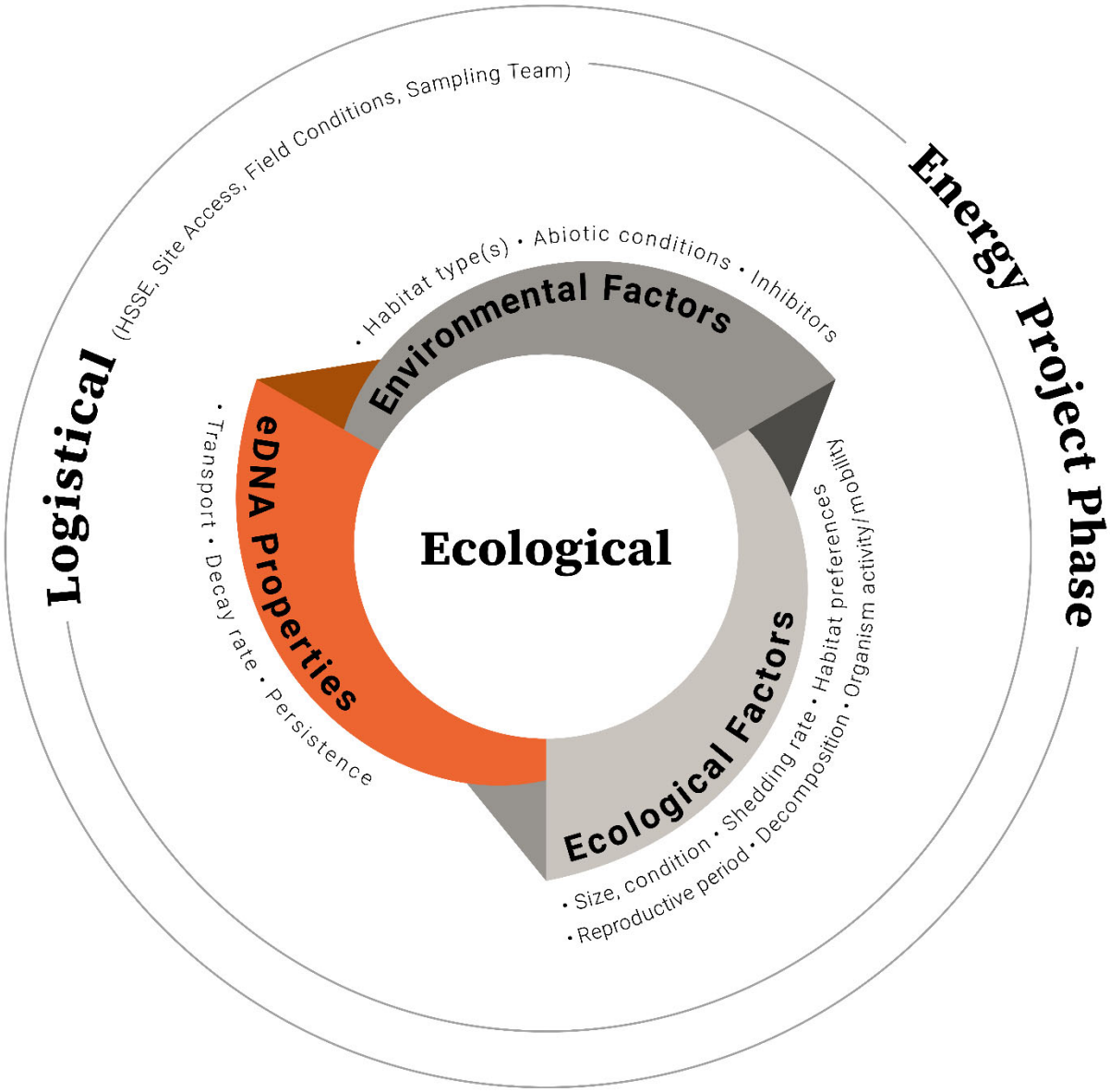

Figure 4-1 Considerations for eDNA study implementation

4.1 Energy Project Phases for eDNA Use

Biodiversity assessments may be required for a proposed energy project during the exploration phase to establish a baseline for assessment, for monitoring during the development and ongoing operational phases, and during decommissioning and site

restoration/remediation/reclamation activities to evaluate progress towards goals for closure. Figure 4-2 illustrates various potential industry-specific applications for eDNA in energy project activities.

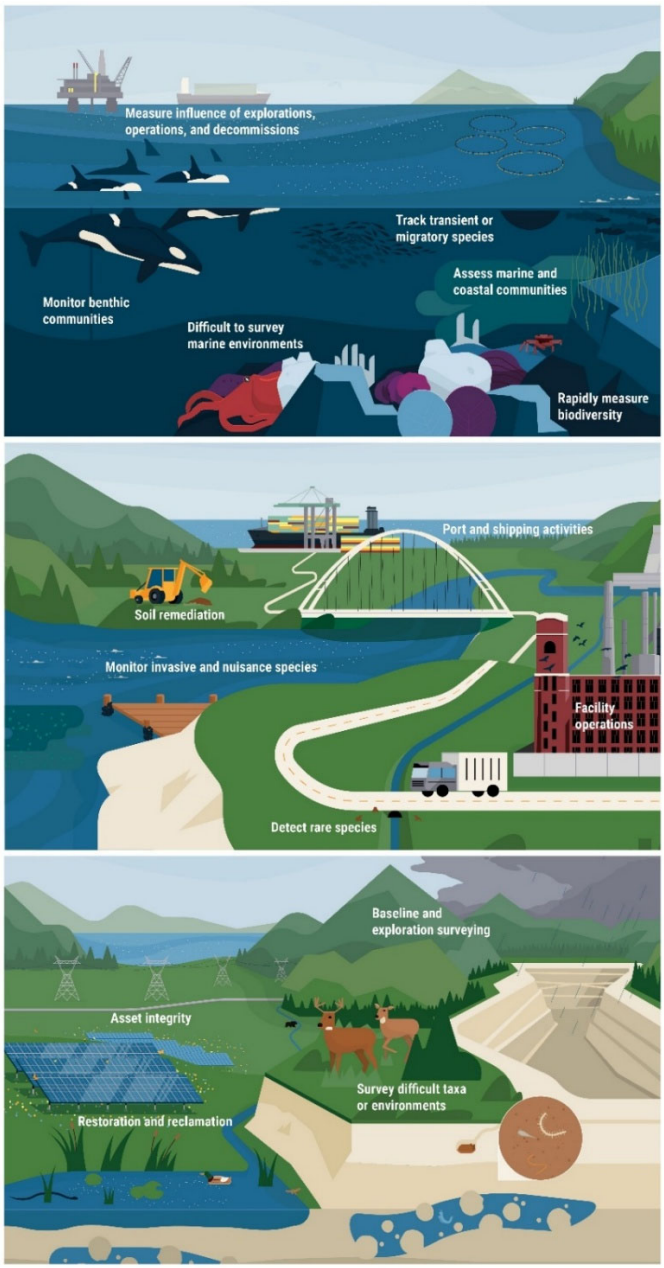

Figure 4-2 An illustration of the sources of eDNA data and its applications to environmental management

4.1.1 Exploration

Baseline assessments are required for the monitoring of environmental effects of energy project activities as they provide a starting point (ideally prior to development) from which to measure

potential environmental change and assess predictions of effects to biodiversity and ecosystem services. This information will be representative of an existing environment in an area that may experience other anthropogenic pressures and will be used to evaluate potential subsequent ecological change or environmental effects from project activities. Without comprehensive baseline assessments, the degree of this potential change over time is more difficult to assess as differences between control and impact sites may have existed prior to activities.

For many energy project activities, exploration will occur within active production areas. Exploration may also occur in "frontier" areas, such as deep-water exploration, where there is limited environmental data, particularly regarding protected species or habitat. Protection of species and habitats is usually regulated through national legislation, requiring an Environmental Impact Assessment for new projects, including establishing an environmental and biodiversity baseline across the exploration area. Measuring biodiversity through eDNA methods is increasingly being incorporated into this process (Hinz et al., 2022).

4.1.2 Development and Production

Energy project activities may affect the surrounding environment. Environmental monitoring of energy project activities is a regulatory requirement in many parts of the world and eDNA techniques are emerging as a potential time and cost-effective supplement or alternative to conventional methods for biodiversity assessment and monitoring. For example, the deposition of drill cuttings resulting from offshore O&G activities can affect nearby benthic communities through physical smothering, organic enrichment and chemical contamination by hydrocarbons, heavy metals, specialty chemicals and sulphide (see Mauffrey et al., 2020; Box 1). The benthic community has been studied using metabarcoding around IOGP members' infrastructure, such as O&G platforms in the Italian Adriatic with Eni (Cordier et al., 2019) as well as offshore windfarms in the UK North Sea with Equinor (Dahlgren et al., 2023) and O&G infrastructure owned by Equinor in Norwegian North Sea (Hestetun et al., 2021;

Box 2). These eDNA-based monitoring techniques can be applied offshore or onshore across a range of media and potential impact sources including the release of produced water (Tiburcio et al., 2021) and unplanned events such as oil spills (Oladi et al., 2022).

Box 1 - Benthic monitoring of oil and gas offshore platforms in the North Sea using environmental DNA metabarcoding.

Authors: Mauffrey et al., 2020 **DOI: https://doi.org/10.1111/mec.15698**

Affiliations: ID-Gene ecodiagnostics; Total

Academic Collaborators: University of Geneva; Polish Academy of Sciences

Objectives: Compare eDNA metabarcoding with conventional macrofauna sampling for identifying alpha and beta diversity trends.

Methodology: The overall aim was to assess environmental pressures associated with drilling cuttings using benthic eukaryotic communities evaluated with eDNA compared to morphology-based macrofauna analyses. A total of 41 stations were sampled at increasing distance from two O&G producing and processing platforms operated by Total E&P Denmark, approximately 200 km from the Danish western coast. This included 7 to 10 surface sediment replicates at each station, leading to a total of 126 sediment samples analysed for eDNA across the project. Seven samples were additionally collected at each station for conventional macrofauna and analysed for the standard suite of hydrocarbons and heavy metals. One COI and two 18S markers were used for metabarcoding sample analysis. The AZTI Marine Biotic Index (AMBI), Shannon diversity index and Infaunal Trophic Index (ITI) were calculated using both the conventional macrofauna and eDNA metabarcoding data.

Results:

- Communities within stations close to the platform (0 to 250 m) were more distinct from those far from the platform (750 m to 3 km) within the eDNA metabarcoding dataset compared to the morphology dataset. These differences with distance from the platform correlated with environmental parameters, such as grain size.
- AMBI values correlated well between metabarcoding and morphology, while Shannon and ITI regression models did not show a significant correlation. AMBI values from metabarcoding and from morphological datasets had a significant decrease (indicating reduced pollution) with increased distance from platforms. Metabarcoding also showed distinct changes in AMBI ecological quality status from medium to good with distance from one platform, whereas morphological data only showed good status.
- Explore new indices and metrics for eDNA metabarcoding data.
- A local de novo pressure index was built using a taxonomy-free approach for station sediments ranked from “good” to “bad” status based on physicochemical variables. Metabarcoding data performed better against this scale than the morphological data.

Key points:

Metabarcoding eukaryotes can provide better taxonomic resolution for community comparisons along pollution gradients than conventional sampling and can be used to generate new useful metrics at a local scale. Diversity metrics derived from metabarcoding and conventional macrofauna data from morphology correlate well with each other, but not perfectly.

Box 2 - Eukaryote metabarcoding for environmental monitoring of marine sediments

Authors: Hestetun et al., 2021 **Link :** https://hdl.handle.net/11250/2829769

Affiliations: Molecular Ecology Research Group (MERG), NORCE Environment, Norwegian Research Council (NRC PETROMAKS2, grant no. 280919); Equinor; Total

Academic Collaborators: AZTI, University of Bergen, Auburn University

Objectives: The overall aim was to assess and advance the maturity level of eDNA-based methods in environmental impact assessment of petroleum extraction activities for marine sediments. This would enable faster and more affordable analyses, providing a more complete and accurate picture of seafloor communities and functional relationships relative to conventional macrofauna methods.

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 metabarcoding data, with verified potential as biological indicators of environmental disturbance related to oil drilling activities.

Develop specific preliminary guidelines for metabarcoding methodology which encompass all project stages, including 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 18S and COI metabarcoding. Obtain and submit to online databases barcodes from specimens representing the most common taxa in the O&G monitoring programs.

Methodology: This study sampled 97 stations for metabarcoding and morphological macrofaunal analysis as representative locations for Norwegian offshore O&G seafloor monitoring. The different method steps from sample replicates within and between grabs, DNA extraction, markers and benthic pollution indices were compared across the dataset and against the conventional data.

Results:

- Newly developed metabarcoding indices could predict environmental impact similarly to the existing morphologically-based biotic index (NSI; Lanzén et al., 2021).
- Sieved macrofauna samples for metabarcoding resulted in biases in estimating relative abundance compared to conventional macrofauna, with crustaceans severely underrepresented and overrepresentation of polychaetes from metabarcoding data (Lanzén et al., 2021).
- *Capitella* species and a haplosporidian species were proposed as possible indicators (Lanzén et al., 2021).
- An intermediate homogenisation program (Precellys homogeniser) significantly increased total DNA that could be extracted from each sample and homogeneity of benthic community data obtained. Optimal number of replicates per grab and station were identified (Hestetun et al., 2021; Figure Box 2-1).

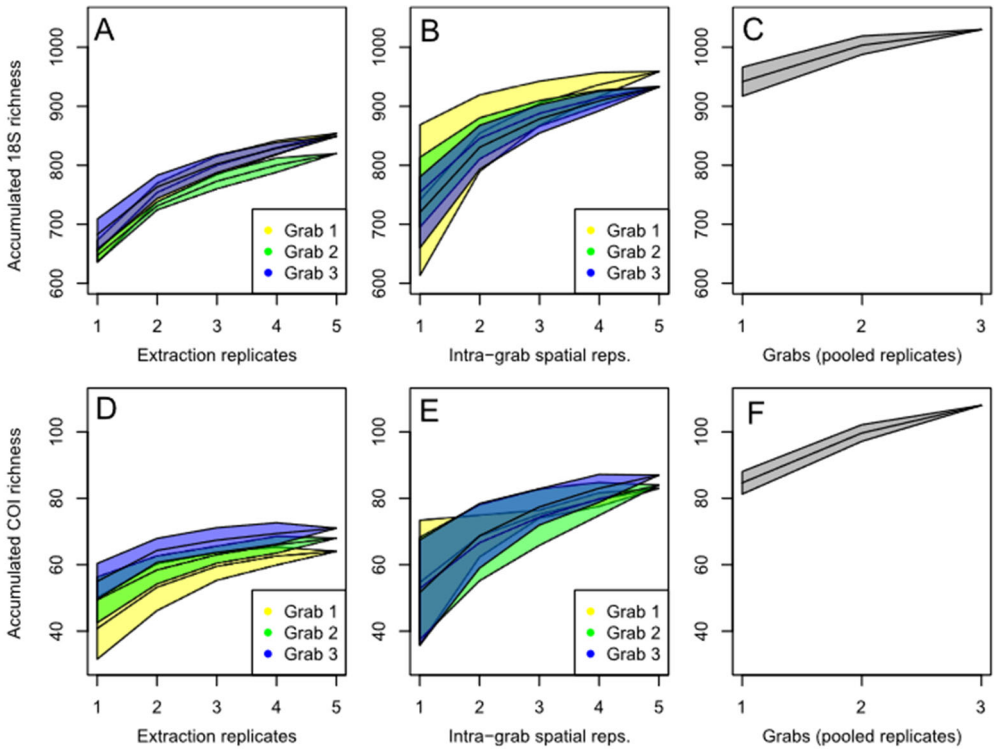

Figure Box 2-1: OTU aggregation curves. Median 18S (A-C) and COI (D-F) richness increase for individual extracts (A,D), and sediment samples per grab (B,E) shown separately for grabs 1-3, and as cumulative total grab data for the whole dataset (C,F). Source credit Hestetun et al., (2021).

- Of 1802 macrofauna taxa in North Sea monitoring region IV, species coverage was 50.4% in Genbank and 42.4% in BOLD for COI; 36.4% in GenBank and 27.1% in SILVA for 18S (Hestetun et al., 2020)
- 96 specimens representing 46 separate polychaete, mollusc and crustacean species were barcoded for COI, 18S and 28S genes (Hestetun et al., 2020).

Key points

A combination of multiple samples within a grab and multiple grabs within a station is recommended to gain a representative sample of station eukaryote diversity. Metabarcoding can be used to consider pollution indices comparably to macrofauna, albeit with different taxonomic biases.

4.1.3 Decommissioning, Site Restoration, Reclamation and Remediation

If the goal is to restore a site to pre-disturbance state or improve conditions to achieve a defined restored state, then monitoring of indicator species will be important. In such cases, baseline characterisation or use of an "undisturbed" reference site nearby will provide information to guide the restoration or reclamation objectives. Baseline characterisation should cover the range of substrates and habitat appropriate for the goal state for detecting target species or communities (Alexander et al., 2023a). Genomic and conventional methods are recommended as they complement each other for taxonomic coverage as well as presenting different forms of biological data (Alexander et al., 2023a). Box 3 highlights an assessment of eDNA for use in biodiversity surveys of decommissioned offshore infrastructure.

Box 3 - Using eDNA to inform decision making around decommissioning alternatives for offshore oil and gas infrastructure

Authors: Alexander et al., 2023a **DOI:** https://doi.org/10.1016/j.scitotenv.2023.165991

Affiliations: Chevron; Department of Conservation New Zealand; PTTEP Energy Development Company Limited

Academic Collaborators: Curtin University; Chulalongkorn University

The overall aim was to assess the versatility and scalability of eDNA metabarcoding to holistically census marine infrastructure. This is being applied to the increasing amount of decommissioned O&G structures, particularly in the Gulf of Thailand, and their potential as artificial reef sites. Objectives were to:

Identify differences in assemblages among sampling media, depths and locations.

Assess role of decommissioned platforms as artificial reefs and impact of their removal

Assess DNA-based methods for detection of key taxa (conservation significance or introduced).

Methodology:

Sampling was carried out at eight platform and five off-platform stations in the Gulf of Thailand.
A total of 156 water samples were collected at 3 depths (10 m, 30 m and 50 m)

A total of 96 biofoul samples were collected at 3 depths (10 m, 30 m and 50 m)

A total of 52 sediment samples were collected

Results:

No single sampling medium can holistically document the entire diversity on or off platform (Alexander et al., 2023a; Koziol et al., 2019). Increased vertical sampling replication may increase detected diversity and provide finer spatial nuance informing decommissioning options. The different taxonomic groups identified at different depths from the varied substrates are illustrated in Figure Box 3-1.

Full removal of the infrastructure could see the local loss of up to 141 identified species that were only detected at platforms, or the potential loss of 36 shallow-water species under a partial removal scenario.

Two of a possible 400 Gulf of Thailand IUCN Red List species were detected, and no introduced species were detected. However, only five introduced species had reference sequences, with only one estimated to have the potential to be amplified with assay used.

Availability of assays and completeness of reference databases should be considered for target or local dominant taxa. The coral assay (ITS2 barcode region) is not recommended for the Gulf of Thailand, due to lack of reference material. Similarly, the broad universal COI assay is not recommended for sediments due to low species resolution with current databases.

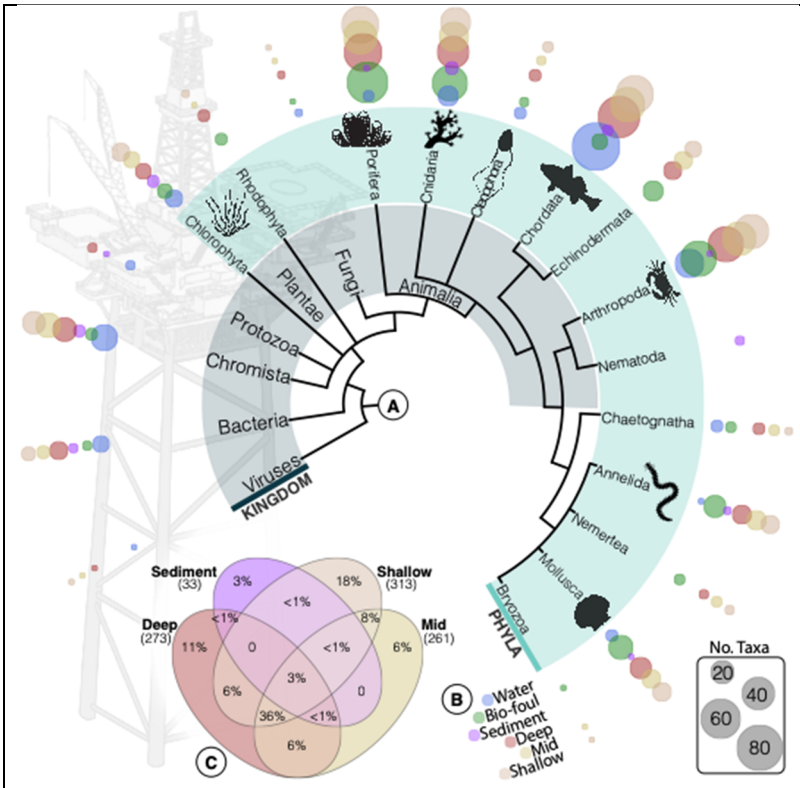

Figure Box 3-1. Biotic kingdoms detected from eight platforms within the Gulf of Thailand, with further phyla level breakdown of detected Plantae and Animalia (A). Circles indicate the number of species level taxa detected per group from each substrate and also each depth (combined water and bio-foul) sampled (shallow, mid and deep) (B), and Venn diagram showing the percentage of taxa common to all depths at platforms utilised in the study (C). Source credit Alexander et al. (2023a).

Key points:

A range of sample media (e.g. sediment, water, and biofoul/marine growth) and water depths should be considered when biomonitoring around O&G infrastructure. The limitations of assays in terms of reference sequence availability and likelihood of successful amplification should be considered when targeting species of interest. O&G infrastructure has the potential to act as an artificial reef for a range of species following decommissioning to enhance biodiversity.

Similarly, tracking the restoration of oil-contaminated ecosystems can be done through monitoring the microbial community and functional groups. This has previously been investigated by assessing changes in soil bacterial community structure following exposure to hydrocarbon fuel (Ruiz et al., 2021). Changes in benthic communities in the Persian Gulf where crude oil spills frequently occur have also been monitored (Oladi et al., 2022), which can drive insights when remedial action has been taken, such as following the use of chemical dispersants (Ankley et al., 2021; Tremblay et al., 2017). Metatranscriptomics using eRNA can be more

informative than eDNA for analysing the impact of contamination as it covers the live/active community uniquely (Greco et al., 2022). However, this method has rarely been used to date due to the increased field and laboratory requirements (Laroche et al., 2016) (but see IOGP-JIP Chapter 3 for guidelines on techniques).

Compared to conventional methods, eDNA methods can improve the detection of invasive or competitive species (Jerde et al., 2013) and biofoul/marine growth (Holman et al., 2019), allowing for early detection (e.g., eggs or larvae presence), and can be used to effectively monitor their presence and persistence in the wake of containment or eradication countermeasures (Morisette et al., 2021). Early detection can allow for control measures to be put into place to prevent or limit the spread of an invasive or competitive species and therefore reduce the potential for environmental effects caused by human activities. For example, following disturbance from energy project activities, the ecological succession process begins and there are advantages to being able to detect newly introduced or competitive species early in the succession process. These advantages include being able to deploy management strategies that prevent the spread or establishment of unwanted species or that can influence the direction of succession and enhance biodiversity. Early detection of species following disturbance can reduce the environmental effect of the disturbance by promoting the recovery of the environment to conditions similar to those prior to the disturbance or to an alternative stable state.

The required levels of survey effort and specificity may vary depending on the project phase in question. Further details on these aspects of eDNA sampling are provided in Section 5.

4.2 Logistical Considerations

4.2.1 Site Access, Field Conditions and HSSE

Energy projects can have vastly differing operational parameters for different habitats and geographic areas. Within onshore/terrestrial projects, activities may be carried out in a tropical rainforest (Codato et al., 2019), wetlands (Kingsford & Walburn, 2022), arid desert (Wang et al., 2023), or temperate climates (Zhou et al., 2022). Marine activities may happen nearshore, or in offshore deepwater locations, almost always requiring a survey vessel or offshore platform, even if deploying remotely operated vehicles (ROVs) or autonomous underwater vehicles (AUVs).

In addition to the above environmental conditions, the project phase of the oil & gas activities will further determine the selected eDNA survey approach. For example, in a greenfield exploration scenario, there may not be existing facilities, such as airstrips, accommodation or access roads, whilst in a mature project, infrastructure such as roads, buildings and facilities may be challenging impediments when developing an ideal survey design (land-based). For offshore exploration projects, port facilities and supply bases may not be available in the vicinity of the project location, whilst in a mature project setting, one may find shipping lanes, commercial fishing, infrastructure, and pipeline routes, intersecting the planned survey area (offshore location).

The above scenarios illustrate the challenges posed by varying operational project settings and phases. In addition to these operational considerations, in all eDNA projects, the sampling crew and their logistics support will have to carry out appropriate HSSE risk assessments for the chosen sampling methods and the resulting risk profile will influence sampling times, sampling approach and turnaround times. This can include steps taken to minimise contamination of samples, such as having a designated area of the vessel to filter samples. Similarly, if using an automated pump, a power supply should be available on the vessel.

When considering eDNA in marine environments, the sampling methods for sediment are comparable to conventional sampling, requiring the deployment of grabs, cores, whilst similar water sampling devices (e.g., Niskin bottles) to those used for water chemistry are required. Sediment and water sampling equipment should only be deployed on a vessel when it is safe to do so, for example, whilst wearing appropriate PPE and when to the grab or bottle is not swinging dangerously. If collecting a biofouling sample for eDNA with an ROV or similar, this is usually faster and less risk-prone than using scientific divers to carry out visual observations. Similarly, a Niskin bottle can be deployed to take an eDNA sample once it reaches the correct depth and recovered immediately, whereas a camera or towed net requires extended deployment at depth.

If freezing samples to preserve their intactness, proximity to a freezer with a power source and backup power to maintain proper temperatures is required. Cold chain transport is also necessary to prevent samples from defrosting and degrading before arrival at the laboratory for processing. Where these two conditions cannot be met, use of a preservation buffer is required. Further detail on preservation methods is given in Section 5.6. The size and weight of samples being preserved should be considered in the planning phase to ensure sufficient storage space during sample collection and for sample transport. In some cases, this may include weight restrictions for transport of samples by air freight.

In any of these potential sampling scenarios, coordination, and supervision of scientific staff by experienced local business line representatives is recommended. In addition to the operational and logistical insights provided, these business line staff are most familiar with the local geography and potential sampling habitats likely to be of most interest to scientific staff.

4.2.2 Sampling Team and Experience

DNA-based surveys can be carried out by a broad range of personnel, provided they are trained in the method for sampling the environmental medium and in the processes of DNA-based sampling, handling and preservation. For example, freshwater eDNA samples can be obtained from ponds by trained citizen scientists if given clear instructions, particularly concerning contamination avoidance (Biggs et al., 2015), although for energy project biomonitoring purposes standardised sampling approaches should be used where possible.

Teams of ecologists who are usually deployed for conventional sampling (such as baseline sampling for environmental impact assessments) are typically also capable of collecting eDNA samples. The skills employed in geotechnical and physico-chemical sampling of sediment and water are generally transferable to eDNA sampling in terms of minimising contamination and

sample preservation. This presents opportunities for offshore geotechnical staff to obtain eDNA samples from sediment cores, seafloor sediments or the water column. Provided there is sufficient training, particularly around minimising contamination, this can be expanded to all industry personnel and contractors. This has the advantage of reducing the need for additional staff in the field to collect biodiversity data. Under this scenario, the sampling staff must be trained and provided with a field plan and sampling design including ecological considerations to allow on-site adjustments, if necessary, due to operational concerns.

For baselining or site exploration, this also means that biodiversity data can be collected earlier in the process. For example, eDNA samples can be collected by the team that deploy the floating LiDAR buoys during the initial characterisation of the site, which generates biological information on the site much earlier than trawl surveys (which can occur 8 – 12 months after buoy deployment). Obtaining this data earlier allows for risk mitigation if specific species are detected and can inform the design of subsequent environmental surveys to make them more time and cost efficient.

A minimum of two people should be considered necessary when undertaking eDNA sampling. This frees one person to record the sample metadata and/or ensure any equipment is held steady when the other is processing the sample, while also monitoring each other's safety. It also reduces the potential for contamination by allowing one person to focus only on handling the sample without needing to touch other equipment for labelling, recording data, or other activities.

Training can be done in person, particularly for well-regulated forms of eDNA sampling such as Great Crested Newt in the UK (Freshwater Habitats Trust). Most companies offering eDNA analysis as a lab service are prepared to run sampling training using their predeveloped sampling kits. Video tutorials combined with written sampling protocols have become more widely produced and disseminated by suppliers, NGOs and regulators (e.g. Smith-Root sampling; UNESCO eDNA sampling; US Fish and Wildlife Service) as well as the lab service providers (NatureMetrics; EnviroDNA). However, group online training sessions are advised to allow for any questions and tailoring of protocols where necessary for the sampling conditions and field equipment available.

4.3 When Should eDNA Sampling Be Conducted?

Species activity and distribution may vary over the day/night cycle, such as with zooplankton which migrate to and from the water surface (Allan et al., 2021). As with conventional sampling, these different sampling windows and their implications in terms of the crew and field team availability should be considered.

Seasonal variation can also alter sampling conditions and subsequent results, as well as species ecology and distributions. For example, freshwater invertebrate communities change seasonally and generally shed less eDNA in the winter months as they are less metabolically active (Reinholdt Jensen et al., 2021). In addition to these variations in DNA presence, poor weather conditions may result in delays in field sampling, which in turn may impact interpretation of sampling results.

A broad range of staff trained in eDNA sampling beyond the environmental surveying team reduces reliance upon specialist availability, thus reducing the pressure on these temporal windows. Where capturing seasonal variation is not practical, sampling during the same season is key when considering interannual variation. A Before-After Control-Impact (BACI) design is generally advised to monitor change over the project lifecycle. This uses reference samples at a location before an impact and includes a reference site sampled at the same temporal intervals as the impact sites, which enables the identification of temporal change across the wider area and can better distinguish broad scale change from signals specific to potential impact sources.

Key timing and location questions to consider when developing an eDNA study plan include:

- What phase of development is the project or facility currently in?

  i.e., a higher degree of data resolution may be required at different project phases

- When is the target species/community active?
  - Need to sample at a time to optimise the potential to collect eDNA from the target species (e.g., time of day, season, life-cycle considerations (e.g., feeding, spawning)
  - Biological activity (spawning events) and eDNA shedding rates may vary across seasons (e.g., rainy season may dilute and transport eDNA, as described in further detail in Section 5)
- Patterns in DNA dispersal and degradation, discussed further in Section 5
  - What area does the sampled eDNA reflect (e.g., dispersal by ocean currents or rivers)
  - What temporal window does the sampled eDNA reflect (e.g., higher degradation rates at higher temperatures)

5 Implementation of an eDNA Study

Environmental practitioners in the Energy sector require the knowledge and ability to provide technical advice on when, where and how to apply eDNA methods. This section provides a practical overview of critical considerations when implementing eDNA methods while highlighting and directing the reader to the resources required to confidently interpret data in the light of the nuances, limitations, and advantages of eDNA. This guidance highlights the minimum ecological factors (Figure 4-1) that need to be considered when designing a sampling program in the context of the operational and logistic requirements presented in Section 4, rather than being definitive or prescriptive. Since eDNA is a rapidly evolving technology, this section is not intended to be complete in content, rather it is deliberately planned to be evergreen. Core concepts of eDNA processes and procedures are presented throughout the section with a tabular summary of practical guidance in Appendix B. Rapidly evolving information is contained in subsequent appendices that are intended to be subsequently updated on a regular basis.

5.1 Study Design

In general terms, an eDNA survey begins with a study design phase. To begin, the first consideration when designing any study or survey is a clear statement of the primary objective. This statement may be framed as an overall study goal, a research question, or formalized hypothesis with subsequent predictions for a priori statistical assessment. For example, “Does this threatened species live in this body of water?” or “Is there a difference in bacterial profile between these disturbed and restored woodlands?”. Regardless of the method of expression, a concise and specific primary study objective is required. Once a research question and a set of hypotheses are established, a robust study design can be developed to ensure appropriate sampling and data collection for the project.

Similar to any ecological study, eDNA studies require robust survey designs to provide adequate sampling (both in terms of number of samples and in spatial or temporal coverage) that can be used with analytical methods to appropriately meet project goals. USFWS (2023) outline several methods of probabilistic sampling which can be followed to provide proper interpretation of results. For example, when no information is known about the ecosystem being surveyed or the target species, a simple random sample design should be implemented to limit biases that may occur during field collection. However, in cases where there is a priori knowledge about the ecosystem, a stratified sampling approach can be implemented to improve coverage while providing representative sampling effort across habitats. Finally, a research project investigating the spatial extent of eDNA detection may want to implement systematic or spatially balanced sample designs. Spatially balanced sample designs are particularly useful when a project is interested in general biodiversity trends across large spatial extents.

After the study design is established, an eDNA survey generally consists of four main implementation steps, which can be visualized as in Figure 5-1:

1. Sample Collection: securing sample media (e.g., water, sediment, soil, or air) and the associated metadata.
2. Sample Processing: laboratory processing steps to isolate and detect target DNA, this includes DNA extraction, purification, and target amplification/sequencing.
3. Quality Control: accuracy of the results is evaluated by incorporating negative and positive controls.
4. Data analysis: the data analysis and interpretation are crucial to evaluate results from an eDNA survey, and this may include incorporating a detection threshold, defining a bioinformatic pipeline, and/or incorporating choice of modelling and/or statistical analyses.

The flowchart in Figure 5-2 outlines these varying considerations (black boxes) and the subsequent influence on the eDNA workflow with a progression through various deduction points ('DP') as illustrated by orange boxes in Figure 5-2.

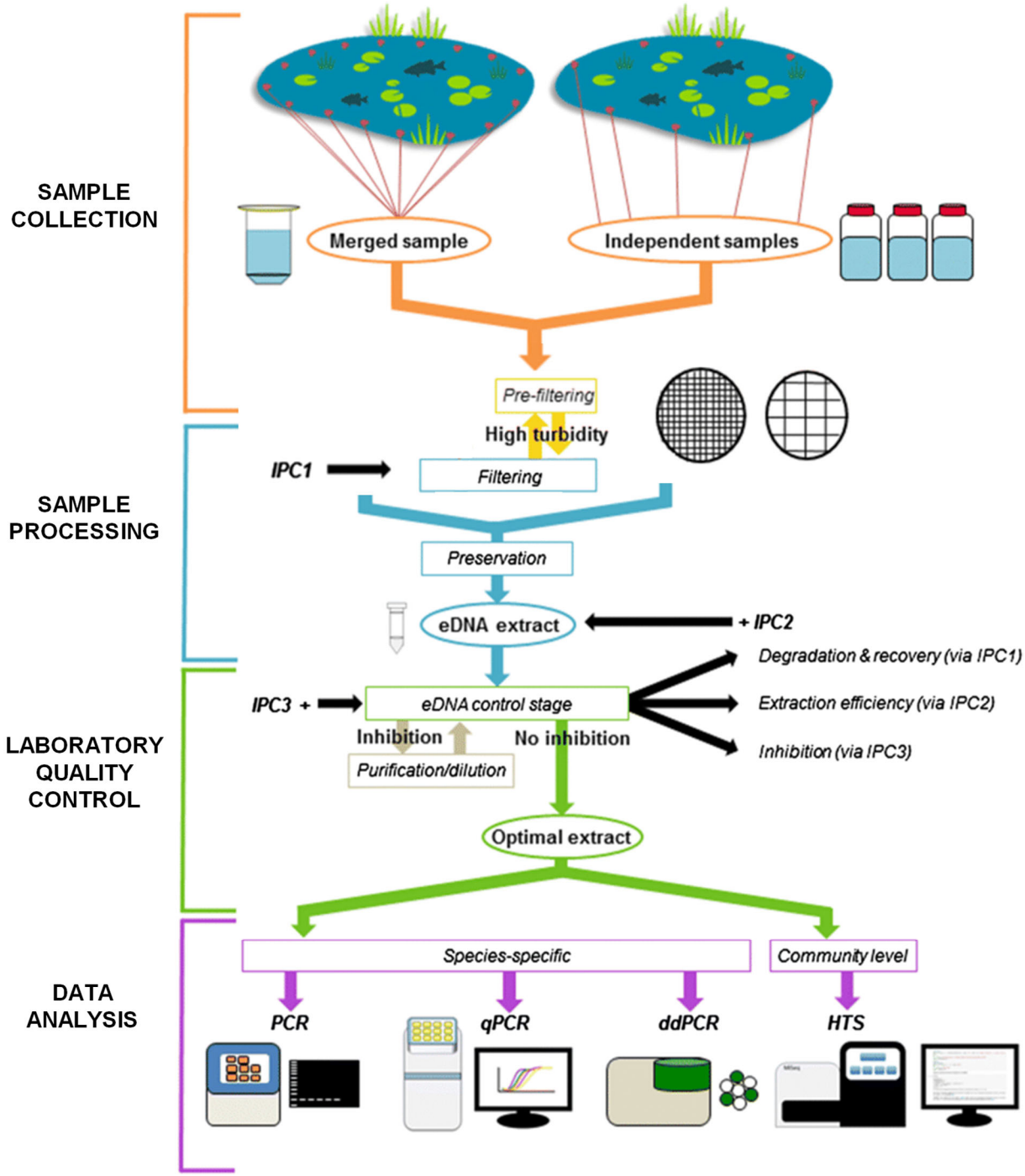

Figure 5-1. Schematic of a generic eDNA study implementation workflow (modified from Harper et al., 2019). IPC = Internal Positive Control, HTS =. High Throughput Sequencing

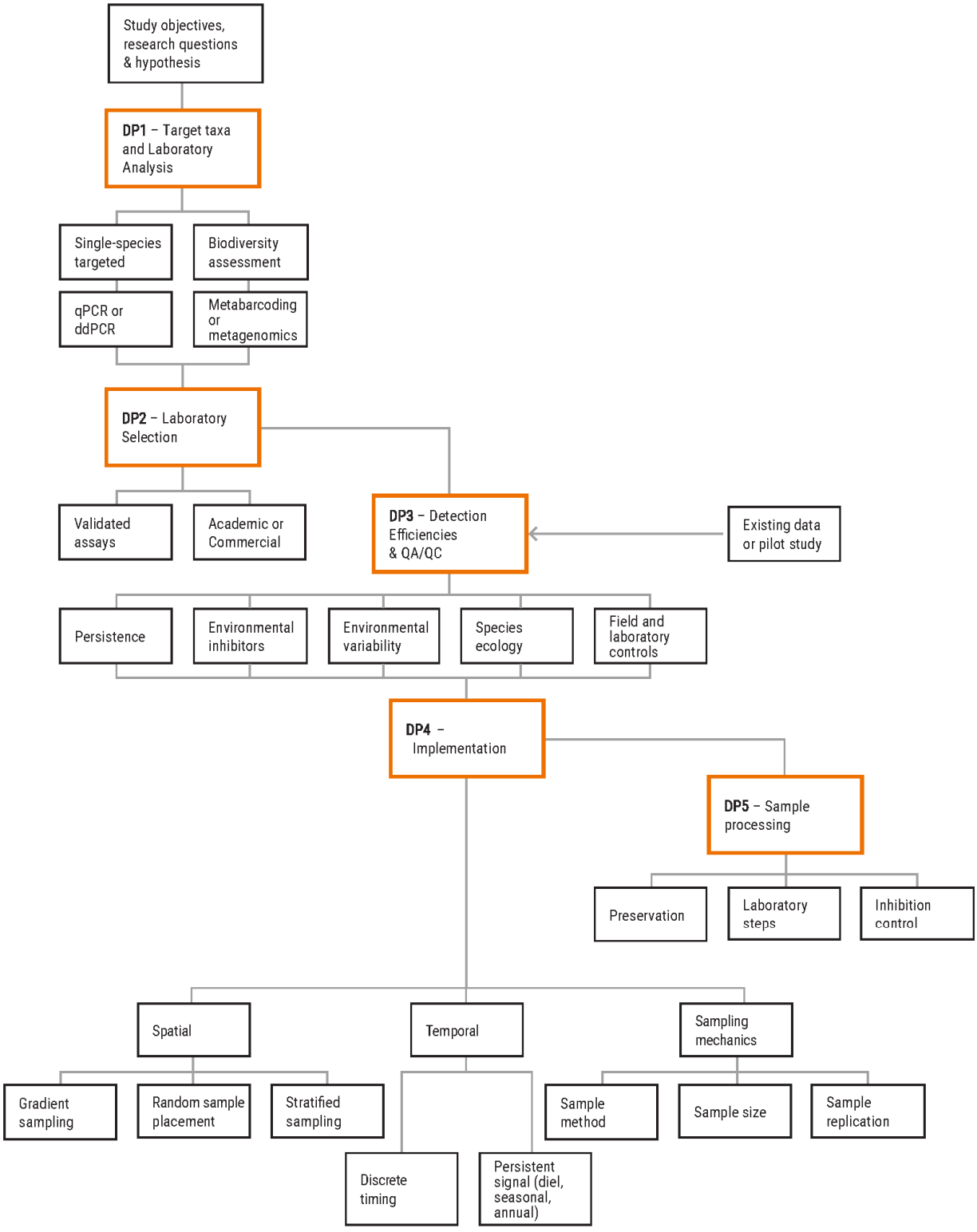

Figure 5-2 Design Considerations for an eDNA study. DP = deduction point

5.2 DP1 – Sample Analysis Type

As noted in Section 5.1, the study objective, research question, or hypothesis is going to dictate both the sampling effort and type(s) of laboratory analysis required to achieve the desired objectives. While the standards and protocols related to laboratory methods and bioinformatics are outside the scope of this report, certain foundational information is required for anyone who aims to conduct/contract eDNA sampling and/or laboratory services from an academic or industrial provider. As outlined in Appendix B (Table B-1), multiple published guidelines detail recommended data analysis and reporting practices. To date, only one known standard for eDNA reporting has been published (CSA W214:21; CSA, 2021), and readers are referred to that document for recommended reporting. IOGP-JIP34 Chapter 4 also provides guidelines for analysis and results reporting.

This report section aims to provide a high-level introduction to those concepts which are necessary to verify that best practices will be implemented to enable client, stakeholder, and regulatory confidence in the results provided and conclusions drawn from single species detection (i.e., targeted qPCR and dPCR) or community-based approaches (i.e., metabarcoding).

5.2.1 Single Species Detection – Targeted qPCR & ddPCR

For species-targeted approaches, qPCR is a preferable process to conventional PCR as it can be used to determine relative quantities of DNA within a sample. In some cases, eDNA concentration can be correlated to organism abundance (Yates et al., 2019), and therefore it can be useful to quantify eDNA across samples and between survey sites. qPCR uses the same process of PCR, however with the addition of real-time fluorescence detection. Fluorescent markers are added to the reaction mixture and emit light when activated. There are many kinds of fluorescent markers used in qPCR analysis, however the most recommended is probe-based qPCR due to its increased sensitivity. Species-specific probes are added into a PCR reaction and bind to the target DNA. During the amplification step, the DNA polymerase enzyme cleaves off the probe, resulting in fluorescence activation. Therefore, the fluorescence signal increases as more DNA is copied.

For any qPCR reaction, the Quantification Cycle (Cq, also called Threshold Cycle [Ct]) value is a key component of interpreting the qPCR results (Figure 5-3). The Cq value indicates the cycle at which the fluorescent dye becomes detectable. This provides the basis for DNA quantification. A sample with higher initial numbers of target DNA molecules will result in fewer qPCR cycles before reaching the detection threshold, thus displaying a lower Cq value. In other words, the more target DNA present in a sample, the lower the Cq value will be (red line in Figure 5-3). Conversely, low numbers of target DNA in a sample will result in higher Cq values (orange line in Figure 5-3). If the target DNA is present at a detectable level, it will be amplified in the PCR and will register as an amplification curve using fluorescence intensity as a function of PCR cycle.

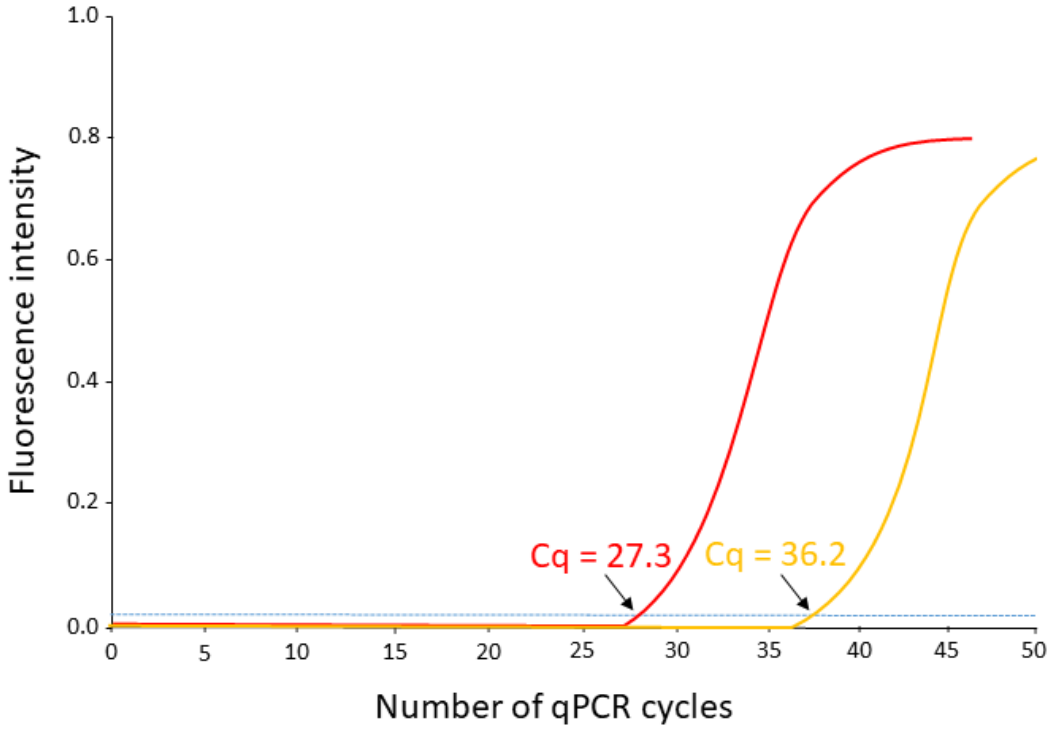

Figure 5-3 *Schematic diagram of a qPCR curve (from Abbott et al., 2021)*

The fluorescent signal intensity (vertical axis) increases above the detection threshold (blue dotted line) at a lower number of qPCR cycles (horizontal axis) in samples with greater DNA concentration and is reported as the quantification cycle (Cq). The number of reaction cycles and fluorescence signal intensity (correlating to DNA concentration) are inversely proportional.

Estimating target DNA concentration requires normalization against a dilution series of a standard with a known concentration (Figure 5-4). Typically, this consists of at least 5 different concentrations of the DNA standard. This standard must contain the identical primer and probe binding sites to that of the target species being tested. As high-concentration positive control DNA poses a contamination risk (especially when working with low template samples such as eDNA), the standard DNA should ideally be created using purified amplicons (e.g., Currier et al., 2018), or double-stranded synthetic amplicons (e.g., gBlocks™; Langlois et al., 2020).

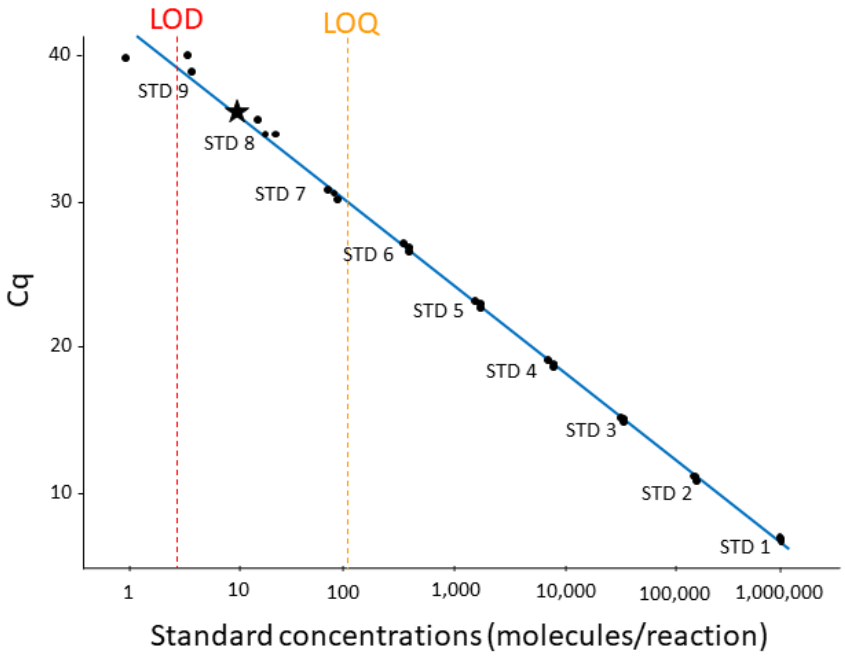

Figure 5-4 A qPCR assay standard curve showing results of solutions with known (circles) and unknown (star) DNA concentrations and limits of detection (LOD, red) and quantification (LOQ, yellow) (from Abbott et al., 2021).

In Figure 5-4, STD 1 through STD 9 represent a dilution series of known DNA concentrations from high to low, respectively. The Cq (quantitation cycle) on the vertical axis is the cycle at which the fluorescent signal is detectable by the qPCR instrument and is plotted against DNA concentration (in log scale) on the horizontal axis. The LOD and LOQ are estimated statistically based on the variance between replicates of standards.

Appropriate interpretation of targeted eDNA assay results is dependent on the accuracy of the assay being used. Therefore, it is crucial to use properly validated assays. A set of five validation levels have been proposed along with clear guidelines for how to both develop new assays and implement previously developed assays (Thalinger et al.,, 2021a) (Figure 5-5). This extensive set of guidelines can be used as the basis on which to standardize reporting guidelines for targeted eDNA assays, facilitating their use for the entire scientific community, including environmental managers.

The process undertaken to validate an eDNA assay should be reported, which includes information on specificity and sensitivity (Figure 5-5). Specificity refers to an assay's likelihood of amplifying non-target DNA. A developed assay should include a list of target and non-target organisms that were examined, the source and number of distinct voucher specimens used, the form and quantity of DNA, and the degree of amplification for non-targets. Sensitivity refers to an assays reliability and repeatability to detect low concentrations of target DNA. A developed

assay should include what concentration dilutions were tested, and the statistical methods used to determine sensitivity.

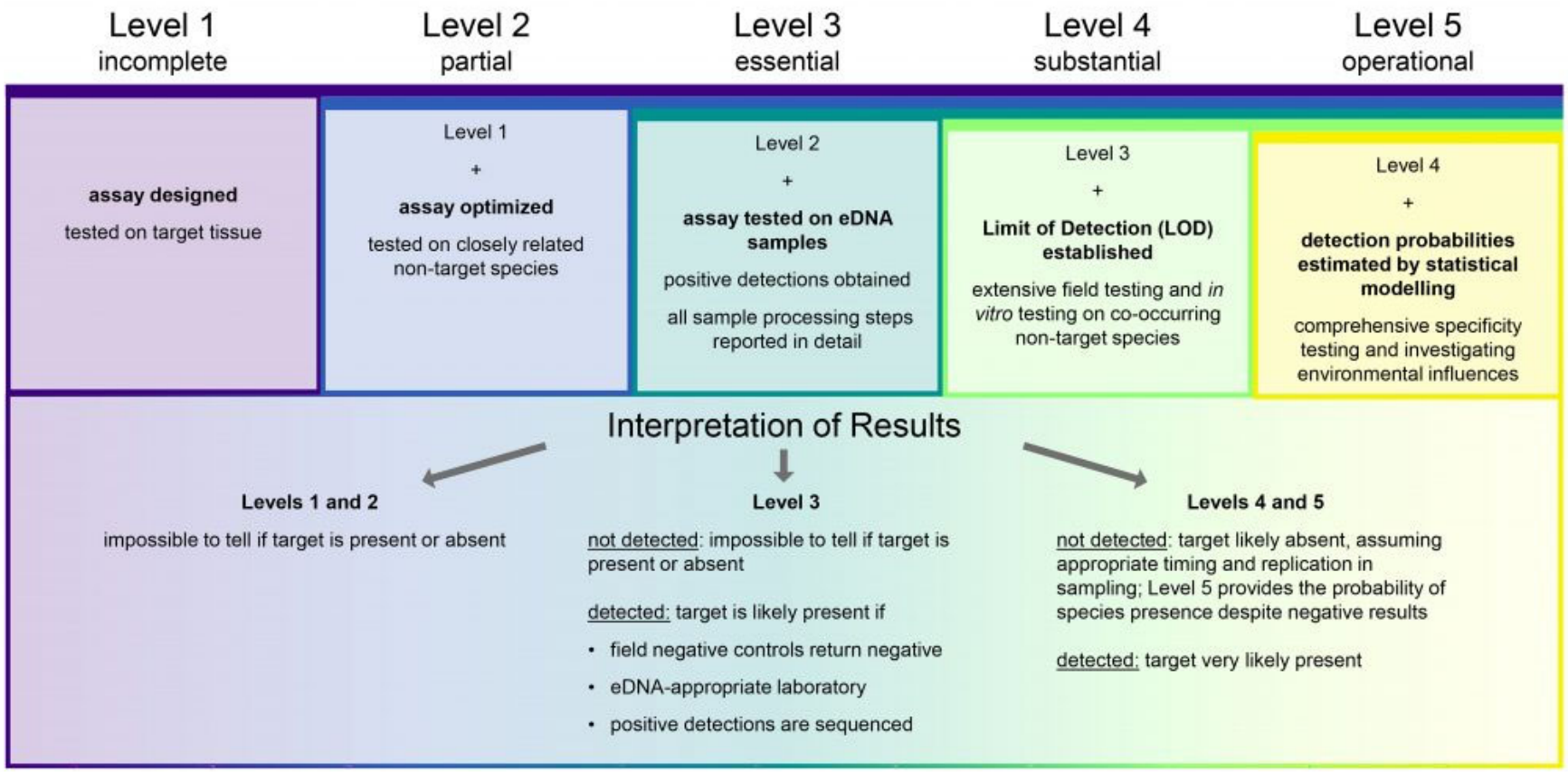

Figure 5-5 Overview of the 5-Level Validation Scale for qPCR assay development (from: https://edna-validation.com)

In order to assess the sensitivity of an assay, the limit of detection (LOD) and limit of quantification (LOQ) should be determined after an assay has been optimized and must be reported for an assay's results to be interpreted correctly (Klymus et al., 2020). The method for determining LOD and LOQ varies across platforms (e.g., qPCR vs ddPCR), however the value of these limits is always calculated using the DNA standards (Figure 5-4). To properly calculate these limits, multiple PCR replicates (5 to 12) are performed for each concentration of the DNA standard. LOD and LOQ can be defined in various ways (e.g., Agersnap et al., 2017; Hunter et al., 2017), and a recent effort to standardize these definitions ultimately defined LOD as being the lowest concentration at which 95% of the technical replicates of the standard amplify, while LOQ was defined as being the lowest concentration for which the coefficient of variation (CV) value is <35% for the used standard DNA (Klymus et al., 2020); definitions also adopted by (Thalinger et al., 2021a). Based on these definitions, it is still possible (indeed common) to detect target DNA at concentrations below the theoretical LOD, especially when multiple technical replicates are run for each environmental sample. Detections below the LOD may be considered at a lower confidence level in interpretation of results but should not be ignored.

For ddPCR, the lowest concentration that can theoretically be detected and measured is one target molecule per reaction (Deprez et al., 2016; Hunter et al., 2017; Thalinger et al., 2021a), and each reaction can include up to 10 µl of DNA template because of the reduced impact of inhibitors compared with in qPCR. The same practical definition of LOD as used in qPCR can be applied to ddPCR (95% positive detections of standard DNA) meaning that it is still possible to obtain valid detections below the LOD. Note that when calculating the mean target concentration of samples, it is important to include non-amplifications as zero-estimates. Droplets beyond the fluorescence threshold are counted as positive events, and those below threshold as negative events.

Laboratory quality controls (positive and negative controls) are essential to successful interpretation of results and their use should be detailed in reporting of methods and results. Reporting requirements regarding the validation of the eDNA assay includes information on specificity (e.g., list of targeted and non-targeted species) and sensitivity (e.g., LOD/LOQ). The results should include the method used to estimate the target eDNA concentration in the environmental media (when appropriate), the confirmation of potential positive detections, the number of detections per total number of technical replicates of a sample and the decision criteria used for determining a positive or negative technical replicate (see Section 5.4.2 for more detail).

5.2.2 Multiple Species Detection - Metabarcoding

For a given metabarcoding project, it is important that all samples are processed with the exact same bioinformatics pipeline, as different bioinformatics pipelines and parameters can potentially give significantly different results from the same raw sequence data. It is also particularly important to consider the need to link together datasets generated from different

sequencing runs. This is especially relevant for taxonomic groups and markers with incomplete reference databases, meaning taxa cannot be linked based on species names and may influence the choice between use of OTUs (operational taxonomic units) and ASVs (Amplicon Sequence Variants; Callahan et al., 2017).

OTUs may correspond to ecological species, niche uniqueness, or biological species (i.e., unique reproductive pools) (Cristescu, 2014). The OTU approach overcomes PCR and sequencing errors that may be present within metabarcoding datasets by clustering together highly similar sequences, with the most dominant sequence from each cluster used for taxonomic assignment. In contrast, ASVs keep each unique sequence separate but filter out potential PCR and sequencing errors based on built-in error models. While overall ecological patterns derived from metabarcoding data tend to be robust to the choice of approach (Glassman & Martiny, 2018), ASVs are more reproducible, and therefore more cross-comparable where linking relies on sequence identity rather than species names (Callahan et al., 2017).

Choice of taxonomic assignment method and taxon acceptance thresholds (i.e., the number or proportion of sequence reads required for an OTU/ASV to be retained in the final dataset) can alter the interpretation of species detections. Optimal parameter choices will depend on the characteristics of the marker used, the completeness of the reference database, and the purpose for which the data is to be used. For instance, if the aim is to assess overall ecological patterns, then more aggressive filtering may be chosen to reduce noise while there is a relatively low cost for inaccurate taxonomic identification. However, if the aim is to detect invasive or endangered species, even very weak detections may be considered, and each species needs to be identified with a high degree of accuracy.

Improvements in taxonomic resolution using eDNA requires robust reference DNA databases (Baker et al., 2018; Everett et al., 2018; Hebert et al., 2003). Reference DNA databases, such as BOLD and GenBank, match curated and verified species to their genotypic sequences (Cowart et al., 2015; Thomsen et al., 2016) and the databases grow as more studies are conducted to contribute verified species. Although databases entries are currently lacking for many species, reference libraries in general are growing (Ratnasingham & Hebert, 2007; Vitecek et al., 2017) and will improve over time. However, many taxonomic groups have little to no representation within reference libraries and, as such, taxonomic assignment from a metabarcoding dataset is currently limited.

Limitations such as insufficient DNA reference libraries could potentially be avoided by skipping the species identification step and assigning ecological values to molecular operational taxonomic units (MOTUs), correlating between species/OTUs occurrence and environmental factors (Apothéloz-Perret-Gentil et al., 2017). This type of taxonomy-free molecular index was tested using a diatom index and showed higher correlation between morphological and molecular indices without taxonomic assignment (Apothéloz-Perret-Gentil et al., 2017). An additional benefit of this approach is that it used 95% of the OTUs in the eDNA sample, as opposed to 35% with the taxonomic assignment approach.

While there are common elements in sample collection and extraction methods shared between targeted (single) species detection and metabarcoding for community analysis, most other aspects regarding sequencing and data analysis are substantially different and hence have different reporting requirements. The minimum information required for the reporting of metabarcoding data analysis to provide sufficient information is conveyed to support data transparency, reproducibility, and review, are set out by the Canadian Standards Association (CSA W214:21; CSA, 2021). Reporting requirements relating to metabarcoding data analysis include, sequence read length, QC criteria, read count per sample before and after quality filtering, software packages and parameter setting used for quality control, sequence assembly, alignment, OTU/ASV assignments, minimal read thresholds, reference libraries used. These minimum reporting requirements will improve confidence in eDNA results and their interpretations and enhance the comparability between multiple studies and between eDNA practitioners. The wider integration of reporting standards will support eDNA data collation, data mining, and meta-analytical approaches for addressing larger-scale environmental questions.

5.3 DP2 - Laboratory Considerations

The breadth of services offered at individual laboratories varies considerably and most laboratories specialize in one or more groups of species and/or types of analyses. Selecting the right eDNA laboratory starts with determining which services are appropriate for addressing your question (as per DP1, Section 5.2). Selection of the eDNA laboratory to use requires up front research, then contacting candidate laboratories to inquire about eDNA services and available assays.

When beginning to look for an eDNA laboratory, it is important to determine the type of eDNA analyses you seek. If you are unsure, then be clear on the question you would like addressed and consult an eDNA advisor or a broader service laboratory to learn about options to address your question. eDNA analyses are typically offered by a specialty commercial laboratory or research laboratory based at academic institutions or agencies that offer commercial services. In addition to eDNA sample analysis, laboratories may offer consulting support in terms of advice on program design and interpretation and may also offer field sampling services.

Commercial laboratories for eDNA analysis are becoming established and, as a business, are expected to have standardized practices for sample handling, analysis, reporting, and turn-around-time for completion of services. Commercial laboratories will also provide a list of available services, a pricing structure, and a mechanism to issue quotations to establish an agreement for scope of services. In comparison, research laboratories may be more flexible and creative in terms of services offered and pricing; given that they are not set up as businesses, research laboratories may not have the same level of technical rigor, QA/QC, turn-around time, reporting, pricing, and contract frame that is provided by commercial laboratories. It is therefore important to do the groundwork to select a suitable laboratory that will address your

needs for services, reporting, pricing, and timelines. Please note that guidelines for laboratories to follow are covered in a separate chapter (Chapter 3: Laboratory Analysis Guidelines and Best Practices for Environmental Genomics Applications Relevant to the Energy Sector).

A good starting point in looking for eDNA laboratories is to conduct an internet search which may reveal laboratory options in your target geography and species of interest. For example, the University of Washington maintains a list of commercial laboratories which is updated periodically (https://ednaresources.science/edna-labs). The scientific literature is also a good place to learn who is publishing eDNA papers on your species of interest and then contact them to discuss options for eDNA analyses (Appendix B).

It is important to contact the laboratory prior to sample collection to determine the laboratory's suitability and to get more detailed information. You may want to ask the following questions:

1. What analyses does your laboratory offer?

   qPCR tests:

   - These should be specific to species (common name, latin name) or species group (e.g., at the genus level); request a list of validated tests by species (validation = demonstrated to meet Minimum Information for the Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines, tested in the laboratory with reports detailing specificity and sensitivity [e.g., published in a primary journal]).
   - Inquire about number of "technical replicates" included (i.e., replicate tests conducted on sub-samples of the eDNA sample extract); typically, 3 at minimum and preferably 4 to 12 technical replicates per eDNA extract is included in the sample cost.
   - New qPCR assay development: if the laboratory does not have the assay developed for the species you are inquiring about, ask if they would develop an assay, and the cost, time and steps that would be involved. This may involve building the assay based on published DNA sequences or application of a published assay in the laboratory to verify that it meets MIQE guidelines. In either case, genomic DNA will be required from that species and closely related conspecifics.

   Metabarcoding:

   - Assays should be specific to a particular group of species using primers that have been developed to discriminate between taxa for the group of species you are interested in.
   - Inquire about their bioinformatics platform and if they will release raw results in the event that you are capable of doing the bioinformatics to parse through the raw data. DNA reference databases are constantly updated with new species vouchers, and old metabarcoding data can be re-analysed in the future potentially identifying new species.

Tissue DNA Barcoding:

- It is often beneficial to provide tissue samples from voucher specimens for the purpose of generating reference genetic databases and/or validating an eDNA assay.

2. What is the laboratory history and qualifications for eDNA analyses? What is their experience with running analyses similar to what is needed?

   Note: This is a nascent service area for which laboratory standards and certifications are in development or newly emerging. Other than standards/certifications that apply to the laboratory's services and jurisdiction/geography, additional qualifications may include published scientific articles and/or references from clients.

3. What is the analysis cost per sample?

   - Is there a cost reduction per test based on number of samples submitted?
   - Is there a minimum number of samples required to perform the analysis?

4. Are sampling supplies provided and, if so, at what cost?

   - Note:
     - Some laboratories may be specific about types of eDNA sample filters and sample types they will accept and may require that you use the supplies they provide.
     - Prices for testing of samples may include these supplies.
     - Account for time for laboratory to ship supplies to you.

5. What quality assurance and quality controls are employed in the laboratory to demonstrate reliability of results?

   - Note:
     - Quality assurance includes use of qualified individuals for sample handling (Section 5.4.1), testing, data analysis and reporting; use of standardized or published procedures; methods used to avoid in-lab contamination of samples; use of high-quality reagents (Section 5.4.2).
     - Quality controls include use and reporting of internal positive controls (IPC), laboratory blanks, technical replication (for qPCR), testing for inhibition.

6. Reporting:

   - What is the turn-around-time to receive results?
     - Note –TAT for a commercial eDNA laboratory may be 2-3 weeks for qPCR and 10-12 weeks for metabarcoding.
   - How will results be reported?
     - Ask for an example of a report for the type of analysis you are seeking.
     - Note: minimum requirements should include list of samples received and media sampled, date of sample receipt and eDNA extraction, date, and description of

methods of extraction and eDNA analysis, results per sample (for qPCR the Cq per technical replicate), results for internal laboratory controls and statement of confidence in the sample results, assay validation information, name and signature of laboratory approver for release of results.
    - Does the lab comply with MIQE guidelines, CSA (2021) or other accepted standard, including reporting on outcomes that affect reliability of results (Section 5.4.2)?
    - A list of key reporting requirements for metabarcoding laboratory reports can be found in Bruce et al. (2021).

7. Review how samples should be collected and submitted (e.g., specific type of filter, preservation method).

    - Will the lab accept samples submitted on only particular type of filter? (Note – check that they will extract eDNA from the type of filter you plan to use to collect eDNA from the media you are sampling).
    - What level of field controls does the lab recommend (note: all eDNA surveys require some level of field negative controls to evaluate potential cross contamination pertaining to sample equipment and sample handling)
    - How should samples be preserved (frozen, desiccant beads, preservation media)?
    - How should the samples be stored prior to extraction and analysis?
    - Do they have a preferred shipment method? (e.g., courier type, shipment method)

8. Review a sample submission (chain of custody) form.

    - Note:
        - The sample submission form provided by the lab or your own should include, at minimum, company/agency name, name of contact person, name of sample collector, date of sampling, list of sample identifiers, matrix sampled (e.g., water, soil, air, swab of tissue), location of sampling, type of filter and packaging used, shipping information to retain sample integrity, shipping method (e.g., courier), chain-of-custody tracking (i.e., signature of sender/receiver for each stage of shipment).
        - The sample submission form should include a place for the laboratory to record condition of samples upon receipt (e.g., damage, missing, temperature).
        - The sample submission form (scanned to PDF) should be included with the laboratory report.

9. Does the lab archive eDNA extracts?

    - Previously collected eDNA samples might be desired for future analysis.
    - Is there a cost for archival storage?

10. Is there a quotation process to form a business agreement for sample analysis including cost, quality, and turn-around time

    - Note: it is important to have a business agreement in place prior to shipment of samples. The business agreement can originate from the purchaser (your company or agency) or the business (laboratory) and should be signed by both parties. It should include standard terms and conditions for provision of services. If the services include development of a novel assay, then the business agreement should include terms regarding who owns the intellectual property for assay development.

Additional considerations for recommendations on the reporting standards from an eDNA laboratory can be found within Appendix B and in CSA (2021).

5.4 DP3 - Probability of Detection & QA/QC

This deduction point (Figure 5-2) assesses factors that influence the probability of eDNA collection and detection of a species if it is truly present in the environment. This information is important to evaluate prior to sample collection, along with factors which may influence data analysis and laboratory detection (Figure 5-1). It is necessary to implement the correct quality assurance/quality control (QA/QC) measures for accurate evaluation of results. It is important to understand what environmental factors may introduce inhibition and reduce the efficiency of an eDNA study. Additionally, for an eDNA study to be effective, it is paramount at the earliest stage to determine the validation status of the intended eDNA assay(s) (Thalinger et al., 2021a), gather up-to-date genetic reference libraries, and determine the persistence/degradation potential of target eDNA.

5.4.1 Sampling Quality Control

As is the case with conventional detection and monitoring methods, eDNA methods have biases and limitations that need to be considered when developing a sampling campaign. eDNA study design should consider vital steps to reduce potential erroneous results due to false positive and false negative detections (Figure 5-6). Field sampling considerations and their associated mitigations are discussed below.

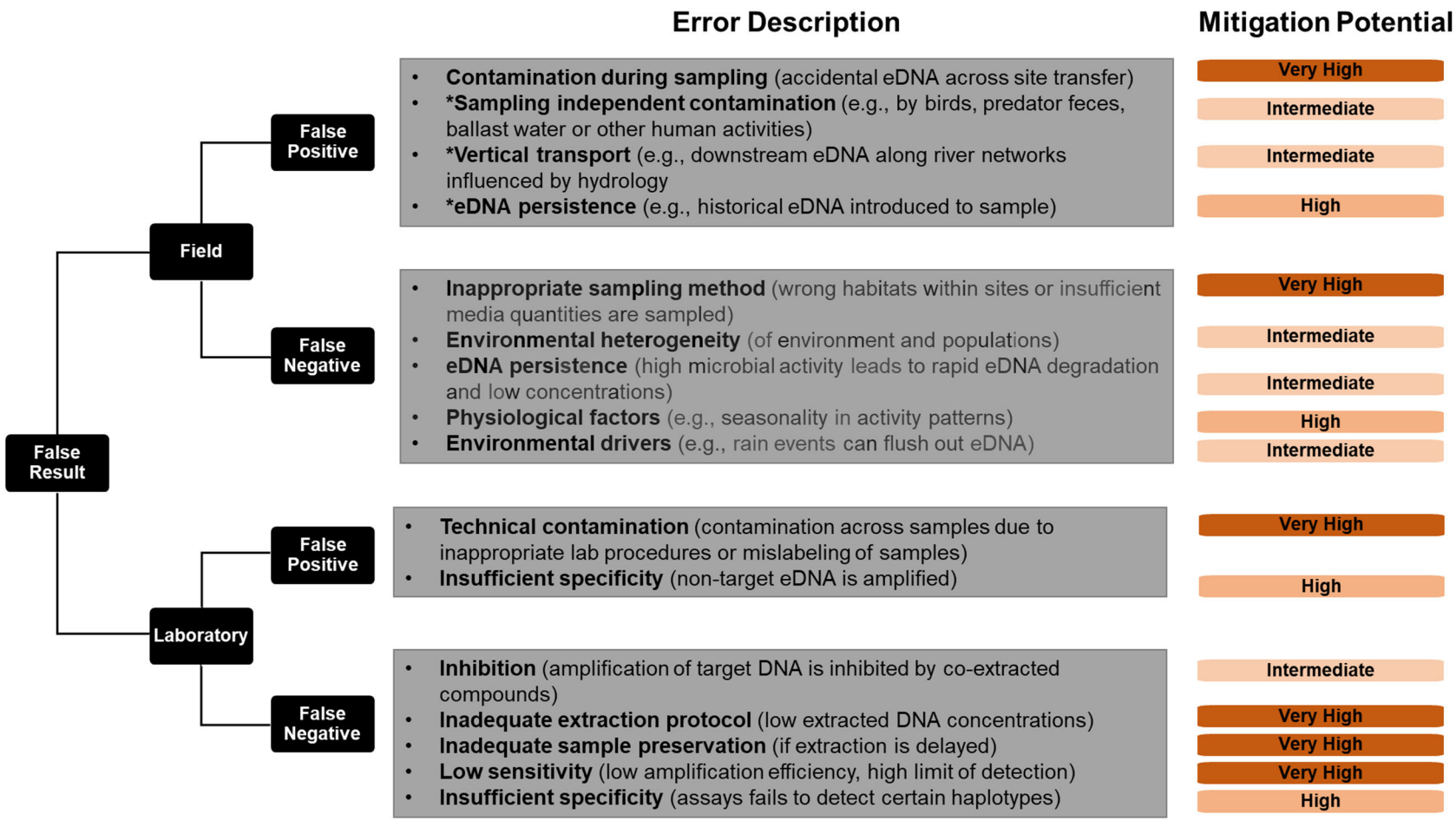

Figure 5-6 Factors influencing a false result (false positive or false negative) introduced during eDNA field collection or laboratory analysis, with the potential for mitigation based on eDNA study design (modified from Burian et al., 2021). * = Errors not considered "true" false positives with explanation in text below

5.4.1.1 Fieldwork-Derived False Positive Detections

A fieldwork-derived false positive detection is defined as an erroneous detection of a molecular target within an environmental sample because of accidental sample contamination in the field, or a series of potential eDNA transport or persistence confounds, as described below.

For a reader to understand the terminology of a false positive, it is first important to state the realistic capabilities of eDNA testing. For any eDNA program, analysis of an eDNA sample will provide information on the detection or probable absence of a DNA molecule. In many cases, the presence of a DNA molecule will represent the close spatial and temporal presence of the species of origin. However, it is possible that detection of a DNA molecule does not represent a nearby organism, and rather the molecule was moved within the environment (e.g., sampling contamination, transport, or prolonged persistence; Figure 5-6). In such cases, the detection of transported DNA should not be considered a "true" false positive detection because the molecular assay was performing for its intended purpose (i.e., detection of a specific DNA molecule within an environmental sample; see Darling et al., 2021).

As such, the mitigation potential for contamination is very high as the required actions are simply to maintain standard operating procedures common to scientific sample collection. Specifics will vary among geographical sampling locations, habitat types, and target taxa; however, the following fundamental principles will apply to all eDNA sampling programs:

1. Use disposable nitrile gloves with glove changes between sampling locations and between samples if samples are not to be combined.
2. Sampling equipment and instruments should be disinfected between sampling locations. Use of commercial products (e.g., ELIMINase) or a 10-50% bleach solution for at least 10 minutes followed by rinses with copious amounts of distilled water (or tap water if no distilled water available) should be used to reduce contamination potential. If tap water is used, it should be tested for target DNA to confirm that no new contamination has occurred.
3. Consider other equipment used to enter the sampling environment and how to disinfect where necessary. Rubber boots are optimal wear to enter a waterbody and can be decontaminated between sites. Decontaminate with the above-mentioned solutions and rinse well with distilled water or tap water.
4. To monitor potential contamination and verify the disinfection protocol, the inclusion of field blanks (clean media samples processed using the same protocol and equipment as well as preserved and processed in the same way as the actual field samples) should also be standard practice.
5. USFWS (2023) outlines multiple places in which a field blank should be included within the field work plan. For example, if filtration occurs on site, a pump field blank should be included where target DNA-free water is filtered following the same process as the environmental samples. Alternatively, if grab samples are collected in bottles in the field and returned to the laboratory, a bottle field blank should be included in which a bottle of target DNA-free water is brought into the field, opened briefly on site, and stored along the environmental samples prior to sample processing. An additional pump blank

can help determine if contamination occurred on-site at the bottle stage, or back in the laboratory during the pump filtration stage.

6. USFWS (2023) recommends a minimum of 10% of total samples collected are some type of field controls.

5.4.1.2 Fieldwork-Derived False Negative Field Detections

A fieldwork-derived false negative occurs when a molecular target is present within the environment; however, the target goes undetected as a result of inappropriate sampling methods/technique or sampling design (lack of spatial or temporal replication), or a series of potential eDNA transport or persistence confounds (Figure 5-6).

In some cases, a lack of detection could have occurred because of insufficient replicates or volume of media sampled, much like what may occur in any conventional monitoring method. Sampling strategy is key to successfully implementing an eDNA survey. How, where, and when an eDNA sample is collected can influence the amount of eDNA captured in the sample. Spatial and temporal availability of eDNA due to target taxa life history (Section 4.3; e.g., seasonal absence from a particular habitat and/or timing window, reduced eDNA shedding rates related to behaviour and metabolic activity), poor choice of sample media/sampling method, or environmental variability have mitigation potentials ranging from intermediate to very high (Figure 5-6). Careful consideration, often requiring consultation with biologists experienced with target taxa and the local habitats are required during the study design phase. Specific recommendations on sample collection from common media are provided in Section 5.5 while considerations and mitigations during sample processing are detailed in Section 5.6.

5.4.2 Laboratory Quality Control

To provide a robust application and interpretation of eDNA assays, laboratories should report on key considerations of the targeted approach. The minimum information required to report are listed by the Canadian Standards Association (CSA W214:21; CSA, 2021). The design of species-specific primers and probes is critical for any targeted single species assay. Therefore, the primers and probe sequences used, including fluorescent dye and quencher type and the position on the probe should be detailed (CSA W214:21). As a guiding principle, the design and implementation of PCR primers should follow the established guidelines for the Minimum Information for the Publication of Quantitative Real-Time PCR Experiments (MIQE), which have been adapted for use in eDNA applications (Bustin et al., 2009; Goldberg et al., 2016; Langlois et al., 2021). Information on PCR assay should incorporate reagents and concentrations, technical replicate reaction volumes, instrument and cycling conditions and multiplex method if used.

As introduced for field sampling in Section 5.4.1, potential biases and limitations of laboratory eDNA procedures require consideration to reduce potential erroneous results due to false positive and false negative detections (Figure 5-6).

5.4.2.1 Laboratory-Derived False Positive Detections

A laboratory-derived false positive detection is defined as an erroneous detection of a molecular target within an environmental sample resulting from:

- accidental laboratory sample contamination
- insufficient specificity in assay design, or
- inadequate reference genetic database for taxonomic assignment of metabarcoding data.

Laboratory sample contamination is defined as the accidental exposure of an environmental sample to DNA sources exogenous to a particular sample. This may occur anywhere in laboratory process, from sample preparation, DNA extraction, amplification, and sequencing in the laboratory (Bruce et al., 2021; Le Port et al., 2018). Due to the ability of PCR to generate billions of DNA copies, contamination that occurs in the laboratory can result in a false positive detection from a non-environmental source, which could have serious implications for the resulting dataset (Le Port et al., 2018).

The mitigation potential for laboratory contamination is very high (Figure 5-6) as standard precautions can be taken in the laboratory (Goldberg et al., 2016) and this is discussed in more detail for laboratories in a separate chapter (Chapter 3: Laboratory Analysis Guidelines and Best Practices for Environmental Genomics Applications Relevant to the Energy Sector). Mitigation includes the use of disposable gloves (changed between every new sample to avoid cross-contamination) and the disinfection/bleaching of sampling devices and laboratory equipment that may come into contact with samples. Additionally, filtration, DNA extraction, and PCR procedures, as well as pre- and post-PCR procedures, must be separated physically within the laboratory to reduce the risk of contamination (Goldberg et al., 2016). Moreover, to monitor potential contamination (i.e., to identify the source of contamination when it occurs), the inclusion of DNA extraction blanks and PCR blanks is essential (De Barba et al., 2014).

Species-specific targeted assays may provide false positives due to low specificity from improperly validated assays. Additionally, when assays are implemented outside the geographic region for which they were originally developed, there is the potential for cross-amplification of sympatric species, leading to false positive detections from species that were not previously validated when the assay was first developed. Thus, the validation status of the primer pair should be considered with great care and additional tests for specificity and sensitivity may be required (Thalinger et al., 2021a). Based on this, the mitigation potential for this issue is high, as it only involves further testing for specificity (Figure 5-6).

For metabarcoding approaches, inadequate reference genetic databases may result in a sequence to be taxonomically assigned to the incorrect species, as is discussed in more detail in a separate chapter (Chapter 4 Industry Guidance on Bioinformatics Analysis Standards and Guidelines for eDNA Data Relevant to Energy Projects). Such misidentification may occur if closely related species have similar or identical sequences within the target DNA region (often occurs within evolutionarily conserved genes). This may be rectified by selecting another gene

region that varies between these species and therefore can be used to discriminate between them. Misidentification may also occur if the DNA sequence for a target species is not represented in a reference database. In this case, the sequence may instead be assigned to a related species that may or may not be present in the geographic area sampled.

Taxonomic assignment can be improved by:

- Evaluating genetic similarity between target species and closely related sympatric species for gene regions amplified with the intended assay(s)
- Supplying tissue samples for up-to-date reference sequences for target taxa within your sampling region

5.4.2.2 Laboratory-Derived False Negative Detections (Non-detection when species are present)

A laboratory-derived false negative is defined as the erroneous non-detection of a molecular target that is truly present within an environmental sample. This may result from presence of inhibition, improper sample preservation/extraction, inadequate assay design, or an inadequate reference genetic database.

PCR inhibition is caused by the presence of chemicals and compounds that interact with the PCR enzyme activity and efficiency (Jane et al., 2015; McKee et al., 2015). Inhibitors have been noted to be present in water samples, but are more commonly an issue within soil, sediment, and fecal samples. Typically, complex humic substances are the main known inhibitor of PCR (Braid et al., 2003), however, phytic and tannic acids, leaf litter, algae, and sediments have all been found to reduce PCR efficiency (Stoeckle et al., 2017; Lance & Guan, 2021). Inhibitors tend to be more prevalent in eutrophic waters than in oligotrophic ones and are also associated with high sediment loads, shallow waters that contain a lot of organic material such as feces from livestock, heavily polluted waters, and samples containing a high concentration of calcium carbonate (e.g., DNA extracted from samples that contain bivalves and gastropods; Schrader et al., 2012).

Thus, it is important to test extracted DNA for inhibition in order to avoid or interpret potential false negative results. This can be achieved using an exogenous IPC DNA added at a known concentration to the extracted DNA (Hoorfar et al., 2004; Furlan et al., 2016; Brys et al., 2021). This DNA, which can be purchased commercially along with the qPCR primers and probes, can be added, in a well-defined concentration to the filter capsule shortly after filtering. If a liquid preservative is used, then the IPC can be pre-mixed into the preservative and efficiently added this way. Note that IPC added to the water prior to filtering may not be captured effectively in the filter since it is not in the same state as eDNA (predominantly cellular, subcellular or particle bound). After DNA extraction, IPC concentration can be quantified using qPCR or ddPCR to check that it is recovered at the expected concentration. Testing should be carried out using the specific sampling and DNA extraction method to be employed, to enable IPC recovery, and to determine the concentration to be added to the sample and the expected results. If amplification of the IPC fails or quantification is lower than expected, the sample should be assumed to be inhibited (Hartman et al., 2005).

Inhibition may be overcome with additional laboratory processing, such as purification steps (using commercial kits such as the Zymo OneStep PCR Inhibitor Removal Kits or Qiagen PowerClean kits), use of chemical enhancers such as bovine serum albumin (BSA) and dimethyl sulphoxide (DMSO) in the PCR reaction, or by dilution of the DNA extract. The inhibition test should be repeated to check that the inhibition has been overcome. While dilution of the DNA to overcome inhibition may reduce the chance of one type of false negative result (inhibition) it can also increase the chance of false negatives occurring due to stochasticity by reducing the concentration of target DNA. This can be especially consequential when working with low concentrations of eDNA. Dilution should therefore be compensated by increasing the number of PCR replicates performed or by increasing the volume of extracted DNA added to each reaction.

False negative results may also arise because eDNA has degraded between sampling in the field and laboratory processing. DNA degradation is a process by which DNA has been damaged to the point that it cannot be amplified using PCR and subsequently detected. The use of preservatives in sampling kits reduces the risk of DNA damage; however, inappropriate storage conditions or a prolonged delay before extractions are conducted may still lead to DNA degradation. If a sample is degraded, a negative qPCR result or absence of metabarcoding sequences could be due to a lack of target species DNA, or because the DNA is present but too degraded to be amplified and subsequently detected. As mentioned previously, use of an IPC can be used to assess DNA degradation. IPC may be added to sampling kits and a qPCR assay specific to the control DNA then can be used to test samples for DNA degradation. DNA degradation is evident if the signal from the IPC is lower than expected or absent.

There are challenges associated with designing and validating species-specific primers that provide consistent specificity and sensitivity across diverse ecological systems. Several guidelines have been suggested for development of qPCR-based environmental assays (Bustin et al. 2009, Goldberg et al., 2016; Langlois et al., 2021; Thalinger et al. 2021a). The bulleted steps below should be addressed by the molecular laboratory to mitigate improper (low specificity or sensitivity) assay design (as also described separately in Chapters 3 and 4):

- Follow the established guidelines for the Minimum Information for the Publication of Quantitative Real-Time PCR Experiments (MIQE)
- Establish a standard curve consisting of a series dilution to calculate a limit of detection and limit of quantification for the assay

False negatives can also occur in metabarcoding approaches if the target species does not have a reference sequence present in the genetic database for the specific gene region analyzed. Metabarcoding assays are typically developed to detect a wide range of species, and development of genetic reference databases enables the evaluation of assay performance for the group of interest. Additionally, many assays that target broad taxonomic groups (e.g., all eukaryotes) can often miss rare DNA targets that occur within the sample, as both PCR and sequencing bias may favor the abundant fragments. To reduce the potential for these false negatives, Cristescu & Hebert (2018) recommended increasing the number of samples per locality, analyzing multiple DNA extracts per sample, increasing the number of PCR cycles, and including positive controls (e.g., mock communities).

Mock communities are defined as synthetic communities and are used as controls when measuring abundance and composition. Mock communities are made up of tissue samples of known quantities of different species that represent a similar complexity to that of the expected environmental samples being analyzed. Mock communities are used to refine eDNA methods by testing the capacity to detect various species and can be analyzed in parallel to estimate rates of false positives/negatives (Cristescu, 2014). This finding demonstrated the utility of mock communities for metabarcoding analysis to identify the requirement to refine primer use to detect the full suite of target species.

5.5 DP4 - Sample Collection

This deduction point (Figure 5-2) extends from DP3 to estimate potential variability around factors influencing the probability of collection of an eDNA target. This will help guide decisions on the spatial and temporal allocation of sampling resources. For example, if an environmental gradient is known or hypothesized to exist (e.g., dilution from a point source along a river or an airborne plume) replicated sampling along such a gradient may be needed. Alternatively, if the project objective is to determine the potential presence of a target taxon within a section of relatively homogenous habitat (e.g., a terrestrial grassland or discrete operations area such as a tailings pond), a systematic sampling approach (e.g., grid sampling or evenly spaced transects) may be employed.

Additionally, sampling considerations may differ depending on the target biota and project objectives. For example, general biodiversity assessments may benefit from random grid sampling to cover a range of potential habitats/taxa, while species-targeted objectives should focus sampling efforts within the preferred habitat for the target species. Similar considerations are necessary in the temporal dimension, as there may be discrete and/or irregular timings of target taxa presence/activity (e.g., seasonal migration, mating season) which is likely to influence the probability of successfully collecting eDNA. Considerations are also required for the mechanics and logistics of eDNA sampling which will guide selection of sample volumes, filter size/type, as well as the necessity or allocation of technical replication. Ultimately, all the above will influence decisions related to any statistical analysis approaches.

Any existing data on the study sites, target taxa, or related ecological information need to be leveraged by the study team. If critical information is missing, a pilot study should be considered to address information gaps and allow for an assessment of adequate project resources; or to re-examine whether the original project objective is currently achievable. An initial pilot study can benefit an eDNA program by providing data to evaluate how, when, and where best to collect samples for a target organism within its environment. Preliminary data or a pilot study can be used to develop occupancy models. Occupancy modelling is often used in ecological surveys to account for the imperfect detection of rare and/or elusive animals, as described in more detail in Appendix C. Such models can estimate the probability of successfully collecting and detecting an eDNA target, providing useful information future sampling design (e.g., what number of samples are required to reach >95% probability of detection). Given that all survey methods are imperfect, occupancy modelling provides information to help tailor the study design to optimise detection.

There are four major considerations for the collection of eDNA samples (see Bruce et al., 2021):

7. What to collect (what sample media is expected to hold eDNA for target species or taxa).

   For example, marine eDNA samples collected from water, sediment, settlement plates, and plankton tows found only 6% of families were detected across all sampling media (Koziol et al., 2019). This suggests eDNA media is a critical consideration at the forefront of an eDNA study design.
8. How to collect (sampling device, number of environmental replicates, volume of medium).
9. Where to collect (considerations along the horizontal and vertical planes, number of sampling locations).
10. When to collect (based on the biology of target taxa and potential stochastic characteristics of the sampled environment).

Additionally, it is important to consider what relevant metadata should be collected throughout the sampling program. This metadata is crucial to evaluate and interpret any eDNA results, as several environmental factors can impact the degradation rate and transport distance of eDNA (e.g., UV radiation, temperature, pH, salinity, discharge, depth).

Common sample metadata to collect includes:

- Sample equipment used (e.g., filter material and pore size), sample ID, geographic coordinates, date of collection, collector information, sample volume collected, and the volume filtered, description of site (site photos), and time of collection.

Environmental metadata may include:

- Water temperature, pH, turbidity, conductivity, total dissolved solids, depth, redox potential, salinity, current speeds, cloud cover or canopy coverage, and other relevant parameters to study questions.

5.5.1 eDNA Sampling Mechanics for Water

Best practices and sampling design protocols for aquatic eDNA are detailed in USFWS (2023). Additionally, in-depth meta-reviews for aquatic eDNA collection from freshwater and marine environments can be found in Tsuji et al. (2019), Shu et al. (2020), Wang et al. (2021), and Xing et al. (2022). Detailed collection protocols are available for several aquatic habitats:

- Headwater streams (Carim et al., 2016; Laramie et al., 2015),
- Ponds and stagnant water (Goldberg & Strickler 2017; Laramie et al., 2015), and
- Large rivers and lakes (Bergman et al., 2016; USFWS, 2023).

Successful detection of an eDNA target within an aquatic environment requires concentrating the DNA from a select volume of water. The two principal methods for concentrating eDNA from water are precipitation or filtration.

In precipitation, a small amount of sodium acetate and ethanol or isopropanol alcohol are added to the water sample to preserve the DNA, and samples are subsequently placed into a centrifuge at high speeds (5,000 - 7,500 x g) for 30 - 60 minutes. This centrifugation process results in the formation of a pellet consisting of organic material that was previously suspended within the water. Centrifugation and precipitation methods are less efficient than filtration because they are limited to small volumes of water (typically <15 - 30mL) (Wang et al., 2021; Xing et al., 2022). The lower volumes processed have been found to reduce detection rates compared to filtration (Muha et al., 2019), and filtration has even been found to outperform precipitation when equal volumes were used (Deiner et al., 2015; Spens et al., 2017). The majority of aquatic eDNA studies and practical guides recommend filtration as the optimal method (see references in Appendix B; Tsuji et al., 2019; Bruce et al., 2021).

Filtration methods involve passing water through a fine porous membrane, with cellular and subcellular material subsequently captured on the membrane. Filtration of water samples can be accomplished with a variety of filter pore-sizes and filter matrices, with majority of aquatic eDNA studies using a pore-size <1 □m (Tsuji et al., 2019; Wang et al., 2021). However, the sampled volume of water and the probability of species detection may increase with an increasing filter pore-size depending on the environment (Fediajevaite et al., 2021).

In addition to variation in pore size, water samples can be filtered using a wide variety of matrix membranes (Figure 5-7), however, glass fiber, cellulose nitrate, and polyethersulfone filters have chemical properties to help adsorb DNA and proteins to their surface, and thus they are the most commonly used (Tsuji et al., 2019; Shu et al., 2020; Wang et al., 2021). These different filter types often require different sets of equipment to complete the filtration process, which includes open filters, housed filters, and enclosed filters (Figure 5-7). In addition, filtration of water can be completed in the laboratory or in the field, however processing in the field will limit potential DNA degradation and reduce chances of sample-to-sample contamination risk. Open filter systems perform the filtration in an open system that is directly exposed to the air. This can be performed with a vacuum pump in the laboratory, or with a peristaltic pump in the field. These filter systems involve the highest risk of contamination, as the collected sample is exposed during the filtration process, the transfer to a preservation vial, and the DNA extraction step. Housed filter systems hold the filter membrane within a solid housing unit that reduces chance of sample filtration-related contamination. However, similar to open filter systems, the membrane still requires handling during preservation and extraction steps, which can introduce contamination. Enclosed filter systems completely enclose the filter membrane inside an outer housing unit that is never opened, and the membrane requires no direct handling. In these systems, preservation methods are added or incorporated directly into the housing unit. This greatly reduces risk of sample collection related contamination. Enclosed systems, such as Millipore Sterivex units and Smith-Root Self-preserving eDNA Filter Packs, typically provide the least effort filtration method for large sampling campaigns and reduced contamination potential, although they also tend to be more expensive than open or housed filters.

Ultimately, the ideal filter matrix, size, and equipment will be dependent on the sampling design chosen based on the project goals and sampled environment. However, the combination of

membrane matrix, filter pore size, and filtration equipment is all important for determining how much volume can be sampled.

Figure 5-7 Water eDNA filter types. 1: Open filters are exposed to the air during filtration either in the field or lab (a and b). 2: Housed filters are a membrane placed in a solid unit during filtration (c). Filters from Open and Housed units need to be removed from the filtration unit and stored in a petri dish or Eppendorf tube until extraction (c and d). 3: Enclosed filters are systems in which the membrane is enclosed within the outer housing (e and f). Extraction is carried out directly from the enclosed filtration unit. (Modified from Bruce et al., 2021).

The question of 'how much to collect?' (e.g., number of environmental replicates per site and volume per sample) often depends on the size of the water body and abundance of the target species. Due to the potential patchy distribution of eDNA within the water column, and the expected low eDNA concentrations for most rare taxa, collecting more volume across several environmental replicates can significantly increase the probability of successfully collecting target eDNA. This is because collecting more volume and more samples will reduce the amount of variation that naturally occurs within the environment. In many riverine studies collecting

three to four environmental replicates per site has been sufficient to exceed a 95% probability of target eDNA detection (Lugg et al., 2018; Strickland & Roberts, 2019; Tingley, 2021; Baudry et al., 2021, Westhoff et al., 2022), and a majority of fish targeted eDNA studies collect between three to six environmental replicates per site (Xing et al., 2022). However, there is no general rule for the required amount of volume or environmental replication within an eDNA survey. In general practice, larger bodies of water typically require more samples. Collection volume and environmental replication will be based on an approach to maximise detection probability while maintaining a reasonable collection efficiency based on project goals and budget. Implementing a pilot study can be helpful to address questions surrounding survey design by evaluating the sampling effort required to reach a desired level of confidence in the data.

Some eDNA protocols have suggested reducing costs by pooling (merging) water samples from multiple locations (Figure 5-1), however, pooling of water samples may reduce species detection and limit spatial resolution (Sato et al., 2017; Zhang et al., 2020). The decision to pool samples will depend on the project goals and budget. For example, a study asking, "Is species X present in pond Y?", might not require a spatial understanding of where species X is present, but rather just a "yes" or "no" if it is present in pond Y.

Similar to increasing the number of samples, increasing the volume sampled can improve detection rates for rare species (Sepulveda et al., 2019; Bessey et al., 2020), and increase biodiversity estimates (Bessey et al., 2020; Govindarajan et al., 2022). Across both freshwater and marine environments, high volumes of water exceeding 100 L have provided greater biodiversity estimates and improved target species detection (Govindarajan et al., 2022; Sepulveda et al., 2019; Schabacker et al., 2020). However, increasing sample volume also increases sampling effort (i.e., filtering 50 L can take ten times the effort compared to filtering 5 L), which may not be feasible for some projects. Several different methods and equipment are commonly used to collect water from the environment and to subsequently push water through a filter matrix (e.g., vampire sampler, peristaltic pump, or vacuum pump). In addition, many freshwater environments have high turbidity and concentrated suspended solids, which greatly restrict the volume that can be filtered. In such cases, a pre-filtration step (e.g., first filter the water through a 50 µm pore size) can be useful to prevent filter clogging and reduce the presence of inhibitors (Takasaki et al., 2021).

'Where to collect' within the environment will depend on the size and movement of water, as well as the habitat of the target organism(s). For example, sampling depth is a crucial consideration within large lakes where thermal stratification can influence where in the water column species occupy, and thus where their eDNA accumulates (Littlefair et al., 2020) (see stratification examples in Figure 5-7). Similarly, depth stratification is likely to occur in large rivers, and therefore sampling programs targeting benthic organisms should consider sampling near river or lake bottom. Sampling depth is likely less critical in streams that display well-mixed water from turbulent flow (Strickland & Roberts, 2019), and thus in lotic systems it is recommended to collect samples based on known habitat of target species and perpendicular to river flow. Within ponds and small lakes sampling from the shoreline can provide high eDNA detection rates, however, eDNA is typically more spatially dispersed within medium and large lakes (Zhang et al., 2020) (see pond and lakes diagram in Figure 5-7). These results suggest

sampling from the shoreline is adequate, however, a greater spatial sampling design is required in larger lakes to fully assess biological communities. Similarly, for marine habitats it is suggested to perform spatial sampling across transect grids, which also includes a depth gradient (Stauffer et al., 2021). This grid sampling design should extend multiple sampling locations along transects that cover the present habitat heterogeneity (see marine diagram in Figure 5-7).

Considerations & Key Points

Important considerations for filter type, pore size, and equipment:

- The ability to filter large volumes of water can be greatly restricted for small pore-size filters, which can quickly clog and limit the amount of eDNA collected.
- Filter clogging is more common in freshwater than marine environments, due to increased turbidity. Increasing sample volume by using a filter pore-size >1 □m is appropriate in many freshwater systems based on turbidity and water chemistry characteristics. A pre-filtration method with a larger pore size can also be advantageous to increase volumes.
- Studies evaluating the size fraction of eDNA have suggested majority of the eDNA material is within the 1-10 □m size (Barnes et al., 2021; Zhao et al., 2021; Cooper et al., 2022). Thus, a filter pore-size >1 □m can increase volumes while still collecting majority of the eDNA present.
- Enclosed filters provide the greatest protection against sample-to-sample contamination but come at a higher cost per sample price.

Important considerations for sample effort – number of sampling sites and environmental replicates:

- Collecting more environmental replicates has a greater influence on the probability of detection than running more qPCR replicates (Lugg et al., 2018; Strickland & Roberts, 2019; Tingley et al., 2021; Baudry et al., 2021), and thus budgetary funds and effort should be focused on collecting more samples where possible.
- The number of sampling sites and replication should reflect the size of the waterbody and the spatial extent the project objectives cover.

Important considerations for sample effort – volume filtered:

- Sample volume should be given similar consideration as to number of samples. Typically, collecting more volume increases the probability of target eDNA detection.
- Sample volumes typically range from 500 mL to 5 L depending on the environment being sampled.
- Sample volumes filtered in marine eDNA studies tend to be higher compared with freshwater studies, as the higher water clarity typically found in marine habitats allows for better filtration rates.

- In some instances, large volumes can lead to the accumulation of PCR inhibitors within the sample, ultimately decreasing the probability of eDNA detection by impeding the downstream analysis (Baudry et al., 2021; Dubreuil et al., 2022).

When considering a sampling design within a lotic system, it is important to consider potential impacts from transport of eDNA from the hydrological dynamics. The potential transport distance of eDNA is strongly associated with river discharge (Wilcox et al., 2016; Van Driessche et al., 2023), with estimated transport distances sometimes exceeding 3 km from the source (Deiner et al., 2014; Wood et al., 2021; Shea et al., 2022; Van Driessche et al., 2023). Therefore, establishing a network of sampling sites along a linear path of a river (see river diagram in Figure 5-7). can provide informative results for interpreting spatial distribution of target organism.

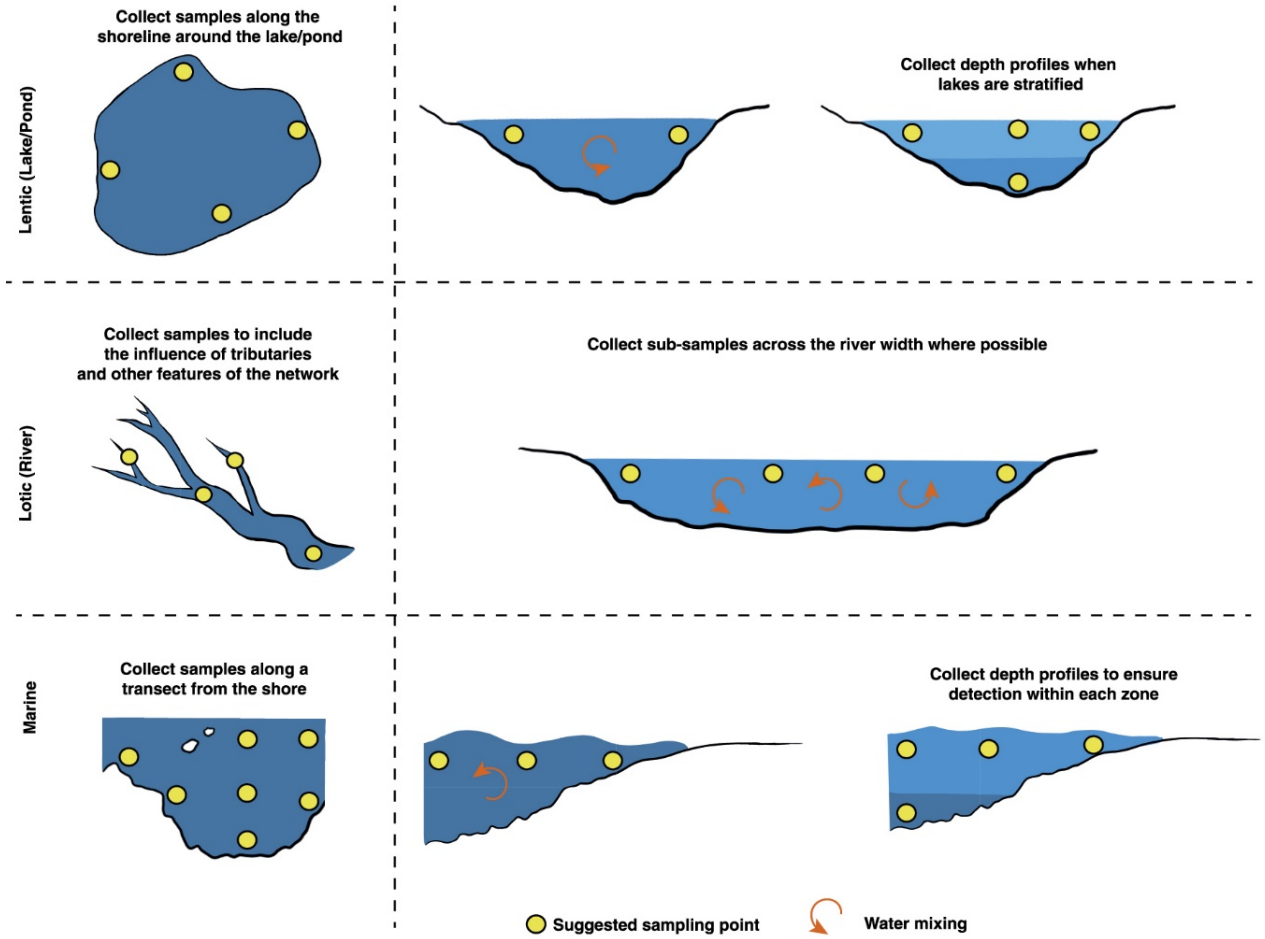

Figure 5-7 Schematic of aquatic eDNA sampling design based on the environment being sampled (From Bruce et al., 2021)

Additionally, a series of spatial sampling points can be established up- and downstream of the site of interest (Figure 5-8a). In this example, the eDNA concentration is evaluated across a linear spatial scale to estimate when detections may be related from transport rather than a nearby organism. In Figure 5-8b, River Transects 4 and 6 may be interpreted as eDNA transport from River Transects 3 and 5, respectively. There are still many unknowns associated with the factors that impact eDNA transport distances, and thus simplified models are not available for widespread implementation across all hydrological regimes. Therefore, an experimental pilot study whereby caged target taxa are placed within the aquatic system of interest (see Van Driessche et al., 2023) may be a useful step for projects concerned with potential downstream transport distances. If the target species is expected to be rare and potentially move throughout the environment, survey programs with multi-seasonal sampling can increase confidence in results.

The most important consideration for determining 'when to sample' is related to the behaviour and ecology of the target organism(s) (discussed in more detail in Section 4). The peak eDNA concentrations are likely to occur when the target species is most active. However, some environmental factors need to be considered for designing when to sample.

- When possible, avoid sampling during the wet season or directly following rain events, to reduce sampling during high discharge. This will reduce both the potential eDNA transport distances and lower detection ability associated with eDNA dilution during high flow (Akre et al., 2019; Curtis et al., 2021).
- Low water temperatures can decrease detection when species are less active, while extreme warm temperatures can decrease detection by increasing microbial and enzyme activity that degrades eDNA.
- Other factors that increase microbial activity or enzymes, such acidic environments or high nutrient inputs may increase eDNA degradation.

Considerations to Reduce Contamination Risk

Several steps within the sampling design can be implemented to reduce or eliminate contamination risks.

- Within lotic systems, surveyors should work in the upstream direction to reduce risk of contamination from downstream transport.
- Any equipment used in sample collection should be downstream of collection points to avoid contamination from gear and equipment.
- Decontaminate any reusable equipment (e.g., bottles, filter holders, pump tubing) and field gear (e.g., boats, waders, sampling poles) before use, and at every sampling site to avoid (cross-) contamination.

Decontamination protocols typically consist of washing equipment in a 10-50% bleach solution. Prior to a bleach wash, equipment must first be thoroughly washed with water to remove any sediment or biofilm material that might trap and hold DNA particles. Following a bleach wash, a

thorough rinse with ultrapure water or water from the sampling site is required to remove residual bleach that can degrade the next set of collected samples.

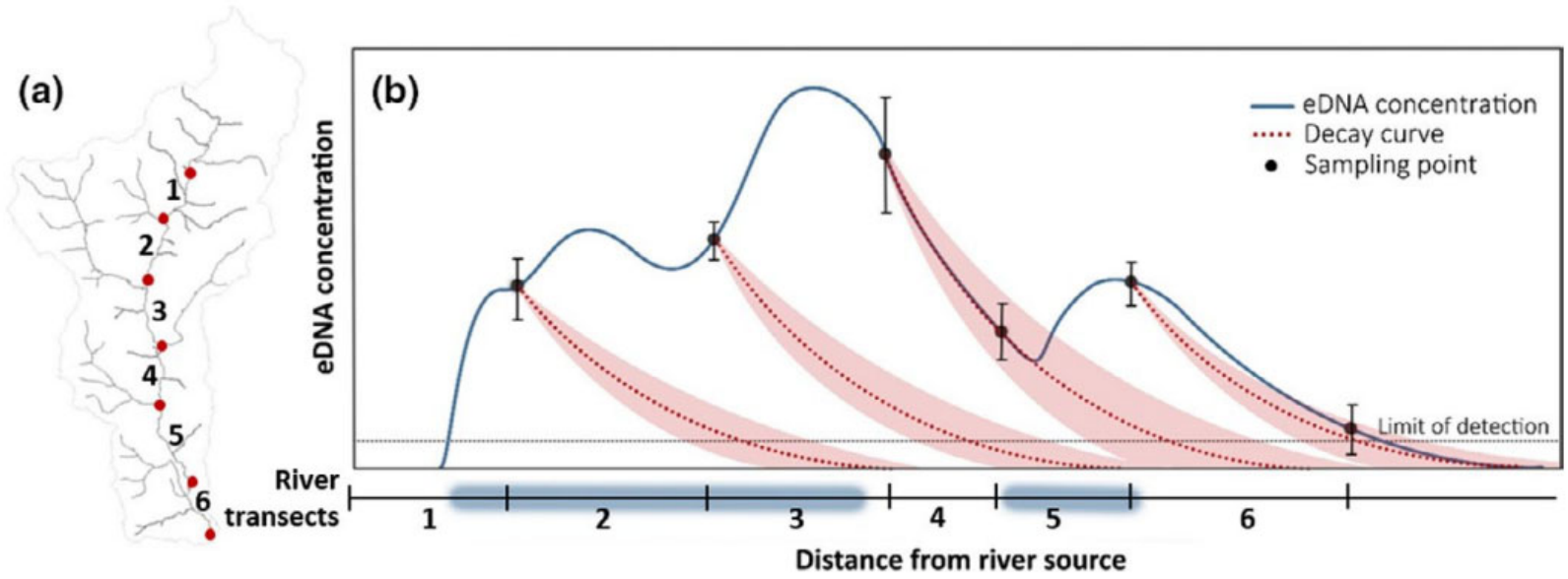

Figure 5-8 Schematic (a) of a lotic sampling plan with (b) associated influence of hydrological flow on potential eDNA transport from upstream population (From Burian et al., 2021)

5.5.2 eDNA Sampling Mechanics for Aquatic Sediment and Terrestrial Soil

Aquatic sediment and terrestrial soil samples typically contain a high diversity of living organisms (organismal DNA) as well as DNA from larger organisms that has been shed into the sediment (eDNA) and DNA from dead or dormant organisms. Soil and sediment metabarcoding that targets metazoa will tend to be dominated by organismal DNA, which is present in much higher concentrations than environmental DNA. In aquatic systems, surface sediments also contain DNA from pelagic organisms or their cells that have settled from the water column. This makes soil and sediment samples a rich source of data across the entire spectrum of biodiversity (Bruce et al., 2021; Weigand & Macher, 2018).

For eDNA sampling, clean tools are essential at every step in the sampling process to prevent DNA contamination between samples. Sampling methods for benthic taxa make use of well-established equipment and techniques to obtain sediment samples from the seabed (Lanzén et al. 2021). There are a range of different options available for sub-sampling and sample preservation, depending on project goals and logistical constraints. General considerations for the implementation of an eDNA survey based on sediment and terrestrial eDNA include the nature of the sediment and type of sampling device, the sampling depth and volume, and the number of samples. The decay rate of sedimentary eDNA is typically lower than that of aqueous eDNA (Turner et al., 2015; Sakata et al., 2020). While the breakdown of eDNA in sediment (and soils) depends on a multitude of factors such as pH, moisture, temperature, sediment or soil texture, and depth, it has been shown that sediments (and other particulate matter) reduce biologically and chemically driven DNA decay by adsorbing both DNases and DNA molecules (Pietramellara et al., 2009; Shogren et al., 2017; Corinaldesi et al., 2011). This means that the detectable period of sedimentary eDNA is typically longer compared to aqueous eDNA (Turner et al., 2015). Under the right conditions DNA captured in soil or sediment can still be detected tens to hundreds of years later, which is why soil samples are not typically used for the purpose of pathogen monitoring, or to determine the presence or absence of a specific species, but rather for the evaluation of community shifts (Nielsen et al., 2007; Foucher et al., 2020). Although it is known that DNA molecules remain for a long time in low-oxygen environments, such as deeper sediments, there is less information on the decay rate of sedimentary eDNA on the surface, such as that from fish, before deposition in the anoxic deep layer. However, the decay rate of sedimentary eDNA has shown to be low even for surface sediment (detected up to 537 days), and the sedimentary eDNA concentration has been shown to be higher than that of aqueous eDNA for the same sample weight (Sakata et al., 2020).

5.5.2.1 Soil and Sediment Collection

Soil and sediment datasets are dominated by organismal DNA and will predominantly comprise meiofauna (small benthic invertebrates typically between 0.45µm and 1mm) rather than macrofauna. This is advantageous from the point of view of maximising the statistical power to show community change in response to impact or land use change, since meiofauna are often more diverse and more abundant than macrofauna, but it makes it difficult to directly compare

metabarcoding results with those obtained from conventional surveys of benthic macrofauna or ground insects.

The overall sampling design (e.g., spatial scale, number of samples, volume per sample, and sediment/soil layer targeted) will be decided according to the portion of biodiversity that is targeted and the spatial scale at which it operates. For microorganisms such as bacteria and single-celled eukaryotes, it is common to collect only very small-volume samples, which can be as small as 0.25g and usually not more than 1g (Pawlowski et al., 2022). These small-volume samples can be easily and cost-effectively preserved and eliminate the need to perform subsampling steps within the laboratory, as most soil DNA extraction kits are designed for low-volume samples. If working with an external laboratory service provider, it is critical to establish their sample volume and any requirements for preservation prior to sampling.

If targeting the meiofauna or macrofauna instead, larger volumes (> 10 g) are recommended to achieve a representative sample (Dopheide et al., 2019; Brandt et al., 2020), and these are somewhat more challenging and expensive to handle. The maximum volume of sediment that can be extracted using commercial kits is 10 g for the Qiagen PowerMax Soil kit. Since DNA does not mix well in sediments and soils, it is usually necessary to collect subsamples from across the area that the sample aims to represent. Porter et al. (2019) found that either collecting more biological replicates (e.g., collecting more samples per plot) or increasing the number of subsamples within a plot (e.g., pooling more subsamples into a single sample) greatly increased richness estimates for soil invertebrates. Subsamples can either be treated separately to maximise statistical power or merged and re-sampled to give a single smaller-volume sample that is representative of a wider area of sediment. The latter approach is more cost effective. Very large volumes of sediment have been used in some studies (1 L or more) to target extracellular or extra-organismal DNA, which is present in very low concentrations in both terrestrial soils and marine sediments (e.g., Taberlet et al., 2012b; Guardiola et al., 2015; Leempoel et al., 2020).

Terrestrial soil samples are typically collected directly from the ground using a shovel or corer (e.g., Andersen et al., 2012; Leempoel et al., 2020; Li et al., 2023). There is no clear consensus as to the ideal vertical depth of the sample, and this may vary between target groups and ecosystems. In terrestrial soils, the vertical distribution of biodiversity is often complex, partly because of the structure provided by plant roots. Therefore, it is more common to sample at greater depths (e.g., Arribas et al., 2016; Treonis et al., 2018) compared to aquatic sediment samples. More research is needed to determine the best way to standardise a sampling strategy for terrestrial soils that balances ease of collection with the structural complexities of this environment. However, the highest metazoan invertebrate richness from eDNA soil samples has been associated with the bryophyte and organic layers, compared to lower levels of diversity within mineral layers (Porter et al., 2019). Similarly, Mundra et al. (2021) found the diversity of Arthropoda was highest within the organic layer, compared to the deeper mineral layers. These results suggest the organic layer is the best location for sampling of soil invertebrate eDNA. Additionally, this implies that a sampling protocol should evaluate soil horizon and layer depth, to confirm that the same soil layers are sampled between sites. However, the exact depth and

layers to be used for a specific project will depend on the desired taxa and environment being sampled, and in some cases the mineral layer may detect key species of interest.

5.5.2.2 Marine Sediment Collection

The choice of sampling device being used to collect sediment has implications for the latter stages of eDNA handling, processing, and results interpretation. For finer sediments, Van Veen or Day grabs are commonly used to collect samples for metabarcoding studies, although box corers may have more success in maintaining the sediment profile. For coarse sediments, a Shipek grab maintains the sediment profile while a Hamon grab does not. Other grab samplers such as Ekman grab for when the bottom consists of soft mud substrate and Ponar grabs for hard clay-like bottoms can also be used. Alternative sampling methods may be suited to different environments, such as multicorers or remotely operated vehicles (ROVs) for the collection of deep-sea samples. For harder substrates, scraping the surface in quadrats is recommended for stations within SCUBA diving range, although a recently developed suction-based method may provide an alternative sampling method, particularly for deeper water (Keeley et al., 2021). For freshwater and nearshore marine sampling, sediments are often collected with a sediment grab (e.g., Van Veen grab) from a small boat (Chariton et al., 2015; Stoeck et al., 2018) or a spade in intertidal locations (Aylagas et al., 2018). Sediment sample collection of eDNA in offshore ocean studies will typically use a corer or a large grab (Kimes et al., 2013; Laroche et al., 2016; Yeung et al., 2011). Once back onboard the vessel, the top (1 - 2 cm) section of cores/grabs are subsampled for eDNA, although subsampling depth has varied depending on the target organism (Wort et al., 2022). Due to the importance of grain size, it should be reported and considered as a potential explanatory variable (Cronin-O'Reilly et al., 2018; Pawlowski et al., 2022).

Responses of microbial communities to aquatic pollution are likely to be concentrated in the surface layer of the sediment (where sediment samples are also collected for physicochemical analysis), so this layer is commonly targeted for microbial assessments in aquatic sediments (Cordier et al., 2019; Fais et al., 2020). Larger organisms (meiofauna and macrofauna) are more mobile and operate at larger spatial scales in both the horizontal and vertical planes (Chen et al., 2017). Therefore, it is common (though by no means universal) to sample to depths of around 5 cm for marine benthic meiofauna (see Wort et al., 2022 for a review). In aquatic sediments, there is an argument for discarding the very surface layer which may contain DNA of more transient species or those that have settled from the water column and are not truly representative of the benthic community. There is also an argument for avoiding sampling from the anoxic sediment layer that sits beneath the oxygenated layer at the surface, since this presents a very different community (composition) (Spedicato et al., 2020; Laroche et al., 2020). The anoxic layer can be recognised by its darker coloration and foul smell and can feasibly be discarded from a transparent corer where the sediment profile has been well retained.

Sediment subsamples are most commonly collected using either:

- A spoon/spatula used to scrape the surface sediment up to 2 cm depth (e.g., Cordier et al., 2019; Stoeck et al., 2018; Cordier et al., 2020).

- A small coring device such as can be fashioned from a syringe (e.g., Laroche et al., 2020; Guardiola et al., 2016; Klunder et al., 2020; He et al., 2021).

Syringe corers are ideal for targeting a deeper sediment profile, and the plunger on the syringe can be used to create suction enabling cores of loose or wet sediment to be collected. When sampling for bacterial and meiofauna, collecting four mini-cores from each grab is recommended to subsample both the surface and sub-surface taxa. This is preferable to the alternative of surface scrapes which would only capture surface taxa, or scoops which are more difficult to regulate sampling depth. However, in some cases the sediment may be too coarse or compacted to easily use a syringe, in which case a scoop is the only viable option, From the homogenised composite subsamples, a smaller subsample is then used for laboratory analyses. If using a Hamon grab, it is recommended that the full sample is thoroughly mixed prior to subsampling.

When sampling for macrofauna, it is recommended that the whole grab is sieved on board the vessel using a 0.5- or 1-mm mesh sieve as appropriate to provide compatibility with morphological macrofauna data (after any subsampling for bacteria and/or meiofauna), or that a minimum of 1,000 cm^3 of unsieved material be collected as a subsample. This approach is broadly compatible with existing morpho taxonomy sampling approaches. It may be possible to use the sediment subsamples directly for DNA-based macrofaunal analysis, but the considerably lower sample volume makes this a poor substitute. It is advisable to take separate macrofauna samples that use a DNA compatible preservation buffer (Wort et al. 2022), rather than the formaldehyde-based preservation buffer used for morphological samples. Freezing is generally accepted to be an effective way of preserving samples; however, various liquid preservative alternatives are available and should be explored based on project goals (Appendix B; Bruce et al., 2021). Tools used for sub-sampling are either pre-sterilized or soaked in 70% ethanol for at least 5 min, then rinsed in sterile, distilled water between coring events (Mason et al., 2014). Subsamples should be taken away from the edges of the corer, targeting the minimally disturbed parts of the sediment that have not come into direct contact with the equipment.

For sediment sampling for general biomonitoring in a coastal environment, sampling stations and sites are commonly arranged along depth and salinity gradients (Lallias et al., 2015; Chariton et al., 2015; Fais et al., 2020; DiBattista et al., 2019). The sampling strategy will be dependent on the availability and resolution of the mapped target habitat distribution.

5.5.3 DNA Sampling Mechanics for Bulk Organisms

Conventional invertebrate monitoring is based on morpho-taxonomy, under a workflow of collection, sorting, manual species identification and analysis of the results (Oliver & Beattie, 1996). Much of the process is time-consuming, with species identification requiring significant expertise - often across multiple taxonomic groups. These same samples can also be analysed using DNA-based methods and are broadly described as bulk samples. The methods to collect invertebrate samples for DNA require the same initial investment as conventional methods, however, morphology-based examination by technical experts is circumvented (Yu et al., 2012; Morinière et al., 2016) and DNA-based methods offer the ability to identify taxa that may be

cryptic or otherwise unidentifiable due to life stage, partial specimens, small size, or morphological similarity with closely related species. Additionally, broader diversity is usually reflected in eDNA analyses with faster turn-around time on results compared to conventional approaches, The results are frequently more in line with conventional methods than water eDNA or soil and sediment sampling, as they undergo the same selective processes (e.g. Djurhuus et al., 2018). For a more in-depth discussion comparing bulk samples analysed using DNA based methods and conventional methods across different environments, please see IOGP JIP34 Chapter 1.

Owing to the great diversity in taxa, there are numerous methods used for sampling terrestrial arthropods. The methodological choices made are often dependent on the taxa or functional group of interest (Lang, 2000; Portman et al., 2020), each varying in effectiveness and efficiency, with no single comprehensive method. In addition to being restricted to sampling during active seasons, factors such as seasonal habitat preferences, life strategies and dispersal of invertebrates also need to be considered in methodological choices (Danks, 2007). Intra-annual replication in sampling is therefore an important factor in monitoring (Didham et al., 2020). The amount of spatial, inter-annual, and within-sample replication are also factors in any monitoring methodology (Montgomery et al., 2021). However, invertebrate capture techniques, are invasive and result in the killing of communities, so the pervasiveness of monitoring should also be considered.

Across all invertebrate monitoring methods, there is also a general lack of standardisation. For example, the time which samples are left between collection points is highly variable, ranging from one day (Thormann et al., 2016) to up to 90 days (Rubink et al., 2003). The most common trapping periods are one or two weeks (Rees, 2022), however, even within studies, timings are inconsistent (Uhler et al., 2021). Though many of the conventional collection approaches for morphological analyses can be employed for the purposes of DNA sampling, they have not been systematically evaluated within a molecular context. A standardisation of methods for use with DNA techniques is essential for long term invertebrate monitoring.

In terrestrial environments, most collection methods are passive, using a variety of techniques (e.g., Malaise traps, pitfall traps, pan traps) though active collection by methods such as light trapping also occurs. The sampling methods used are dependent on the target taxa for collection. For example, Malaise traps are appropriate for airborne invertebrates, whereas pitfall traps are most efficient in targeting ground dwelling taxa. There are also technical considerations to be made in terms of site characteristics and study objectives. For a small study area, it may be less appropriate to survey for those invertebrates which are most mobile/aerially active using Malaise traps since they show decreased local site fidelity in comparison to more sedentary organisms. Shorter ranging taxa also show stronger recovery trajectories and therefore may be most appropriate to target where projects dictate shorter monitoring periods (van der Heyde et al., 2022). To increase efficiency and taxonomic breadth collected, a combination of multiple methods may be required, depending on study objectives (Hausmann et al., 2020).

In addition to bulk invertebrate samples, DNA analysis can be useful for the description of both zooplankton (Djurhuus et al., 2018) and ichthyoplankton samples (Carvalho, 2022). These complex samples are collected with small-mesh plankton nets, and can contain numerous cryptic taxa, with individuals typically becoming damaged during collection which results in difficult or impossible morphological identifications. No matter the sampling methodology or taxonomic group, once the bulk sample is collected and returned to the laboratory, the entirety of the sample can be homogenised to obtain a representative DNA mixture from all the present organisms.

Bulk invertebrate tissue can also be used to indirectly detect terrestrial vertebrates that were not sampled. As an emerging technique, the association with vertebrate blood, carrion, or faeces by hematophagous, sarcophagous, and coprophagous invertebrates is used to directly sample species DNA, termed invertebrate-derived DNA (Calvignac-Spencer et al., 2013; Drinkwater et al., 2021; Gogarten et al., 2020; Massey et al., 2022). The preservative in which invertebrates are stored following collection has recently been used as the material itself from which to extract DNA (Nielsen et al., 2019). Although some results have been promising, biases occur towards softer, less sclerotized taxa (Martins et al., 2021; Zizka et al., 2019). Further work on both above is required to determine its effectiveness and efficiencies in terrestrial monitoring strategies. Sampling bulk invertebrates is still an invasive approach, requiring high resource usage (Kirse et al., 2021).

5.6 DP5 - Sample Processing - Preservation & Extraction

There are many different preservation methods available, and each provides advantages or limitations for the sampled media. It is best practice to discuss with the laboratory which preservative is optimal for their downstream sample processing.

5.6.1 Aquatic Filter Samples

5.6.1.1 In-field Processing

Although it is preferable to filter water samples at the collection site, if this is not practicable, it is recommended to store sampled water under cool (e.g., 4°C) and dark conditions and to perform filtration as soon as possible (no more than 24 hours, but less time is best).

After filtration, the sample can continue through to DNA extraction, or, in most field work scenarios, preserved prior to processing. To limit DNA degradation prior to extraction and analysis in the laboratory, eDNA on filters is preserved for transportation and storage by either freezing, drying, or adding liquid preservative to the filter. It is ideal for the laboratory to process samples for DNA extraction as soon as possible. Long delays can result in eDNA degradation, even when stored in -80°C.

Storing filters at -80°C is effective at halting eDNA degradation but requires immediate access to cooling equipment and the ability to keep the samples cooled or frozen during transportation to the laboratory. Freezing may have a positive effect on eDNA recovery compared with samples that are extracted immediately after filtering, possibly due to a lysis effect resulting from cell

bursting (Mauvisseau et al., 2021), but multiple freeze-thaw cycles should be avoided. This is most applicable to samples that have been filtered in the laboratory.

Drying the filter membrane requires either silica gel, a desiccator, or paper that absorbs water. This is a less commonly used preservation approach but is attractive in its ease and simplicity as it allows storage at room temperature for several weeks or even months (Bakker et al., 2017). It is challenging to completely dry the membrane within an enclosed, disc-shaped filter capsule, so alternative approaches are advisable if working with this type of filter. Some currently available enclosed filter units provide self-preservation methods to reduce potential contamination (e.g., Smith-Root's Self-preserving eDNA Filter Packs).

Preservation solutions can be broadly assigned to two different categories: pure preservatives such as ethanol and RNAlaterTM, or lysis agents, including Longmire's buffer (Longmire et al., 1997, Wegleitner et al., 2015) and Sarkosyl buffer (Civade et al., 2016), which release DNA into solution while also preventing degradation. Note that the use of ethanol as a preservative requires non-denatured ethanol, as the denatured form can often contain chemicals that inhibit the downstream laboratory processing steps. It is also important to consider whether the microbial diversity in the sample will be analysed. If microbes are targeted then it will be important to arrest microbial growth at the time of preservation, and not all preservative solutions will achieve this. An advantage of using preservative solutions is that a positive control of DNA can be incorporated into the solution and used to check DNA preservation and extraction (see Section 5.4.1). It is also critical to know whether the buffer used is lysing the cells or organelle membranes, since this will affect how to approach the early stages of the DNA extraction process in the laboratory. Ethanol can be an inexpensive option for preservation, but it is often difficult to ship and travel with due to its classification as a hazardous material. RNAlater™ can be easier to obtain and take into the field for a sampling program.

5.6.1.2 In-Laboratory Processing

In the laboratory, pure preservatives such ethanol and RNAlater™ can be discarded, leaving the material containing DNA on the filter membrane ready for subsequent lysis (Spens et al., 2017). With ethanol, complete evaporation is essential to prevent inhibition of later stages in the extraction. To maximise DNA recovery from samples contained in ethanol it is possible to carry out a precipitation step on the ethanol in addition to extraction from the filter membrane, although this will increase costs. By contrast, preservation in lysis buffers (e.g., Longmire's or Sarkosyl buffer) cause much of the DNA to be in solution by the time extraction begins in the lab. In this case, discarding the storage solution will result in catastrophic loss of DNA. Instead, the filter should be incubated in the storage solution, which is then used as lysate for the next stages of the DNA extraction. Note that some DNA extraction kits require particular salt concentrations in the lysis buffer, so it is important to check compatibility between the buffer solution and kit chosen.

In the laboratory, eDNA extraction protocols from filters can be based on several different commercial DNA extraction kits (e.g., Qiagen DNeasy PowerWater or PowerSoil kits, Macherey Nagel NucleoMag Water kits), custom column-based methods (Sellers et al., 2018), or liquid

phase methods (Renshaw et al., 2015; Deiner et al., 2018). For routine monitoring applications in regulatory or industry contexts, the use of reagents from commercial DNA extraction kits is generally advised since they are expected to be standardised and certified as DNA-free. Protocols such as liquid phase extractions with phenol-chloroform-isoamyl are effective and produce greater DNA yields (Deiner et al., 2015), but they carry significant health and safety concerns that most commercial laboratories will seek to avoid. Initial steps in extracting DNA from filters must be optimised according to the type of filter and preservation strategy used and the biological targets for analysis. First, the target group(s) must be considered in the selection of a lysis method. Chemical lysis is sufficient for extraction of animal DNA, but mechanical lysis is required for disrupting cell walls of some unicellular groups such as diatoms. Since mechanical lysis cannot easily be applied to enclosed filters, open or housed filters are recommended if you plan to target such groups in your samples, and a DNA extraction that includes bead beating (a type of mechanical lysis using beads to break down cell walls) or equivalent is required. Second, the preservative solution and filter type will influence the lysis procedure.

An additional consideration is that not all extraction methods will release DNA bound to particles such as clay. If it is suspected that much of the DNA in a sample is particle bound (e.g., in highly turbid waters), then discuss extraction options with the laboratory. There are kits optimized for soil extraction (e.g., Qiagen PowerSoil) and lysis buffers containing trisodium phosphate that are used to release adsorbed DNA (Ogram et al., 1987; Sellers et al., 2018). If not considered, then adsorbed DNA will not be extracted, and a false negative may be the result.

Another consideration is removal of inhibitors that may interfere with eDNA detection. For example, organic compounds co-extracted from the sample along with the DNA may inhibit downstream PCR reactions. This particularly affects samples from turbid waters and small water bodies containing lots of rotting leaves, which introduce tannins and other dissolved organic compounds to the water. Other sources of organic material that may cause inhibition include faeces from livestock (e.g., cattle; Wilson, 1997; Rapp, 2010). Extraction kits designed for use on water, soil or faecal samples often incorporate inhibitor removal as part of the standard process. Others - including those designed for use of pure biological samples of tissue or blood - do not, and a separate step may be required to remove inhibitors post-ex- traction using kits such as the Zymo OneStep PCR inhibitor removal kit or Qiagen PowerClean kit. Inhibition can also sometimes be overcome by dilution of the DNA extract. This reduces the concentration of inhibitors but also reduces the concentration of target DNA, which can affect detection probability. A common approach is to run a dilution series to determine the minimum level of dilution that can counteract the inhibition. Note that the use of clean-up kits also results in some DNA loss, so these measures should only be applied to eDNA samples that are affected by inhibition. Extraction efficiency and the presence of inhibitors can be assessed by including internal positive control (IPC) DNA in the lysis buffer and checking via qPCR that it is recovered in the expected quantity after DNA extraction.

5.6.1.3 Robotic extractions

Several commercial and academic laboratories implement the use of robotic workstations for the extraction and purification of nucleic acids for an automated hands-free sample processing (Loeffler et al., 2022). This process eliminates potential human error that can occur during this sample processing, including error such as mis-labeling sample tubes, misused or forgotten reagents, or cross-contamination through lack of sterile methodology. Robot automation is commonly used within molecular analysis for criminal forensics and medical diagnosis, and the same equipment and methodology can apply to the processing of eDNA samples.

5.6.2 Soil and Sediment Samples

5.6.2.1 Separation of Organisms from Soils and Sediments

One way to process large volume soil or sediment samples is to separate the organisms (macrofauna and/or meiofauna) from the soil or sediment. The need to perform this separation will be dependent upon the research goals. Sieving the material to have a representative sample of only the large organisms will eliminate non-target amplification of microeukaryotes during the metabarcoding stage. If sieving was not performed, metazoan organisms may be missed during sequencing due to the overabundance of microeukaryotic DNA in the total DNA pool. Projects seeking to compare sediment eDNA to conventional macroinvertebrate community indices may benefit from the isolation of macrofauna.

The separation of organisms is usually achieved through a series of flotation, decanting and sieving steps. Soil faunae are also typically separated from soil using Tullgren funnels (arthropods) or Baermann funnels (nematodes) (Creer et al., 2010; Fonseca et al., 2011; Creer et al., 2016; Haenel et al., 2017). The organisms can then be processed as bulk samples, as described above. This is a labour-intensive process with initial steps typically carried out in the field, which potentially limits the number of samples that can be collected. Extracting DNA directly from the soil or sediment itself is preferable in many ways since it makes the process more readily standardised and scalable and requires less handling of the sample - reducing the risk of contamination.

If a sorting approach is taken, it is important to consider and mitigate the risk of cross-contamination from the equipment used to clean and sort the sample. The method used for separating the organisms from the sediment significantly affects community composition of the sample (Haenel et al., 2017), and should therefore be maintained throughout the monitoring program, particularly if results are being compared to morphological data.

5.6.2.2 Preservation and Extraction of Soils, Sediments and Snow

Samples must be preserved for transportation to the laboratory and storage prior to DNA extraction. Rapid and effective preservation of soil/sediment samples is particularly important if either eDNA, RNA, or microorganisms are targeted. Common preservation strategies include freezing at -80°C, and the use of preservative solutions such as ethanol or Qiagen's LifeGuard Soil Preservation Solution (Figure 5-9).

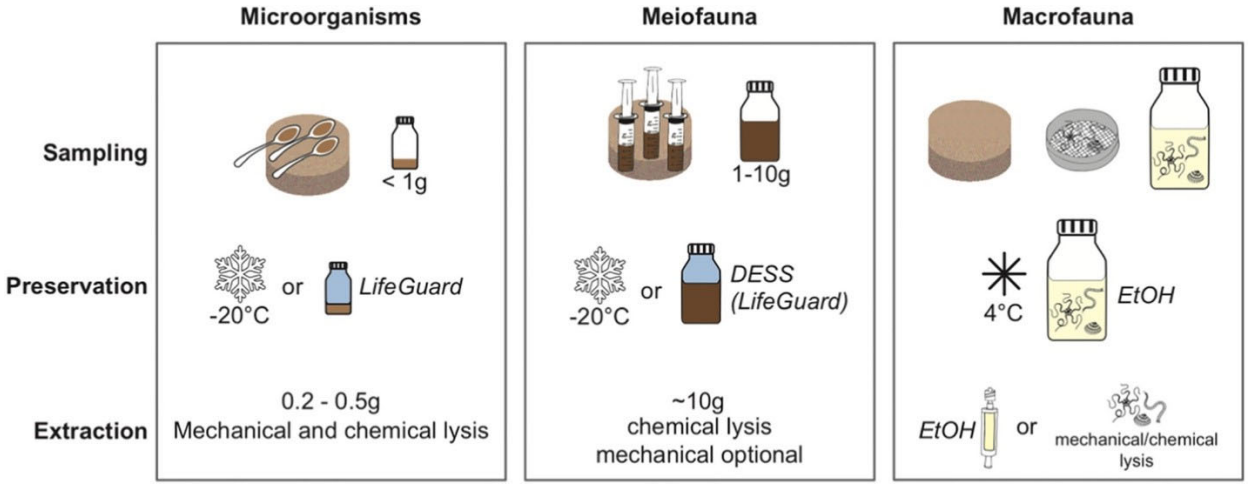

Figure 5-9 Recommended methods for sampling, preservation and extraction of sediment DNA depending on the target group of organisms (from Pawlowski et al., 2022)

Cold storage is recommended for sample preservation targeting bacteria and meiofauna. If samples are to be delivered to the laboratory within two days of sampling, they can be stored in a fridge (4°C). If storage time will be longer, samples should be transferred to a frost-free freezer as soon as possible after sampling. To maintain their integrity, samples must be either frozen at -20 °C (e.g., Laroche et al., 2016; Turner et al., 2015) or -80 °C (e.g., Kimes et al., 2013), stored in 95% ethanol, or exposed to a desiccant until analysis (Lear et al. 2018). Terrestrial soil samples can be stored temporarily at 4°C, if needed, then frozen for long term storage at -20°C or -40°C (e.g., Andersen et al., 2012; Leempoel et al., 2020), or samples can be sieved and stored at -80°C (e.g., Lloyd-Jones & Hunter, 2001). When collecting eDNA samples from snow, samples may also be kept frozen in the outside temperatures until sampling is complete and then transferred to the lab, thawed, and analysed (Franklin et al., 2019).

If samples are frozen, they must remain frozen (ideally a non-frost-free freezer) until arrival at the laboratory as freeze/thaw cycles will affect the results. For transportation to the laboratory, samples should be packed in a cool box with ice packs leaving minimal headspace. Freezing may be an impractical choice for large-scale monitoring programs in which a range of different parties will be collecting samples, and not all may have ready access to freezers.

Non-denatured ethanol is a good preservative, but for the effective preservation of DNA from sediment samples (which have a high-water content) it should ideally be changed once, targeting a final concentration of greater than 80% ethanol. It is important to note that ethanol presents logistical and safety challenges when applied to routine or industrial use and interferes with the chemistry of the DNA extraction process if not completely removed from the sample. Salt-based lysis solutions such as DESS (Yoder et al., 2006; Tatangelo et al., 2014) are effective at preserving metazoan DNA but require the addition of sodium azide or similar chemicals to arrest growth of some microbial groups, which brings health and safety concerns in the field

and in the laboratory. Further systematic testing of the effectiveness of different solutions for preserving communities of various groups of organisms in sediments is required.

RNAlater™ has been advocated against as a preservative for soil and sediment samples because storage in this medium has been shown to lead to changes in composition of microbial communities and loss of diversity (Nilsson et al., 2019; Delavaux et al., 2020). However, other sources show that, depending on the target taxa, RNAlater™ is appropriate to use for soil and sediment sample preservation (Brauer et al., 2022; Premke et al., 2022).

The initial steps in DNA extraction will depend on the volume of soil or sediment collected, the target group for analysis, and the preservation method used. If a preservation liquid such as ethanol or LifeGuard™ has been used for sample preservation then this must first be removed from the sample, usually by centrifugation and discarding the supernatant. Subsequent wash steps may be needed to help remove all traces of preservative as they may interfere with the chemistry of the extraction kit. This is especially important with sample preserved in ethanol. If a salt-based buffer such as DESS has been used to preserve DNA then the sample can sometimes be introduced more directly into an extraction process (Fonseca et al., 2011; van der Loos & Nijland, 2021).

The maximum volume of soil or sediment that can be directly extracted in commercial kits is currently 10 g. Where a larger volume has been collected, thorough mixing prior to subsampling for extraction will help provide an accurate representative of the sample for DNA extraction. With large volumes of soil, it may be advantageous to have a preliminary test where multiple DNA extractions per sample are run to determine how representative a single DNA extraction is of the whole sample. Soils and sediments typically contain PCR inhibitors, and heavily polluted sediments are often associated with particularly high levels of inhibition. Therefore, most extraction protocols need to include an inhibitor removal step. This is incorporated into commercial kit protocols designed for soils and sediments, but a specific clean-up step will almost certainly need to be incorporated where custom extraction protocols are used (e.g., Sellers et al., 2018). DNA from very large volume sediment samples is typically extracted using a phosphate buffer approach as outlined by (Taberlet et al., 2012b), although note that this specifically targets extracellular DNA.

5.6.3 Bulk Organisms

To facilitate DNA-based identification, bulk samples need to be sufficiently preserved from the point of collection through to laboratory analysis. This may require preserving samples while traps are still in situ (e.g., for terrestrial invertebrate samples) or following removal from a trap or collection device. In many cases, it can be days to weeks before samples are returned to the laboratory for processing. Preservation has conventionally been achieved using non-denatured ethanol in differing concentrations. For DNA analysis, this is typically >95% (Liu et al., 2020) in comparison to lower concentrations of 70% for morphological identification which can lead to DNA degradation (Carew et al., 2018). Due to the logistical difficulties associated with transporting hazardous substances, post-sampling alternatives such as propylene glycol, RNAlater™, or storing samples dry, have been explored (Patrick et al., 2016; Liu et al., 2020). For

optimal DNA preservation, temperature is a key factor, with most studies storing bulk invertebrate samples in freezing temperatures post collection (Liu et al., 2020). Room temperature storage is only suitable for a very short timeframe, however, appropriate combinations of killing solution, preservative fluid, and trap type, can facilitate trapping periods of several days to weeks prior to processing (Dopheide et al., 2019; Rees, 2022).

Of note, as an alternative or additional DNA source, the ethanol used to preserve the sample can be filtered to collect eDNA particles from the collected organisms. However, previous studies comparing DNA analysis from homogenised bulk samples, ethanol preservative eDNA, and soil eDNA have found inconsistencies in community detection, suggesting each DNA source has some biases and limitations (Marquina et al., 2019).

6 Current Limitations, Knowledge Gaps, and Next Steps

In general, there is persistent and growing optimism for environmental genomics to advance concepts in biodiversity and ecosystem structure and function based upon a series of initial successes in moving beyond the academic sphere towards practical application within industry. Yet limitations remain which are barriers to regulatory uptake, resulting in a constrained application for supporting permitting approvals. Evaluation, prioritisation and developing means of addressing these knowledge, understanding and methodology gaps are key to countering regulator uncertainty and reluctance to employ environmental genomics in decision-making processes. This section discusses critical knowledge gaps identified and provides high-level recommendations for addressing these gaps towards the goal of fostering regulatory uptake and confidence.

6.1 Regulatory Engagement

The principal international commissions and conventions related to monitoring environmental compliance and development of best practices for the offshore O&G industry are generally devoid of specific considerations of environmental genomics. In 2020, Stantec consulted a series of international regulatory agencies and found that all were aware of environmental genomics and the potential application to resource development decision-making, but some jurisdictions were more advanced in their confidence level with this technology and their subsequent willingness to employ it in regulatory approval processes. These jurisdictions exist along a gradient of potential for regulatory appetite. The most accepting of these regulators actively cultivate the advancement of environmental genomics in regulatory decision-making for industrial proponents. In contrast, most regulators do not generally rely on this technology for the assessment of industrial proposals yet will readily utilise environmental genomics to satisfy internal mandates related to biodiversity surveys, invasive species detection, or effects assessments. Lastly, several regulatory jurisdictions have relatively little or no previous experience with industrial permitting requests proposing genomic tools but are generally receptive to these approaches, particularly for geographies or ecological groups which are data poor or previously unstudied.

Current Limitations and Knowledge Gaps

- Various concurrent initiatives to understand and address barriers to regulatory confidence in and uptake of environmental genomics. Recently, the Second US National Conference on Marine eDNA gathered over 300 academics as well as research scientists from state and federal agencies with the aim to identify the necessary next steps to move eDNA methods into the management application mainstream (Stepien et al., 2022). The consensus among participants was that communication between scientists and resource managers remained the largest barrier to broader adoption. While scientists continue to voice caution related to method limitations, resource managers communicated the need for pathways/timelines for method adoption, including standard operating procedures, lab accreditation, and unified sequence libraries. Similar sentiments were also communicated during the 6th annual Environmental DNA (eDNA) Technical Exchange Workshop (Stepien et al., 2023) which concluded that future regulatory research directions should include studies of DNA fate and transport, autonomous sampling/sample processing, and reference library curation.
- Similar conclusions were mirrored by Stantec (2020) which also identified five intersecting knowledge gaps related to: understanding environmental eDNA persistence and dispersal; large-scale integration of eDNA data with different data types; improvement of reference library databases; molecular refinement of taxonomic indices; and standardisation.

Recommendations

- Preliminary consensus from Stepien et al. (2022) was that DNAqua-Net protocols currently provided the best template for North American regulators to follow (Stepien et al., 2022).
- The Bruce et al. (2021) publication described in Appendix B is derived from DNAqua-Net protocols and is highly relevant to practitioners in energy companies, regardless of geography, as it summarises the scientific consensus relating to every step of the field and laboratory workflows involved in the most common types of samples and analyses. Leveraging opportunities in the above-described domains, energy companies should incorporate DNAqua-Net protocols as being the foundation to any biodiversity assessment whenever possible and layer additional, bespoke protocols, derived with regulatory input whenever necessary.
- The burgeoning offshore wind sector is primed for the application of eDNA in environmental surveys. The potential for faster survey methods is particularly relevant as the transition to new energy is frequently time bound at the corporate and government level (e.g., 50 GW for the UK by 2030; Department for Energy Security and Net Zero and Department for Business and Trade, 2023). This has made it a field suited to rapid calibration and adoption of new methods through combined

government and industry research, potentially generating more ecological data and reducing consenting times (Elliot et al., 2023; Ray et al., 2023).

6.2 New Techniques and Methods

6.2.1 Collection and Analysis of Environmental RNA (eRNA)

Much like eDNA, eRNA can be readily collected within various environmental media.

Potential Advantages

- Due to the lower stability of the structure of RNA, some gene targets display faster degradation rates for eRNA compared to eDNA (Marshall et al., 2021). Thus, eRNA may provide a smaller time window snapshot of biodiversity by surveying the recently released genetic material.
- Similarly, eRNA metabarcoding may be more informative than eDNA for considering environmental functions such as analysing the impacts of contamination and soil remediation as it better targets the live/active community (Greco et al., 2022).
- Unlike DNA, RNA analysis provides an opportunity to evaluate the transcriptome and protein synthesis, providing insight into ecological data beyond species presence or absence. For example, gene targets expressed during larval metamorphosis of amphibians have been successful at distinguishing adult populations from larval populations when analysing eRNA (Parsley & Goldberg 2024).
- With the development of well validated assays, eRNA has the potential to assess physiological status of organisms and the ecological health of those populations and communities (Yates et al., 2021).

Current Limitations or Knowledge Gaps

- eRNA is less stable than eDNA, requiring more complex or expensive sample preservation and logistics prior to arrival at a laboratory for analysis. Although preservation buffers such as RNALater™ are available, freezing at -80°C is also commonly applied upon sample arrival at a laboratory (Knapik et al., 2020; Tremblay et al., 2019)
- eRNA degradation rates are likely to be impacted by environmental parameters in a similar fashion to eDNA; however, continued work needs to investigate these dynamics (Cristescu et al., 2019).
- Unlike degradation rates, eRNA shedding rates may vary widely across gene targets. In previous mesocosm experiments with dreissenid mussels, a gene target associated with cell mitosis (H2B) was found to have significantly lower concentration in eRNA than its eDNA counterpart, while for an rRNA gene target (16S) eRNA was found to have significantly higher concentration than its eDNA counterpart (Marshall et al., 2021). These dynamics likely relate to cell function, as rRNA is a crucial component

of all protein synthesis and consequently comprises >80% of total RNAs within a eukaryotic cell. Future research needs to intertwine concepts of cell function with eRNA shedding rates to better evaluate its true application.

Recommendations

- While transcriptomic methods are well established for microbial samples and individual tissue samples, a mixed genomic sample should be obtained from the environment in the case of more complex media. Careful development of assay validation is required for accurate and appropriate interpretation of results. Current limitations in genomic and transcriptomic databases may impede the development of species-specific eRNA assays.
- Gene transcripts specifically targeted for life-history stages, sexes, assessing population health or phenotypes within a species may not be sequenced for the various species present within the environment. This increases the risk of having non-specific amplification leading to inappropriate interpretations. eDNA protocols detail the development of assays to target well-established barcode regions, similarly, a major effort will be required to understand which gene targets are best suited for different eRNA applications (Stevens & Parsley, 2023).

6.2.2 In-Field Analysis

In-field analysis of environmental DNA (eDNA) is emerging as a powerful tool to reduce the lag time between sample collection and result generation. Traditionally, eDNA samples require laboratory processing, but recent advances in miniaturised molecular devices now allow for real-time, on-site analysis. These technologies are particularly valuable in remote or time-sensitive scenarios.

Technologies and Applications

- **MinION Sequencer (Oxford Nanopore Technologies):** A portable, USB-powered device capable of real-time sequencing. It has been successfully deployed in diverse environments including rainforests, polar regions, and mountainous terrains (Pomerantz et al., 2018; Goordial et al., 2017; Parker et al., 2017).

- **Isothermal Amplification Methods:**

    - LAMP (Loop-mediated Isothermal Amplification): Enables rapid species detection without the need for DNA extraction or thermal cycling (Williams et al., 2017, 2019).
    - CRISPR-based detection: Offers potential for highly specific, field-deployable assays (Phelps, 2023).

- **Portable Lab Setups:** Devices like Bento Lab, Liberty16, and Biomeme qPCR systems allow for DNA extraction, amplification, and sequencing in the field. These setups have

been used for barcoding nematodes, vertebrates, marine metazoa, and detecting invasive or trafficked species (Cardeñosa et al., 2019; Chang et al., 2020) (see Table 6-1).

Table 6-1 Summary of recent miniaturised molecular devices

Type of equipment	Product name	Company	Price range*	Website
All-in-one DNA laboratory - PCR	Bento Lab	Bento Lab	$1,600 - 2,000	https://www.bento.bio
Gel electrophoresis & visualisation system	blueGel / GELATO	Amplyus	$350 - 890	https://www.minipcr.com/product-category/gel-electrophoresis/
PCR	mini8 / mini16	Amplyus	$650 - 840	https://www.minipcr.com/products/minipcr/
qPCR	Liberty16	Ubiquitome	$6,000	http://www.ubiquitomebio.com
qPCR	Mini RT-PCR	Mygo	$9,200	http://www.mygopcr.com
qPCR	Open qPCR	Chai	$4,500 - 6,500	https://www.chaibio.com/openqpcr
qPCR	Franklin one9 / two9 / three9	Biomeme	$5,950 - 9,950	https://info.biomeme.com/mobile-qpcr-thermocyclers
Sequencer	MinION / Mk1C	Oxford Nanopore Technologies	$1,000 - 4,900	https://nanoporetech.com/products
Sequencing flow cells	Flongle / Flow cell	Oxford Nanopore Technologies	$90 - 900	https://store.nanoporetech.com/us/flowcells.html

- * = Price range is meant as an indication only. True prices will be subject to factors such as exchange rates and shipment costs (from Piel et 2021)

Potential Advantages

- Real-time results enable rapid decision-making in the field.
- Reduced equipment requirements compared to traditional PCR.
- Enables research in remote or logistically challenging environments.
- Supports rapid screening for invasive or endangered species.

Current Limitations and Knowledge Gaps

- Lower sensitivity and higher inhibition risk compared to benchtop qPCR protocols.
- Limited throughput makes large-scale field analysis inefficient.
- Higher sequencing error rates in portable devices like MinION require robust bioinformatics pipelines (Baloglu et al., 2020).

- Field-based detection may not be suitable when high accuracy or large sample volumes are required.

Recommendations

- Use in-field analysis for time-sensitive or remote applications where rapid feedback is critical.
- For routine or high-accuracy needs, continue using validated laboratory protocols.
- Support further R&D to improve DNA extraction kits, reduce inhibition, and validate field-based methods against lab standards (e.g., MiSeq vs. MinION).
- Encourage partnerships between technology developers and eDNA service providers to accelerate adoption and standardisation.

6.2.3 Aquatic Auto-Sampling Technology

The use of automated eDNA samplers is expanding rapidly across marine and freshwater environments, driven by efforts to validate the technology and compare results with conventional sampling methods. These samplers are often deployed alongside environmental and geophysical surveys to support integrated monitoring strategies.

Deployment Methods

Deployment methods vary widely:

- Fixed-point installations (e.g., offshore buoys, harbours) for long-term monitoring (Mynott, 2019).
- Remotely Operated Vehicles (ROVs) for sampling across multiple locations (Everett & Park, 2018).
- Autonomous Underwater Vehicles (AUVs) coupled with environmental sample processors to maximize data collection (Yamahara et al., 2019).
- Freshwater systems, where autosamplers have also proven effective (Searcy et al., 2022).
- Concurrent sampling with CTD/Rosette casts, although traditional Niskin bottle arrays remain common (Easson et al., 2020).

The autonomy of these systems allows for static deployments and continuous surveillance, reducing reliance on vessel-based operations. As technology advances, equipment is expected to become more compact and compatible with ROV/AUV platforms, further minimizing offshore vessel and personnel time.

Potential Advantages

- Reduced HSSE exposure due to unattended operation over extended periods.
- Non-invasive sampling, aside from initial installation.
- Flexible sampling intervals (weekly/monthly/quarterly).
- Lower operational costs through fewer field visits and potential use of existing infrastructure.
- Simplified sample collection, storage, and transport via integrated sample cassettes.

Current Limitations and Knowledge Gaps

- Supply of autosamplers by companies (e.g., Dartmouth Ocean Technologies, McLane Research Laboratories) and research institutes (MBARI, WHOI) does not yet meet demand.
- DNA samples still require manual retrieval for metabarcoding analysis.
- Maintenance frequency and battery life vary; innovations like seafloor charging stations may help (Lin et al., 2022).

Recommendations

- Energy companies should foster partnerships between autosampler manufacturers and eDNA service providers (e.g., NatureMetrics and Dartmouth Ocean Technologies).
- IOGP members should consider integrating eDNA sampling capabilities when procuring AUVs/ROVs for broader monitoring and surveying applications.

6.2.4 Airborne eDNA Sampling

Airborne environmental DNA (eDNA) is an emerging tool for surveying terrestrial biodiversity. Initially developed through studies on pollen and airborne plant DNA (Longhi et al., 2009; Johnson et al., 2019), the technique has since expanded to include fungal and vertebrate taxa. Proof-of-concept studies in controlled environments such as zoos and enclosures have demonstrated its potential for detecting vertebrate species (Serrao et al., 2021; Clare et al., 2022; Lynggaard et al., 2022). However, its effectiveness in natural environments remains under investigation.

Sampling Techniques

Airborne eDNA can be collected using active or passive sampling methods (Figure 6-1):

- Active Sampling: Involves drawing air through filters (e.g., 0.22 µm Sterivex or F8 fibrous filters) using powered devices. Filters are then processed either in the field or lab (Clare et al., 2021; Lynggaard et al., 2022).

- Passive Sampling: Relies on wind to carry airborne particles into containers or dust traps filled with sterile water, which is later filtered (Johnson et al., 2019; Klepke et al., 2022).

Each method has trade-offs. Active samplers offer higher throughput but require power, while passive samplers are more suitable for remote, long-term deployments but may collect less DNA.

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 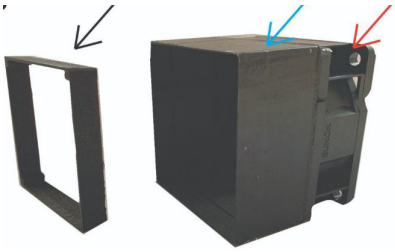 </td>
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<td>Big Sping Number Eight dust trap used to passively collect airborne eDNA (Johnson et al., 2019)</td>
<td>5 V and 12 V active filter air samplers. a) Frontal view of the air sampler where the radial blower fan can be seen. b) Side view of the air sampler. Black arrow indicates the filter holder, blue arrow indicates the3D-printed filter housing and the red arrow the radial blower fan. Design, including 3Dblueprint of the filter holder and the 3D-printed filter housing is outlined in Lynggaard et al. (2022).</td>
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Figure 6-1 Examples of eDNA samplers used to capture particulate matter from air

Potential Advantages

- Enables detection of macrofauna in terrestrial habitats where soil eDNA may be less effective.
- Can leverage existing air quality monitoring infrastructure and protocols.
- Offers a non-invasive, scalable approach to biodiversity monitoring.

Current Limitations and Knowledge Gaps

- Environmental Variables: DNA degradation is influenced by temperature, humidity, and UV exposure. The persistence and decay rates of airborne DNA are not well understood.
- Detection Sensitivity: DNA concentrations in natural air samples may be too low for reliable detection, especially for vertebrates.
- Sampling Optimization: Key questions remain about optimal air volumes, filter types, and sampling durations.
- Preservation and Contamination: Most studies rely on freezing filters at -20°C, which may not be feasible in remote settings. Sterility protocols and field blanks are essential to avoid contamination.
- Comparative Performance: Airborne eDNA has shown lower species detection rates compared to conventional methods (Roger et al., 2022).

Recommendations

- Conduct field trials in diverse natural habitats to evaluate detection probabilities across taxa and environmental conditions.
- Optimize equipment for collecting biological replicates without increasing sampling effort (e.g., 3D-printed samplers by Clare et al., 2022).
- Investigate alternative preservation methods for remote deployments.
- Continue comparing passive vs. active sampling methods to determine context-specific best practices.
- Develop standardized protocols to improve reproducibility and scalability of airborne eDNA monitoring.

6.2.5 Passive eDNA Samplers

There is growing interest in developing sampling methods that involve passive filtration, as these approaches remove the need to use pumps to actively collect samples through mechanical filtration.

Potential Advantages

- This has the potential to reduce labour effort while increasing detection rates due to the ability to sample large volumes. Samplers can be left out for hours to days (Bessey et al., 2022), taking advantage of lotic flows or marine tides to collect transported eDNA that captures a larger spatial scale than can be achieved with active filtration samples at a single point in time.
- The ease of passive sampling approaches allows for increased biological replication as several sets of samples can be placed at a site without much increased effort.

- If the passive sampler is composed of a mesh or gauze material then it can be easily attached to other sampling gear to increase survey efficiencies, such as passive samplers designed for invasive dreissenid mussels in Lake Superior (Larson et al., 2022) or gauze material attached to marine fish trawls to complement conventional surveys (Maiello et al., 2022).

Current Limitations or Knowledge Gaps

- Many questions remain on which materials are most suited for passive collection, and it is unknown how environmental parameters (e.g., total dissolved solids, pH, salinity, flow) will impact collection rates across these materials. However, different sampling methods using a wide variety of materials (Figures 6-2, 6-3) and protocols are beginning to be evaluated (Bessey et al. 2021, 2022; Chen et al., 2022; Jeunen et al., 2022; Verdier et al., 2022).
- While studies have demonstrated passive samplers can be left out for hours to days, it is still unknown what the optimal timeframe is. It is possible that filter materials become saturated within a small timeframe, there is the potential confounding influences of eDNA degradation, and longer sampling events may not increase sample coverage.

Recommendations

- Projects directly evaluating passive and active filtrations methods within a variety of habitats will allow for a more robust understanding of where and when passive filtration can be an appropriate survey method.
- Studies utilising marine flow and current models should be combined with passive sampling approaches to evaluate the potential temporal or spatial coverage which a passive sample collection achieves.
- The optimal passive filtration material for a marine environment might not be as optimal in a highly eutrophic freshwater environment that is rich in algae and dissolved solids. Additionally, studies should investigate the applicability of passive sampling in small to medium lentic systems that have naturally little to no water movement.

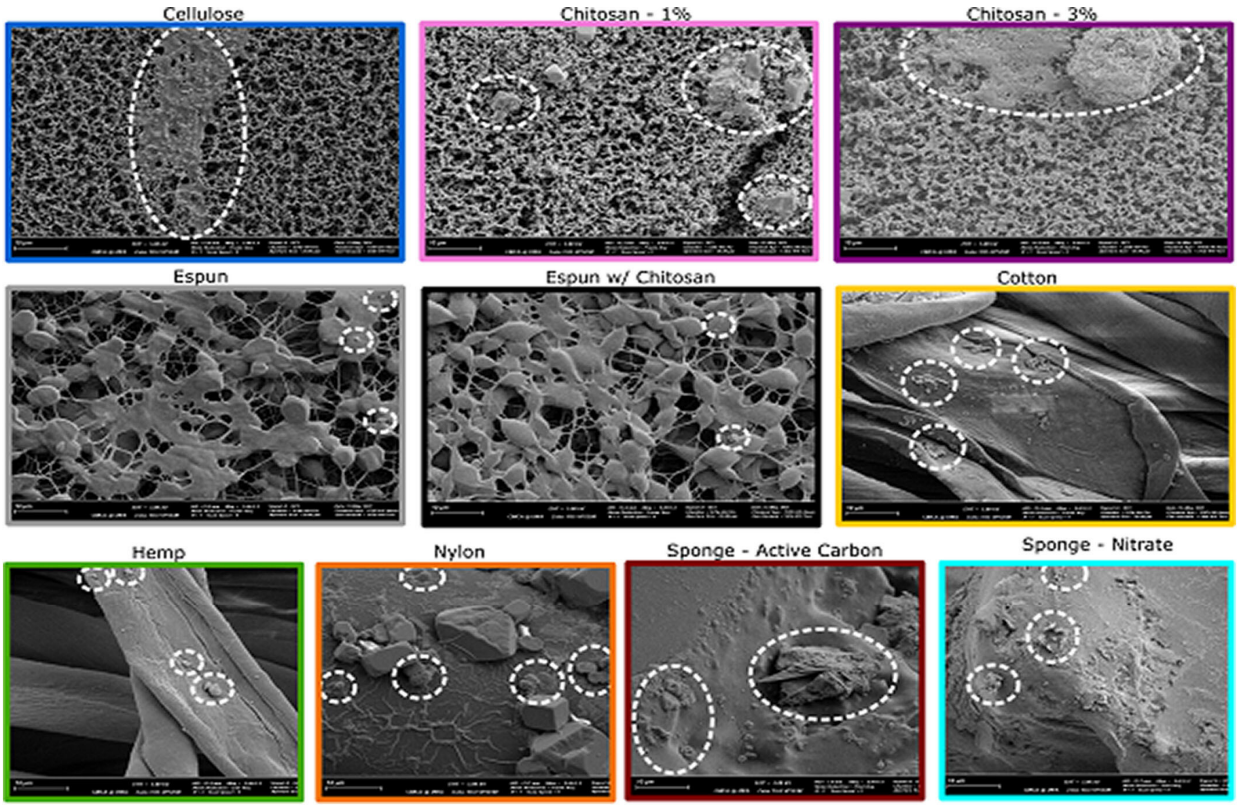

Figure 6-2 Scanning electron micrographs of materials trialed for passive eDNA collection in water Scanning electron micrographs of materials; 5000× magnification; dashed circles identify biological matter (from Bessey et al., 2022).

Figure 6-3. Passive eDNA collection experimental apparatus containing filter membranes that were submerged 1 m below the ocean surface from the bow of a boat (Bessey et al., 2021)

6.2.6 Substrate Swab eDNA Sampling

Similar to passive filtration, there has been recent developments in methodologies to collect eDNA by swabbing surfaces of various substrates that a target species may come into contact with. An example for sampling snake eDNA is shown in Figure 6-4.

Figure 6-41. Field site photos from Matthias et al. (2021) demonstrating Artificial Cover Object physical surveying (left) and moistening of a finger cot with 70% isopropanol alcohol for taking a swab of the artificial cover object for snake eDNA

Potential Advantages

- These methods improve the ability to survey macrofauna from terrestrial habitats, where soil eDNA may be an inefficient method for detection.
- The methods take advantage of low-cost easy to obtain materials such as paint rollers for surveying arthropods (Allen et al., 2023) and vertebrate taxa (Kyle et al., 2022; Newton et al., 2023; Allen et al., 2023) or cotton finger cots to swab artificial cover boards targeting snake eDNA (Matthias et al., 2021).
- In marine environments, the swabbing method also generates a higher diversity than epibenthic scrapes (Alexander et al., 2023b)

Current Limitations or Knowledge Gaps

- While some studies have shown promise in testing materials and methods for swab-based detection, a lack of robust data limits standardisation and recommendations for protocols. However, some basic concepts of eDNA degradation can be applied to swab-based survey design, such as avoiding swabbing substrates that are directly under UV sunlight or following a rain event that likely washes away any present eDNA.
- Further testing of materials that can entrap eDNA will help facilitate this methodology for terrestrial biodiversity surveys. Additionally, similar to swabbing artificial cover boards for snake eDNA, future research evaluating approaches to passively collect eDNA as terrestrial organisms pass over the substrate will advance these tools.

6.2.7 Snow and Ice collection

Snow and ice represent promising alternative media to sample for eDNA study in cold environments. Snow will accumulate and preserve eDNA over time in the compacted layers of snow and be at higher density in snow tracks (Dalén et al., 2007, Hinlo et al., 2017, Franklin et al., 2019, Howel et al., 2021). To date, eDNA studies from snow and ice have mainly focused on vertebrates and microorganisms (Howel et al., 2021) and protocols to collect eDNA from snow and ice are still in development. Vertebrates have been surveyed from snow directly under and near an animal snow track collected using a variety of methods and sample volumes across studies. For example, plastic scoops were used by Franklin et al. (2019) to collect 2 L of packed snow in a Nalgene bottle. Barber-Meyer et al. (2020) filled two 120 mL specimen cups per trail of tracks for a total of ten tracks per sample (five tracks per cups). In this case, the edge of the

specimen cup was used to directly scoop the snow. In another study, sample of fresh snow were collected in 50 mL Falcon tubes (Dalén et al., 2007). Kinoshita et al., (2019) collected surface snow (1-3 cm) from five sequential footprints as one sample for each track. Field controls for most studies consisted of undisturbed, flat snow to provide a level of background DNA (Dalén et al., 2007, Kinoshita et al., 2019). Ice samples can be collected as cores or blocks and stored at -20°C, then thawed and treated as water for extraction and detection of eDNA (Bayless et al., 2015). For example, bacterial diversity has been investigated from cryoconite holes and ice cores collected with Kovacs ice corer powered by motor or electrical drill and (Webster-Brown et al., 2015, Weisleitner et al., 2019).

6.3 Metabarcoding Reference Libraries

Where species level identifications are necessary, such as with the detection of protected or invasive species, targeted barcoding campaigns as described in Hestetun et al., (2020) may be necessary. For some indicator groups used for health indices such as diatoms, taxonomy-free approaches may mean that barcoding is less important (Apothéloz-Perret-Gentil et al., 2017).

Current Limitations or Knowledge Gaps

- New markers may be necessary for certain key biological groups such as coral which continue to prove difficult to detect using eDNA (Alexander et al., 2023b).
- For some taxonomic groups, detections may not be limited by markers or reference libraries, but by the sampling methods (Alexander et al., 2023b; Marquina et al., 2019). Determining whether this is the case is essential to avoid wasting resources on developing reference libraries on a new gene region.

Recommendations

- When barcoding tissue samples, DNA should be bio-banked (preserved for long term storage) for any future barcoding that may target different gene regions.
- For barcoding campaigns, the genes that are most commonly used for eDNA and therefore should be targeted are:
    - 18S and COI for invertebrates (e.g., Hestetun et al., 2020; Elbrecht & Leese, 2017)
    - 12S (Miya et al., 2020; Riaz et al., 2011), 16S (e.g. Valsecchi et al., 2020; Sakata et al., 2022) and less frequently cyt-B (e.g. Blanco et al., 2020) and COI (e.g. Breitbart et al., 2023) for vertebrates
    - ITS for fungi (Kauserud, 2023)
    - rbcL for photosynthetic organisms (Duarte et al., 2020)
    - 16S for bacteria (Caporaso et al., 2011)

6.4 Summary

To maximise regulatory uptake and confidence, the energy industry must address five intersecting scientific and procedural knowledge gaps related to: environmental DNA persistence and dispersal, large-scale integration of eDNA data with different data types, improvement of reference library databases, molecular refinement of taxonomic indices, and procedure standardisation (Stantec 2020).

The subsequent aim of the current document is to provide guidance to environmental practitioners on minimum sampling design requirements required to confidently employ environmental genomics technology in contexts within the energy industry.

The current report accomplished this aim by guiding end-users through a description of eDNA methods (Section 2), summary of existing standards and guidelines (Section 3), sampling considerations specific to oil & gas applications (Section 4), and guidance on implementing an eDNA study (Section 5).

In summarising current limitations and knowledge gaps, the current report highlights the following key findings and considerations:

- There is currently a developing consensus on scientific best-practice in many areas, but this is not readily discernible from the now-extensive and ever-growing body of research literature.
- While sampling guides exist, few have immediate utility for the energy sector, given the breadth of geography, habitats, and taxa to be assessed.
- Presently there are no definitive global standards or regulations that govern the use of environmental DNA-based applications and their interpretation.
- This lack of standardised approaches within industry continues to hamper communication with regulators and stakeholders.
- Dialogue between government, industry, and academia at an international scale has resulted in a shared understanding of what would be needed at a minimum to constitute a reliable approach incorporating eDNA data into an environmental monitoring framework.

To facilitate regulatory uptake we have determined that, in concert with the Energy Industry-focused guidance provided in the current report, the Bruce et al. (2021) guidance derived from DNAqua-Net protocols is most complementary and broadly amenable to accomplishing the objectives desired by environmental practitioners in the energy sector. Similar to our aim in the current report, the Bruce et al. (2021) guidance is oriented towards scientific “First Principles” and synthesises generalised scientific consensus derived from extensive reviews of scientific literature and practical considerations provided by both expert and non-expert end-users of environmental genomics.

Beyond the additive benefits of the compilation of technical knowledge and advice, amalgamating the above approaches of the current report and the Bruce et al. (2021) guidance when possible and feasible further leverages the recognised scientific rigour of the COST (European Cooperation in Science and Technology) Action DNAqua-Net activities that underpin both approaches. Incorporating the DNAqua-Net protocols as the foundation for biodiversity assessments while adding practical guidance from Bruce et al. (2021), and layering the additional considerations presented in the current report would provide a tiered approach from scientific “First Principles” through energy-specific practicality necessary to raise regulatory and stakeholder confidence. Similarly, the industry use of guidance which is underpinned by third-party academic expertise provides regulatory agencies with objective criteria for their own assessment and verification as well as with their own stakeholder engagements when considering and communicating decisions on environmental permitting applications in the energy industry.

Glossary

Environmental Genomics	Also known as ecological genomics, broadly refers to the study of genetic material recovered directly from environmental samples, and encompasses DNA barcoding, quantitative polymerase chain reaction (qPCR), metabarcoding, metagenomics, and transcriptomics.
DNA barcoding	The use of a short segment of DNA from a specific gene to identify a unique, individual species or taxon. The most commonly-used segment for DNA barcoding is a ~600 base pair (bp) sequence of the mitochondrial gene Cytochrome Oxidase I (COI)
Environmental DNA	Genetic material that has detached or sloughed off from an organism (in either intra- or extracellular form) into non-living components of an ecosystem such as air, water, or sediment. For eDNA analysis, generally short fragments (~100 – 150 bp) are used as they mostly consist of released DNA as opposed to tissue samples used in DNA barcoding.
Assay	Also known as a primer set, is defined as two short, single-stranded nucleic acid molecules (typically 20 bp or longer) consisting of a sequence of DNA bases that are designed to match the target DNA at a particular point in the genome. PCR usually requires a pair of primers (or primer set), one matching the target DNA at either end of the barcode region to be amplified.
Reference Database	A library of DNA sequences derived from specimens of known identity. Sequence data obtained from test samples (e.g., via metabarcoding) can be matched against a reference database to assign taxonomic names to the sequences

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Appendix A Industry-Specific Applications of Interest Matrix

A.1 Primary Industry-Specific Applications of Interest

INDUSTRY SURVEY																						
		Project Phase				Setting			Target			Technology Status										
Potential Industry Specific Applications	Industry Focus	Exploration & Appraisal	Development (site selection & construction)	Operations	Decommissioning/post-decommissioning	Onshore	Offshore	Coastal	Single species	Indicators (single or multiple species)	Overall Biodiversity	Regulatory Acceptance	Technology Readiness (TRL)	Shell Priority	Eni Priority	TOTAL Priority	Chevron Priority	ExxonMobil Priority	Hess	Equinor Priority	Overall Priority	Application Avge
Rapid assessment of potential invasive species (e.g. ballast water, biofouling, etc..)	Accuracy of results (false positives, negatives, poor quality data, persistence of DNA)	X	X	X	X	-	X	X	X	X	X	2	2	2	3	1	3	3	3	1	2.3	1.8
	Real-time on-site measurement and analysis	X	X	X	X	-	X	X	X	X	X	2	1	2	1	1	3	1	1	1	1.4	
	Species viability (live vs dead, function, etc)	X	X	X	X	-	X	X	X	-	-	1	2	2	1	1	3	2	2	1	1.7	
	Species abundance (how much is there)	X	X	X	X	-	X	X	X	X	-	1	1	2	1	1	3	1	2	1	1.6	
Early detection of newly introduced invasive or competitive species (not specific to ballast water or biofouling)	Accuracy of results (false positives, negatives, poor quality data, persistence of DNA)	-	X	X	-	X	X	X	X	-	-	2	2	2	3	2	3	3	3	3	2.7	2.4
	Distribution (where is it)	-	X	X	-	X	X	X	X	-	-	2	2	2	2	2	2	1	2	3	2.0	
Detection of key species (RTEs, commercial species)	Accuracy of results (false positives, negatives, poor quality data, persistence of DNA)	X	X	X	X	X	X	X	X	-	-	2	2	3	2	3	3	3	3	2	2.7	2.4
	Distribution (where is it)	X	X	X	X	X	X	X	X	-	-	2	2	3	1	3	3	1	2	2	2.1	
	Ecological change (temporal or spatial variation)	X	X	X	X	X	X	X	X	-	-	2	2	3	2	3	3	3	3	3	2.9	
	Species abundance (how much is there)	X	X	X	X	X	X	X	X	-	-	1	1	2	2	3	1	2	2	2	2.0	
Population Status & Dynamics (single species)	Species abundance (how much is there)	X	X	X	X	X	X	X	X	-	-	1	1	3	2	3	1	2	2	3	2.3	2.4
	Distribution (where is it)	X	X	X	X	X	X	X	X	-	-	2	2	3	2	3	1	2	2	3	2.3	
	Ecological change (temporal or spatial variation)	X	X	X	X	X	X	X	X	-	-	1	2	3	3	3	1	3	3	3	2.7	
	Data interpretation and comparability (indices, communication)	X	X	X	X	X	X	X	X	-	-	2	2	3	3	3	1	3	2	3	2.6	
	Species viability (live vs dead, function, etc)	X	X	X	X	X	X	X	X	-	-	2	1	1	3	3	1	2	3	3	2.3	
Habitat Delineation (identifying potential habitats based on biological characteristics)	Community representation (what's there)	X	X	X	X	X	X	X	-	X	X	1	2	2	1	2	1	3	1	3	1.9	1.8
	Distribution (where is it)	X	X	X	X	X	X	X	-	X	X	2	1	2	1	2	1	1	1	3	1.6	
	Data interpretation and comparability (indices, communication)	X	X	X	X	X	X	X	-	X	X	2	1	1	2	3	1	3	2	3	2.1	
	Species abundance (how much is there)	X	X	X	X	X	X	X	-	X	X	1	1	1	2	2	1	1	2	3	1.7	

Potential Industry Specific Applications	Industry Focus	Project Phase				Setting			Target			Technology Status										
		Exploration & Appraisal	Development (site selection & construction)	Operations	Decommissioning/post-decommissioning	Onshore	Offshore	Coastal	Single species	Indicators (single or multiple species)	Overall Biodiversity	Regulatory Acceptance	Technology Readiness (TRL)	Shell Priority	Eni Priority	TOTAL Priority	Chevron Priority	ExxonMobil Priority	Hess	Equinor Priority	Overall Priority	Application Avge
Baseline assessments (establishing pre-existing conditions)	Community representation (what's there)	X	X	-	-	X	X	X	-	X	X	2	3	3	1	3	3	3	3	3	2.7	2.7
	Species abundance (how much is there)	X	X	-	-	X	X	X	-	X	X	1	1	3	1	3	2	2	3	3	2.4	
	Distribution (where is it)	X	X	-	-	X	X	X	-	X	X	2	2	3	1	3	3	2	2	3	2.4	
	Accuracy of results (false positives, negatives, poor quality data, persistence of DNA)	X	X	-	-	X	X	X	-	X	X	2	2	3	1	3	3	3	3	3	2.7	
	Ecological change (temporal or spatial variation)	X	X	-	-	X	X	X	-	X	X	2	2	3	2	3	3	3	2	2	2.6	
	Sampling procedure & design	X	X	-	-	X	X	X	-	X	X	2	2	3	2	3	3	3	3	3	2.9	
	Data interpretation and comparability (indices, communication)	X	X	-	-	X	X	X	-	X	X	1	2	3	2	3	3	3	3	3	2.9	
Monitoring of Environmental Effects of oil & gas activities (natural or engineered environments)	Ecological change (temporal or spatial variation)	-	-	X	X	X	X	X	X	X	X	1	2	3	3	3	3	3	3	3	3.0	2.5
	Data interpretation and comparability (indices, communication)	-	-	X	X	X	X	X	X	X	X	1	2	3	3	3	3	3	3	3	3.0	
	Distribution (where is it)	-	-	X	X	X	X	X	X	X	X	2	2	3	3	3	3	1	2	2	2.4	
	Sampling procedure & design	-	-	X	X	X	X	X	X	X	X	2	1	3	3	3	3	3	3	1	2.7	
	Species abundance (how much is there)	-	-	X	X	X	X	X	X	X	X	1	1	3	2	2	3	1	3	2	2.3	
	Real-time on-site measurement and analysis	-	-	X	X	X	X	X	X	X	X	1	1	1	2	2	2	1	1	1	1.4	
	Accuracy of results (false positives, negatives, poor quality data, persistence of DNA)	-	-	X	X	X	X	X	X	X	X	2	2	3	3	3	3	3	3	2	2.9	
	Species viability (live vs dead, function, etc)	-	-	X	X	X	X	X	X	X	X	1	2	3	3	2	3	1	2	3	2.4	
Remediation / Restoration	Species abundance (how much is there)	-	-	X	X	X	X	X	-	X	X	1	1	1	1	2	2	2	2	2	1.7	2.1
	Distribution (where is it)	-	-	X	X	X	X	X	-	X	X	2	2	1	2	2	2	2	2	2	1.9	
	Ecological change (temporal or spatial variation)	-	-	X	X	X	X	X	-	X	X	2	2	1	3	1	2	3	3	2	2.1	
	Community representation (what's there)	-	-	X	X	X	X	X	-	X	X	2	2	1	1	3	2	3	3	2	2.1	
	Data interpretation and comparability (indices, communication)	-	-	X	X	X	X	X	-	X	X	1	2	1	2	2	2	3	3	2	2.1	
	Sampling procedure & design	-	-	X	X	X	X	X	-	X	X	2	2	1	2	3	2	3	2	3	2.3	
	Accuracy of results (false positives, negatives, poor quality data, persistence of DNA)	-	-	X	X	X	X	X	-	X	X	2	2	1	2	3	2	3	3	3	2.4	
	Species viability (live vs dead, function, etc)	-	-	X	X	X	X	X	-	X	X	2	2	1	2	2	2	2	2	2	1.9	

A.2 Potential Applications Based on Recent Review

INDUSTRY SURVEY																						
		Project Phase				Setting			Target			Technology Status										
Potential Industry Specific Applications	Industry Focus	Exploration & Appraisal	Development (site selection & construction)	Operations	Decommissioning/post-decommissioning	Onshore	Offshore	Coastal	Single species	Indicators (single or multiple species)	Overall Biodiversity	Regulatory Acceptance	Technology Readiness (TRL)	Shell Priority	Eni Priority	TOTAL Priority	Chevron Priority	ExxonMobil Priority	Hess	Equinor Priority	Overall Priority	Application Avge
Bio-Corrosion	Species abundance (how much is there)	-	-	X	-	X	X	X	X	X	-			1	1	1	1	1	1	1	1.0	1.3
	Community representation (what's there)	-	-	X	-	X	X	X	X	X	-			1	1	3	3	1	1	1	1.6	
	Distribution (where is it)	-	-	X	-	X	X	X	X	X	-			1	2	1	1	1	1	1	1.1	
	Ecological change (temporal or spatial variation)	-	-	X	-	X	X	X	X	X	-			1	2	1	1	1	1	1	1.1	
	Species viability (live vs dead, function, etc)	-	-	X	-	X	X	X	X	X	-			1	3	2	1	1	2	1	1.6	
	Real-time on-site measurement and analysis	-	-	X	-	X	X	X	X	X	-			1	1	1	1	1	1	1	1.0	
	Accuracy of results (false positives, negatives, poor quality data, persistence of DNA)	-	-	X	-	X	X	X	X	X	-			1	2	2	2	1	2	1	1.6	
Early warning and micro-leakage detection	Ecological change (temporal or spatial variation)	-	-	X	-	X	X	X	-	X	X	1	2	1	3	1	2	1	2	1	1.6	1.3
	Data interpretation and comparability (indices, communication)	-	-	X	-	X	X	X	-	X	X	1	1	1	2	1	2	1	2	1	1.4	
	Real-time on-site measurement and analysis	-	-	X	-	X	X	X	-	X	X	1	2	1	1	1	1	1	1	1	1.0	

Appendix B Summary of Existing/Developing Standards and Guidelines

B.1 The Need for Standardisation

The successful mainstreaming of environmental genomics will require standardisation of protocols across industry, academic, and regulatory bodies, and a rethinking of some fundamental concepts. A dearth of standardised approaches (e.g., for sampling methods, reporting accuracy of results, and describing limitations to interpretation and reliance) has hampered communication with regulators and stakeholders. Some level of standardisation is needed to permit greater confidence in, and reliability upon, the results of environmental genomics data.

The field of environmental DNA (eDNA) technology has developed fast and eDNA methods have been applied to a wide range of research and monitoring projects globally. Non-invasive rapid, sensitive, and scalable detection of practically any species in any environment, including cryptic, rare, elusive, and microscopic taxa, makes eDNA an incredibly useful tool for biomonitoring. However, over the years, a high level of methodological variation has been introduced, and still exists at all stages of the workflow. There is now a developing level of consensus on scientific best-practice in many areas, but this is not readily discernible from the now-extensive body of research literature. There are no definitive global standards or regulations governing the use of DNA-based applications for biomonitoring.

B.2 How Standardisation Is Evolving

As environmental practitioners and policy makers are increasingly starting to integrate DNA-based methods into routine monitoring applications, various national and international efforts have been undertaken to standardise methods and integrate them into monitoring frameworks (Pilliod et al., 2019; Loeza-Quintana et al., 2020; Minamoto et al., 2021; Pawlowski et al., 2020).

In many countries, there is an increasing body of eDNA guidelines based on consultation between government, industry, and academia in the form of working groups or initiatives, such as DNAqua Net1 in Europe, the US Federal Government eDNA Working Group (GeDWG)2, and the eDNA Society3 in Japan. While these currently do not result in agreed standards, the dialogue between these different groups at an international scale has resulted in a shared understanding of what is important and what would at a minimum need to be included to constitute a standard (Bruce et al., 2021). Principles of standardisation that have been agreed upon include that standardisation should be derived from a scientific consensus for field and laboratory processes, whilst being practicable in terms of cost, logistics, safety, and quality

[1] https://dnaqua.net/

[2] https://doimspp.sharepoint.com/sites/usgs-CDI-eDNA-Community-of-Practice

[3] https://ednasociety.org/en/about-2/

assurance. These standard approaches should allow robust planning of DNA-based monitoring in a way that is replicable (Bruce et al., 2021).

Additionally, there are efforts underway by several global organizations, such as the International Organization for Standardization (ISO), to harmonise the many biodiversity metrics and indicators currently in use to streamline reporting standards for biodiversity assessments. One key set of standards under development is ISO/CD 17805 for "Water quality – Sampling, capture and preservation of environmental DNA from water" as part of ISO/TC 147/SC 5/WG. Another is the Technical Committee of ISO/TC 331 for Biodiversity created in 2020, which is aiming for 'Standardization in the field of Biodiversity to develop principles, framework, requirements, guidance and supporting tools in a holistic and global approach for all organizations, to enhance their contribution to Sustainable Development'. This standard is currently developing (in the preparatory phase) the terms and definitions in the field of biodiversity, including terms related to biodiversity loss (e.g., climate change, pollution, invasive species, soil degradation, deforestation) and means to protect biodiversity (measurement, monitoring and assessment, restoration, conservation and protection, sustainable use). This will cover genetic diversity, species diversity and ecosystem diversity, all of which can be measured using DNA based methods. We would therefore advise monitoring the outputs from this group over the coming years.

One of the first standards applied to eDNA was the method for the detection of the Great Crested Newt (GCN), which is a formally regulated eDNA standard in the UK (Biggs et al., 2014). This standard applies the ethanol precipitation technique to store the water samples and preserve the eDNA, which is now widely regarded as an inefficient sample storage method (Tsuji et al., 2019). Water filtration is currently the most widely used and accepted method for collecting and storing eDNA from aquatic samples. Similarly, the US Fish and Wildlife Service (USFWS) developed a protocol for eDNA detection for the detection of invasive Carp in 2012 (Jerde et al., 2013), which used ethanol precipitation centrifugation rather than filtration for the separation of eDNA from the water samples. This protocol is now being revised to align with the scientific consensus on filtration as a more effective method in terms of sensitivity, contamination risk, logistics, and safety.

The above examples demonstrate how standards in a continually evolving field such as that of environmental genomics need to be regularly reviewed to be updated in line with the latest best practice.

Examples of new technologies that are currently applied in academia but will soon become developed sufficiently for industrial application are:

- The MinION device from Oxford Nanopore Technologies for in-field sequencing, both for barcoding and metabarcoding (Pomerantz et al., 2018; Davidov et al., 2020; Hatfield et al., 2020)
- CRISPR as a method for rapid detection of species in the field (Williams et al., 2019, 2020), which may ultimately supplant qPCR

The relative merits, caveats, and barriers to standardisation of these methods are discussed in Section 6.7 of the main report.

B.3 Summary of Existing Standards Published to Date

The information in this section covers the most relevant and most recent publications on eDNA standards and guidelines, but also incorporates SOPs (Standard Operating Procedures) and best practices. Section B.4 covers the related efforts and developments in eDNA application standards that are not yet formally implemented.

There are standards that specifically cover regional or local eDNA sampling scenarios for local species such as the Great Crested Newt (GCN, *Triturus cristatus*) in the UK (Biggs et al. 2014). However, many eDNA standards and guidelines are widely applicable across geographies and habitats cover the full process of the eDNA applications, including:

- Sampling Design
- Sampling Methods
- Preservation and Storage
- Laboratory Procedures
- Data Analysis and Reporting

A key resource on DNA-based methods, for both in the field and in the lab, for practitioners in the energy industry is 'A practical guide to DNA-based methods for biodiversity assessment' (Bruce et al., 2021). This document is the main output of many years of consensus-building through the EU DNAqua-Net program. The practical guide summarises the scientific consensus on field and laboratory workflows relating to the different types of samples and analyses. The emphasis is on robustness, replicability, traceability, and ease-of-use, highlighting key decisions to be made and the inherent trade-offs associated with the various options, to help navigate the key considerations associated with planning and evaluating DNA-based monitoring programmes.

The Bruce et al. (2021) document covers topics that are universally applicable to eDNA-based methods, such as guidelines on (cross-) contamination mitigation and the use of negative field and laboratory samples but also measuring the quality and quantity of extracted DNA from any source. Additionally, specific guidelines on the capture, preservation, and extraction of multiple sample media are discussed. Both target species detection through qPCR, and community assessment through metabarcoding are considered.

Bruce et al. (2021) also explain that sampling strategies for eDNA are frequently a compromise between experimental design, field effort (particularly vessel time for offshore projects) and cost. To be effective, eDNA sampling should account for:

1. Physicochemical properties of the sampled matrix
2. Environmental variability
3. Ecology of the target species

Standard operating procedures (SOPs) that have been developed for conventional biomonitoring techniques such as for sediment macrofauna (Rumohr 2009), can also be applicable to eDNA sample collection. Particularly in the areas of equipment selection and sampling design. SOPs might be generated internationally, for example by ICES, The International Council for the Exploration of the Sea [(Rumohr 2009) and nationally, such as by DEFRA, the Department for Environment, Food and Rural Affairs in the UK (McNiven and Gilchrist, 2016)]. Consequently, practitioners are advised to look for any SOPs relevant to the geographic location of sampling for their project. Table B-1 below gives a list of existing standards and guidelines and their relevance to IOGP members. Table B-2 provides a summary of recent peer-reviewed literature judged to be of most relevance to IOGP members with potential to inform eDNA standards and guidelines development or implementation.

Table B-1 Existing eDNA Standards and Guidelines

Title	Author, Year (Country of Publication)	Sample Medium	Type of Document	Description	Relevance to IOGP	Sampling Design	Sampling Methods	Preservation and Storage	Laboratory Procedures	Data Analysis and Reporting
Analytical and methodological development for improved surveillance of the Great Crested Newt, and other pond vertebrates – WC1067	Biggs et al., 2014 (United Kingdom)	Freshwater	Standard	DEFRA published report setting ***field and lab standards*** for using eDNA to report ***Great Crested Newt*** presence/absence in UK freshwater.	Low • Includes sample design good practice for ponds • Very specific to one species • Uses ethanol precipitation which is no longer considered best practice	✓	✓	✓	✓	✓
Environmental DNA Sampling Protocol—Filtering Water to Capture DNA from Aquatic Organisms	U.S. Department of the Interior U.S. Geological Survey, 2014 (United States)	Freshwater	Best practice Guideline	A sampling workflow diagram and three sampling protocols are included as well as a list of suggested supplies	Medium • All areas are relevant to practitioners in the energy industry • Has been updated in more recent guideline documents		✓	✓		
A practical guide to DNA-based methods for biodiversity assessment	Bruce et al., 2021	Freshwater Soils Sediments Biofilm	Best practice Guideline	Summary of the scientific consensus relating to every step of the ***field and laboratory workflows*** involved in the most ***common types of samples*** and analyses.	High • All areas are relevant to practitioners in the energy industry • Universally applicable guidelines • In-depth coverage of sample media and sequencing methods	✓	✓	✓	✓	
Environmental DNA applications in biomonitoring and bioassessment of aquatic ecosystems	Pawlowski, 2020	Freshwater Sediment Biofilm	Best practice Guideline	Provides detailed protocols and best practices for sampling design and ***processing*** of eDNA samples.	Medium • All areas are relevant to practitioners in the energy industry	✓	✓	✓	✓	✓
Review of DNA-based marine benthic monitoring protocols	NatureMetrics, 2022	Marine	Standard protocol/best practice guideline	Standard protocol for ***collection and preservation*** of samples from the ***subtidal benthic*** environment for DNA analysis by Natural England/JNCC.	High • All areas are relevant to practitioners in the energy industry operating in a marine habitat • State of the field in obtaining and processing sediment samples from the seabed	✓	✓	✓		
Environmental Genomics Applications for Environmental Management Activities in the Oil and Gas Industry– White paper for IOGP	Stantec, 2020	Freshwater Marine Soils Sediments	White paper	State of the art review and ***future research needs.***	High • All areas are relevant to practitioners in the energy industry • State of knowledge overview, produced for IOGP	✓	✓	✓	✓	✓
ISO/IEC 17025	ISO	General	Standard	'***Standardisation*** in the field of Biodiversity to develop principles, framework, requirements, guidance and supporting tools in a holistic and global approach for all organizations, to enhance their contribution to Sustainable Development'. It may include ***sections on eDNA*** as a monitoring tool.	Low • Potential relevance for high level consideration of biodiversity. • Low relevance for eDNA work at present.					
Environmental DNA standardisation needs for fish and wildlife population assessments and monitoring	Canadian Standards Association, 2019 (Canada)	General	Common Practice	Based on consultation with practitioners. Describes current ***common practices*** for implementation of eDNA methods, identifies ***knowledge gaps*** and critical considerations during implementation, and evaluates the motivation for standardisation of various aspects of these methods.	Medium State of knowledge Outlines standardisation needs					
Environmental DNA (eDNA) reporting requirements and terminology	Canadian Standards Association, 2021 (CSA W214:21) (Canada)	General	Reporting guidelines	To provide a robust application and interpretation of eDNA assays, laboratories should report on key considerations of the sample collection and laboratory methodology.	Medium • Relevant to data reporting reproducibility • Useful information on laboratory data interpretation	✓			✓	✓

Table B-1 Existing eDNA Standards and Guidelines

Title	Author, Year (Country of Publication)	Sample Medium	Type of Document	Description	Relevance to IOGP	Sampling Design	Sampling Methods	Preservation and Storage	Laboratory Procedures	Data Analysis and Reporting
Performance criteria for the analyses of environmental DNA by targeted quantitative polymerase chain reaction	Canadian Standards Association, 2023 (CSA W219:23) (Canada)	General	Best practice Laboratory Guidelines	Reporting and laboratory sample processing guidelines specifically for qPCR analysis	Low • Relevant for commercial and academic laboratories • Could be useful for understanding qPCR data interpretation and for evaluating laboratory reports				✓	✓
Guidance on the use of targeted environmental DNA (eDNA) analysis for the management of aquatic invasive species and species at risk	Abbott et al., 2021 (Canada)	General	Standard protocol/Best practice Guidelines	Canadian federal Fisheries and Oceans review. Focused on ***qPCR***. Supports the science advice, first step towards providing national guidance on eDNA by promoting more consistent ***reporting and communication*** between eDNA service providers and ***Aquatic Invasive Species (AIS) and Species at Risk (SAR)*** program managers.	Medium • Relevant for invasive species and species at risk considerations	✓	✓	✓	✓	✓
Criteria for depositing eDNA samples and data, including vouchered specimens	Norwegian Environmental Agency (NEA), 2020	General	Reporting guidelines	***Reporting*** data and metadata, and ***storing samples*** from projects where data are generated from environmental DNA analysis.	Medium Relevant to data reporting reproducibility					✓
Quality assurance project plan, eDNA monitoring of bighead and silver carps	U.S. Fish and Wildlife Service, 2022 (United States)	Freshwater	Standards for monitoring program	A guide to ***field and laboratory methods for invasive carp*** eDNA monitoring programs.	Low • Only relevant for invasive carp species	✓	✓	✓	✓	✓
Environmental DNA (eDNA) monitoring and surveillance: field and laboratory standard operating procedures	Aquatic Research and Monitoring Section Ontario Ministry of Natural Resources and Forestry, 2014 (Canada)	Freshwater	Technical report	Focused on ***qPCR***. Describes the ***field and laboratory*** procedures employed and/or developed by the Ontario Ministry of Natural Resources and Forestry (OMNRF) Aquatic Research and Monitoring Section Genetics Laboratory, from field sampling and processing of environmental water samples to testing and validation of DNA surveillance results.	Medium Relevant for a single species approach	✓	✓	✓	✓	✓
Standard Operating Procedure for environmental DNA field sample collection.	Northern Australia Environmental Resources (NAER) Hub, 2021 (Australia)	Freshwater	Standard Operating Procedures	***Standard operating procedure*** that provides a step-by-step guide to a simple eDNA water ***sample collection*** method developed for use by non-scientists.	Low Relevant for sample collection	✓	✓	✓		
Best Management Practice guidelines for salmon farms in the Marlborough Sounds (NZ): Benthic environmental quality standards and monitoring protocol	Benthic Standards Working Group	Marine	Best practice guidelines	Guidance document to inform the development and implementation of ***benthic monitoring*** programmes for ***salmon farms*** in the Marlborough Sounds (NZ). Review of management practices. Contains the benthic standards and monitoring protocol.	Low Requirements for benthic monitoring and management of salmon farms	✓				
Environmental DNA protocol development guide for biomonitoring	De Brauwer et al. 2022a (Australia and New Zealand)	General	Quality control and minimum standard operating procedures for implementing eDNA surveys	Comprehensive guide for the development and use of eDNA/eRNA test protocols, as recommended and curated by experts, stakeholders and end users in Australia and New Zealand	High All areas are relevant to practitioners in the energy industry Universally applicable guidelines In-depth coverage of sample media and sequencing methods	✓	✓	✓	✓	✓
Environmental DNA test validation guidelines	De Brauwer et al. 2022b (Australia and New Zealand)	N/A	Quality control and minimum standard considerations for developing and validating assays	Guidance document to inform the development and implementation of qPCR and metabarcoding eDNA assays.	Low Relevant for molecular testing laboratories Still useful to understand downstream data interpretation				✓	✓

Table B-1 Existing eDNA Standards and Guidelines

Title	Author, Year (Country of Publication)	Sample Medium	Type of Document	Description	Relevance to IOGP	Sampling Design	Sampling Methods	Preservation and Storage	Laboratory Procedures	Data Analysis and Reporting
Environmental DNA (eDNA) Best Management Practices for Project Planning, Deployment, and Application	USFWS 2023 (United States)	Freshwater	Best practice guidelines and Standard Operating Procedures	Guidance document on each step of the eDNA survey process within a freshwater ecosystem.	High All areas are relevant to practitioners in the energy industry Universally applicable guidelines In-depth coverage of survey design and sampling collection	✓	✓	✓	✓	✓

Note: For each table entry the relevance to IOGP has been qualified either Low, Medium, or High, based on the topics covered in the document. Qualification assignments are based on criteria such as whether the document covers a broad spectrum of eDNA applications, as opposed to being focused on (local) single species detection, and whether the document include specific sections on sampling design, and field, laboratory, and analysis methods in addition to a review of current knowledge.

Table B-2 eDNA Academic Review Papers

Title	Author, Year	Description	Relevance to IOGP members	Sampling Design	Sampling Methods	Preservation and Storage	Laboratory Procedures	Data Analysis and Reporting
Scaling up: A guide to high-throughput genomic approaches for biodiversity analysis	Porter & Hajibabaei, 2018	Presents the **most common and emerging DNA-based methods** used to generate data for biodiversity and biomonitoring studies.	Medium Considerations for producing DNA-based indicators Guide to methods used in biodiversity genomics focused on upscaling				✓	✓
Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA	Ruppert et al., 2019	**Review of the basic methodology, benefits, and concerns** of eDNA metabarcoding. Future applications of the method as well as expected technological advances.	Medium Covers the applications of eDNA methods in global ecology Overview of current and future applications					
Environmental DNA metabarcoding: Transforming how we survey animal and plant communities	Deiner et al., 2017	**Review of the use of eDNA metabarcoding** for surveying animal and plant richness, and the challenges relating to the estimation of relative abundance.	Medium Ability of different eDNA sample types to approximate richness in space and across time Guiding questions for study design	✓				✓
Critical considerations for the application of environmental DNA methods to detect aquatic species	Goldberg et al., 2016	Set of **guidelines and considerations** for implementing eDNA methods to detect aquatic macroorganisms.	Medium Synthesis of knowledge on eDNA applications	✓		✓	✓	✓

Note: For each table entry the relevance to IOGP members has been qualified either Low, Medium, or High, based on the topics covered in the document. Qualification assignments are based on criteria such as whether the document covers a broad spectrum of eDNA applications, as opposed to being focused on (local) single species detection, and whether the document include specific sections on sampling design, and field, laboratory, and analysis methods in addition to a review of current knowledge.

B.4 Summary Of Emerging Standards and Other Developments

Leading examples of national and international application of eDNA to environmental monitoring and the organizations who are driving them are set out below based primarily on Morisette et al. (2021). Broadly speaking, eDNA "knowledge hubs" can be found in Europe, North/South America, and Asia/Oceania. Organizations in these regions are developing standards and guidelines in "real time". However, there are other countries who have emerging efforts in this field who are also noted below. Practitioners in the energy industry are advised to check for updates from the below organizations or initiatives in the region in which they and their projects are situated.

B.4.1 Europe

The main recent developments or organizations working in the field of eDNA standardisation in Europe are listed below.

- The international working group EU COST Action DNAqua-Net (Leese et al., 2018) aims to incorporate molecular monitoring tools for Biological Quality Elements (e.g., fish, macroinvertebrates and phytoplankton-benthos) into the Water Framework Directive (WFD, 2000/60/EC) and the Marine Strategy Framework Directive (MSFD, 2008/56/EC). The recent Bruce et al. (2021) publication described in Table B-1 is a result of the work of this group and is highly relevant to practitioners in the energy industry.
- A formal working group within the European Committee for Standardization (CEN) (CEN/TC230/WG28) in collaboration with DNAqua-Net and ECOSTAT has published methods for sampling of benthic diatoms for metabarcoding to examine water quality (CEN/TR 17245:2018). A publication covering sampling, capture and preservation of eDNA from water is in progress (prEN 17805). Each of these products are highly relevant to practitioners in the energy industry.

Key regulators and governmental bodies are:

- The Offshore Petroleum Regulator for Environment and Decommissioning (OPRED) in the UK is responsible for regulating offshore O&G industries to ensure sustainable development.
- OPRED enforces compliance with regulatory requirements and OSPAR recommendations during environmental assessment and monitoring.
- Any environmental monitoring method used to provide data to support an EIA application must provide a basis for assessing the degree of potential impacts while understanding any potential data limitations. As practice, OPRED does not stipulate

what environmental monitoring methods should be undertaken in support of EIA applications, but methods must be relevant to what is being assessed and an application is considered on a case-by-case basis, considering the quality of and limitations of data used to support such an application. As things currently stand OPRED has no intention of developing guidance on the use of environmental genomics or encouraging proponents in its use (Stantec, 2020).

- OSPAR is the legislative body regulating international cooperation on environmental protection in the North-East Atlantic. The OSPAR Convention, includes non-polluting human activities that can adversely affect the sea, focusing on biodiversity and ecosystems (OSPAR Commission, 2020). This organization has monitored progress in eDNA research considering invasive species present in ballast water but has not gone beyond.

The DEFRA Centre of Excellence for DNA Methods was launched in the UK in 2020, which seeks progress on implementation of eDNA approaches to achieve various government priorities. That group is aligned with both the recently established Scottish DNA hub and the UK DNA network. As noted in Table B-1, DEFRA has developed eDNA standards which may be of some relevance to practitioners in the energy industry.

- The Norwegian Environmental Agency (NEA) is responsible for ensuring that water in marine areas is of such a quality that it both preserves species and ecosystems and promotes human health and well-being.
- The 2015 NEA environmental monitoring guidelines do not discuss the use of DNA-based approaches other than to assess DNA damage to fish, but the government does fund genomic research to determine its potential for future monitoring programs.
- NEA commissioned the NTNU Science Museum, as head of the Norwegian Barcode of Life network, to review standardisation criteria for the use of DNA barcoding and environmental DNA. The report[4] with the English title “Criteria for depositing eDNA samples and data, including vouchered specimens”, aims to ensure the necessary reproducibility when reporting data and metadata, as well as storing samples from projects where data are generated from environmental DNA analysis. While the document does not address technical requirements for the design of studies or technical details around laboratory protocols and analysis, it may be moderately

[4] https://www.miljodirektoratet.no/publikasjoner/2020/mars-2020/kriterier-for-lagring-av-miljo-dna-prover-og-data-herunder-henvisning-til-referansemateriale/

relevant to practitioners in the energy industry. This report involves setting requirements for:

- An agreed minimum standard for reference to and use of publicly available reference material
- A common platform for reporting and making publicly available DNA sequences and associated metadata
- Retention of collected samples, which are not analysed immediately, over a long period of time to ensure that these are available for future research and management, regardless of the original contracting institution.

B.4.2 North America

The main recent developments in the field of eDNA standardisation in North and South America are listed below.

- The Canadian Standards Association (CSA) Group is a global not-for-profit organization dedicated to standards development and in testing, inspection and certification around the world including Canada, the U.S., Europe and Asia.
- The CSA Group has recently initiated a project to develop a National Standard of Canada related to eDNA survey data and interpretations with the goal of enhancing regulator and public confidence.
    - The proposed standard aims to define consistent eDNA use across applications and sectors. The initial phase consisted of a research paper (CSA Group, 2019) which examined eDNA implementation methods through a lens of potential standardisation.
    - In the second project phase, technical experts from academic, government and industry drafted a standard on eDNA reporting requirements and terminology requirements (CSA W214:21; CSA, 2021) but did not include requirements for conducting eDNA analysis. The standard defines minimum requirements for the reporting of methods, data, and results, including possible sources of errors.
- The Canadian Standards Association (CSA) have also already set or are actively developing standards for certain other aspects of DNA-based methods:
    - Soil quality – Direct extraction of soil DNA (ISO 11063:2020)
    - Soil quality – Estimation of abundance of selected microbial gene sequences by quantitative PCR from DNA directly extracted from soil
    - Genomics informatics – Quality control metrics for DNA sequencing (ISO/TS 22692:2020)

- Each of these products are moderately to highly relevant to practitioners in the energy industry. The United States National Environmental Policy Act (NEPA) promotes the enhancement of the environment and requires federal and independent agencies, such as Bureau of Ocean Energy Management (BOEM) and the Environmental Protection Agency (EPA), among others, to manage national resources and assess environmental effects of proposed projects. Examples of US American initiatives for genomics methods standardisation are:
- The California Water Quality Monitoring Council's Molecular Methods Workgroup whose objective is to guide adoption of environmental genomics via consensus building between researchers and stakeholders, and the standardisation of molecular methods for biomonitoring applications5. A lack of standardised approaches for DNA-based environmental monitoring has impeded the adoption of these methods for regulatory application. Therefore, the end goal of the working group is to create guidance documentation for use of environmental genomics.

Key regulators and governmental bodies are listed below.

- Canada's Department of Fisheries and Oceans (DFO) has promoted several eDNA research projects, frequently funded by the Genomics Research and Development Initiative (GRDI), such as the following:
- A publication on monitoring aquatic invasive species as well as species at risk, marine protected areas, fisheries, aquaculture, and biodiversity assessments. (Baillie et al. 2019).
- The sponsorship of the National eDNA Technical Working Group and two national eDNA workshops.
- Developing science advice for aquatic invasive species and species at risk (initiated by the Canadian Federal and Provincial National Aquatic Invasive Species Committee) on minimum reporting standards, terminology, and guidance on interpretation of data (Abbott et al., 2021).
- Engaging on eDNA standardisation and technical development with academic and industry partners through the Pathway to Increase Standards and Competency of eDNA Surveys (PISCeS) semi-annual conference and working group (Loeza-Quintana et al., 2020).

[5] https://github.com/stheroux/MMWG/

- In the US, the outcomes of the 1st iteration of the US National Conference on Marine eDNA[6] laid the groundwork for this planned regulatory engagement. The participants pushed for dialogue on the impact of marine eDNA becoming established as a credible ecosystem census indicator on existing statutes, regulations, and permitting/licensing processes. Examples include:
- NEPA, Magnuson-Stevens Fishery Conservation and Management Act
- Marine Mammal Protection Act, Endangered Species Act
- Recently, the Second US National Conference on Marine eDNA gathered over 300 academics as well as research scientists from state and federal agencies with the aim to identify the necessary next steps to move eDNA methods into the management application mainstream (Stepien et al., 2022). The consensus among participants was that communication between scientists and resource managers remained the largest barrier to broader adoption. While scientists continue to voice caution related to method limitations, resource managers communicated the need for pathways/timelines for method adoption, including standard operating procedures, lab accreditation, and unified sequence libraries. Similar sentiments were also communicated during the 6th annual Environmental DNA (eDNA) Technical Exchange Workshop (Stepien et al. 2023) which concluded that future regulatory research directions should include studies of fate and transport, autonomous sampling/sample processing, and reference library curation.
- The ISO/IEC 17025 has been developed for laboratories to demonstrate they can operate competently and generate valid results, which has been applied to qPCR eDNA analyses, although it was not specifically designed for eDNA analysis. This standard has a low direct relevance to practitioners in the energy industry.

B.4.3 Asia/Oceania

The main recent developments in the field of eDNA standardisation in Asia/Oceania are listed below.

- In New Zealand, standardisation of sampling protocols and sample curation, which has been driven by the oil & gas and energy sectors, are being prioritised.
- The Cawthron Institute completed a long-term study to assess metabarcoding approaches for monitoring the integrity of marine benthic environments. The outcome was a fully standardised protocol, which will be applied unchanged during a 'phase-in' period of up

[6] https://phe.rockefeller.edu/eDNAmarine2018/docs/MURU_eDNA_Conference_final_report.pdf

to five years as part of regular compliance monitoring. This protocol is highly relevant to practitioners in the energy industry.

- The Cawthron Institute also produced and published a molecular-based tool for assessing benthic impacts associated with salmon farming practices in New Zealand. The document contains industry and regulatory guidance on eDNA for aquaculture, and the monitoring of sediment enrichment, which is very relevant to the energy sector as the method is also applicable to other sources of sediment enrichment.

Key regulators and governmental bodies are:

- In Australia, the National Offshore Petroleum Safety and Environmental Management Authority (NOPSEMA) is the offshore energy regulator for health and safety, environmental management for activities in Commonwealth waters.
- NOPSEMA encourages industry to explore standards they can define for environmental genomics and would consider use of approaches/methodologies if these standards are more broadly accepted, including in other jurisdictions (Stantec, 2020).
- With respect to environmental genomics, no approach or method is prescribed. Any industry proposal, whatever the method, needs to demonstrate it is appropriate to ensure that environmental impacts and risks will be managed to an acceptable level and reduced to as low as reasonably practicable.
- NOPSEMA recognises there are certain benefits to standardisation of any methods and specifically highlighted that standardisation could even facilitate comparison of multiple datasets across jurisdictions.
- NOPSEMA has noted if research is performed on how to apply standardisation in environmental genomics, they would be keen to provide some assistance in identifying the types of environmental management outcomes (Stantec, 2020). They state that any advances that could decrease project EIA and regulatory uncertainty has potential to increase efficiency and minimise delays in approval timelines.

New Zealand's petroleum regulators (different responsibilities and areas of expertise) include:

- New Zealand Petroleum and Minerals (NZP&M), Territorial Authorities
- Environmental Protection Authority
- Department of Conservation (DoC)
- Maritime New Zealand (MNZ)
- WorkSafe New Zealand (WorkSafe)

Japanese researchers established the eDNA Society in 2018 (Minamoto et al., 2020). A key product of the society has been a standardised protocol[7] for eDNA sampling, analysis, and interpretation which has already been implemented for several academic and government projects within the country. This protocol is highly relevant to practitioners in the energy industry.

B.4.4 Other Countries

Initial developments towards eDNA standardisation in other countries are listed below.

- In Brazil, Environmental Impact Assessment Studies (EIA) and Environmental Impact Reports (RIMA) are reviewed by the Brazilian Institute of the Environment and Renewable Natural Resources (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis [IBAMA]) (Petrobras 2020). IBAMA is linked to the Ministry of the Environment as an agency of the federal government and is responsible for issuing environmental licenses, controlling the quality of the environment, and overseeing the use of natural resources.
- Stantec (2020) discussions with IBAMA noted only one project involving environmental genomics had been proposed for a permit application for O&G drilling in a specific region. IBAMA indicated to the proponent that they should utilise metabarcoding to characterise a large marine area containing coral reef fauna, deep water corals, and other sensitive benthic habitats; however, they did not make and recommendations on specific genomic methodology.
- The Environmental Protection Agency (EPA) of Guyana was legally established by the *Environmental Protection Act* in 1996, tasked with the responsibility to manage, conserve, protect and improve environment. The EPA Oil and Gas Unit, formed in August 2019, is responsible for authorizing, monitoring, and managing projects in the O&G sector.

Stantec (2020) discussions with EPA representatives indicated that while they do not have much regulatory experience with eDNA, they see value for its use in baseline survey validations. Regulators feel actual biodiversity is being underreported by conventional survey techniques and thereby undermining management objectives.

- Esso Exploration and Production Guyana Limited (EEPGL) submitted an EIA in 2019 which utilised water and sediment eDNA samples collected in 2016 and 2017 for baseline characterisation of marine habitats for the Liza Phase 2 Project. While not directly related

[7] https://ednasociety.org/

to standardisation, this study demonstrates the utility of using robust, validated approaches to quantifying biodiversity, such as eDNA in frontier O&G jurisdictions.

B.4.5 Intergovernmental

The North Pacific Marine Science Organization (PICES) (countries include Canada, Japan, China, the Republic of Korea, Russia, and United States) through the Advisory Panel on Marine Non-Indigenous Species (AP-NIS) is scheduled to host a workshop related to discussing this topic amongst the "North Pacific" countries at an upcoming meeting[8].

Arctic countries (countries include Canada, Finland, Iceland, Norway, Sweden, Russia, and the United States) have developed the Arctic Invasive Alien Species (ARIAS) Strategy and Action Plan and through its implementation are now leveraging eDNA tools to monitor invasive species[9].

Appendix B References

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[8] https://meetings.pices.int/members/advisory-panels/AP-NIS https://meetings.pices.int/

[9] https://www.caff.is/strategies-series/415-arctic-invasive-alien-species-strategy-and-action-plan#:~:text=The%20Arctic%20Invasive%20Alien%20Species,impacts%20of%20invasive%20alien%20species

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Appendix C Occupancy Models

eDNA surveys typically involve collection of multiple samples per site location (e.g., environmental replicates) and laboratory analysis includes subsampling of the eDNA extract from each individual sample (e.g., qPCR technical replicates). Therefore, eDNA surveys typically include three nested levels of sampling (Figure C.1):

- Locations or sites (primary sample units) within a study area,
- Environmental samples (secondary sample units) collected form each location, and
- Subsamples (replicate observations – qPCR) taken within each environmental sample

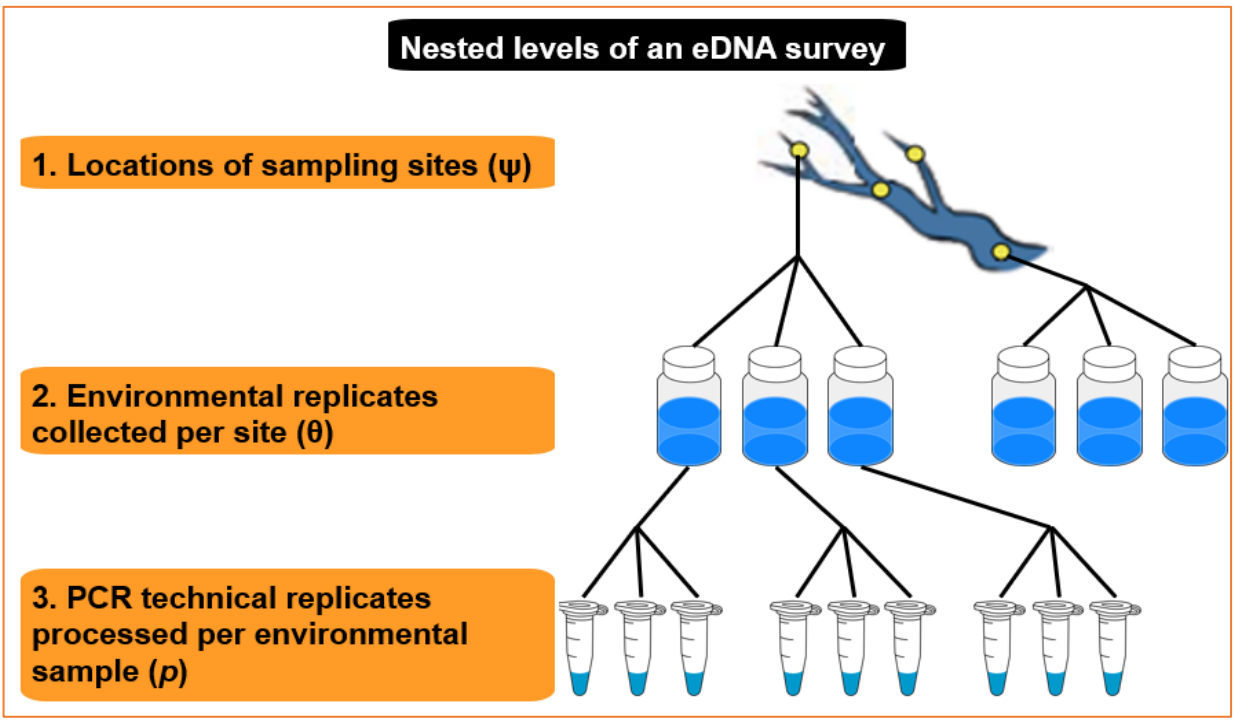

Figure C.1 Diagram depicting the nested levels typically implemented in an eDNA survey. The first level indicates sampling locations or sites where eDNA is collected. The second level indicates multiple environmental replicates collected per site. The third level indicates multiple PCR replicates processed per environmental sample collected.

Therefore, a multiscale occupancy model can be implemented to estimate (Figure C.2):

1. the probability of target species occurrence at the location (ψ, *psi*),
2. the conditional probability of target eDNA occurrence in an environmental sample given that the target species is present at that location (ϑ, *theta*), and
3. the conditional probability of positive detection in a qPCR replicate given that the target eDNA is present in the environmental sample (p).

The simplest model for this multiscale occupancy ($\psi(.)$, $\vartheta(.)$, $p(.)$) estimates the mean probability the species is present across any of the sampling locations (ψ), the mean probability target eDNA was collected in a sample if the target species was present (ϑ), and the mean probability eDNA was detected in a qPCR replicate if the target eDNA was collected (p).

- Using the equation $1-(1-\vartheta)^n \geq 0.95$, where ϑ is the probability of eDNA occurrence and n is the number of water samples, the number of water samples required to surpass 95% probability of successful eDNA collection can be calculated,
- Using the equation $1-(1-p)^n \geq 0.95$, where p is the probability of qPCR detection and n is the number of qPCR replicates, the number of qPCR replicates required to surpass 95% probability of detection within a water sample can be calculated.

Both the probability of eDNA collection (ϑ) and the probability of qPCR detection (p) will influence the cumulative probability of eDNA detection (Figure C.2). For example, a qPCR cannot detect an eDNA molecule that was never successfully collected, no matter how many qPCR replicates are performed. Collecting more eDNA samples can improve the probability of eDNA collection and performing more qPCR analysis can increase the probability of eDNA detection within a qPCR. A pilot study performed at sites of known species occurrence can help to evaluate what level of effort (both environmental and qPCR replicates) is required to confidently conclude presence or probable absence from an eDNA survey.

A species may have a low probability of eDNA occurrence in a water sample (ϑ) if it is rare, displays a low eDNA shed rate, occurs in a habitat with large water volume (causing eDNA dilution effects), or if samples aren't collected near individuals (e.g., surface water samples collected targeting a benthic organism). Furthermore, differences in environmental covariates across sampling sites are likely to influence the probability of eDNA occurrence in a water sample (ϑ), for example turbidity (reducing the amount volume filtered), water discharge/flow, river width or depth, and many more.

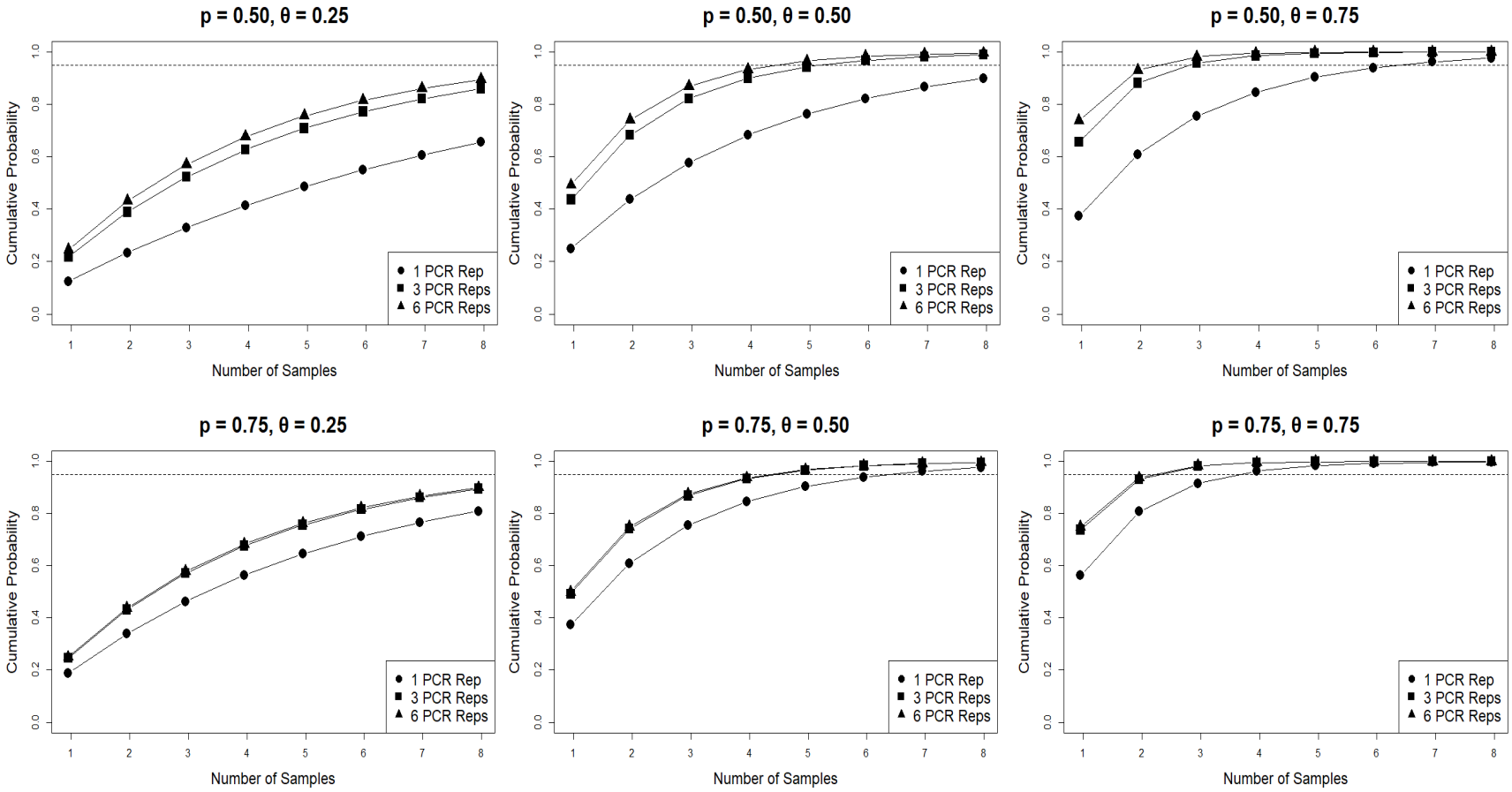

Figure C.2 Changes to the cumulative probability of eDNA detection based on the number of environmental samples collected. Each plot displays differences across an increasing number of PCR replicates (1, 3, or 6). The top three plots display probabilities based on a PCR detection of p = 0.50 and the bottom three display probabilities based on PCR detection of p = 0.75. Plots from left to right indicate cumulative probabilities based on increasing probability of eDNA collection (ϑ = 0.25, 0.50, or 0.75). Dashed line indicates a 95% probability of eDNA detection.

Through occupancy models, the ϑ and p probabilities can be estimated, and the sampling design can be evaluated to ensure sufficient sampling to reach a target probability of species detection. This can provide confidence to an eDNA study program. Let's take an example where the probability of eDNA collection within a sample (ϑ) is 0.50 and the probability of eDNA detection with a PCR replicate (p) is 0.75 (Figure C.2). With these parameters, we can see how changing the number of samples collected (the x-axis of Figure C.2) or changing the number of PCR replicates (the three curves in Figure C.2) impact the cumulative probability of detection. In this scenario, it is required to collect five eDNA samples and analyze at least three PCR replicates to achieve a >95% probability of detection (dashed line in Figure C.3).

Several software packages have been developed for occupancy modeling analysis of eDNA data:

- eDNAoccupancy R package (Dorazio & Erickson, 2017) – most commonly used,
- msocc R package (Stratton et al., 2020),
- https://seak.shinyapps.io/eDNA/ (Griffin et al., 2020) – recently developed to incorporate probability of false positives.

These programs typically perform a Bayesian multiscale occupancy model to estimate posterior summaries of occurrence and detection probabilities. Furthermore, a user can evaluate how occurrence and detection probabilities are impacted from environmental and sampling covariates. This allows users to evaluate if certain environmental or sampling covariates are impacting the probability of species presence (ψ) and/or the probability of successful detection (θ and p). This information can help to both understand what factors impact the distribution of a species across sites/locations, and what factors impact the successful collection and detection of eDNA for the target species. Furthermore, analysis of occupancy models can provide estimates of probability of collection within a water sample (θ) and probability of detection within a qPCR replicate (p), to inform if appropriate sampling effort was undertaken. It is important to collect the necessary environmental metadata during sampling that an end user wants to evaluate in an occupancy model.

Dorazio, R. M., & Erickson, R. A. 2018. ednaoccupancy: An r package for multiscale occupancy modelling of environmental DNA data. *Molecular Ecology Resources,* **18**(2), 368-380. **https://doi.org/10.1111/1755-0998.12735**

Stratton, C., Sepulveda, A. J., & Hoegh, A. 2020. msocc: Fit and analyse computationally efficient multi-scale occupancy models in R. *Methods in Ecology and Evolution,* **11**(9), 1113-1120. **https://doi.org/10.1111/2041-210X.13442**

Griffin, J. E., Matechou, E., Buxton, A. S., Bormpoudakis, D., & Griffiths, R. A. (2020). Modelling environmental DNA data; Bayesian variable selection accounting for false positive and false negative errors. *Journal of the Royal Statistical Society Series C: Applied Statistics,* **69**(2), 377-392. **https://doi.org/10.1111/rssc.12390**