Long-read sequencing for biodiversity analyses - a comprehensive guide

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Abstract

1. DNA-based monitoring of biodiversity has revolutionised our ability to describe communities and rapidly assess anthropogenic impacts on biodiversity. Currently established molecular methods for biomonitoring rely heavily on classic metabarcoding utilising short reads, mostly through Illumina data. However, increasingly more studies use long-read sequencing technologies, such as Oxford Nanopore Technologies (ONT) and Pacific Biosciences (PacBio), for analyses of environmental DNA (eDNA) and DNA barcoding. These long-read sequencing approaches can be advantageous over existing methods by providing increased information content and opening new avenues for understanding biodiversity at a larger scale.
2. In this review we provide a comprehensive overview of all studies to date using long-read sequencing platforms for biodiversity analyses of eukaryotes from eDNA and community metabarcoding, and DNA barcoding. We also give detailed information on each step required for sample processing, data generation and analysis of long-read sequencing datasets for biodiversity applications. Even though the number of studies using long-read sequencing technologies is rapidly increasing, clear established guidelines for sample preparation or analysis of such datasets are lacking. Furthermore, there is no existing overview of eukaryote monitoring applications for both ONT and PacBio technologies across different sample types.
3. Long-read sequencing platforms provide possibilities for metabarcoding (PCR-based) of both short and long fragments, shotgun sequencing (PCR-free), or DNA barcoding. ONT platforms in particular can also allow real time data acquisition while the portability of the MinION instrument can support sequencing in the field. PacBio on the other hand can provide highly accurate reads and can be used to reliably address open questions in ecology and evolution for difficult to characterise taxa such as microbial eukaryotes. Streamlining the use of these technologies could enable sequencing of whole organelles or population level assessments from environmental data, which would be a step-change for DNA based monitoring.
4. Here we review current applications, applied methodology, and future perspectives in the field. Overall we aim to facilitate the use of long-read sequencing technologies by the wider community, promote best practices for data generation and standardised data reporting that supports reproducibility, and to encourage open data policies and tools.

Keywords: biodiversity monitoring, DNA barcoding, eDNA, long-read sequencing, metabarcoding, ONT, PacBio, shotgun

1. Introduction

Traditional methods for biodiversity assessment are based on taxonomic identification of species, and can require highly specialized taxonomic expertise while they are generally low in throughput, costly, and potentially invasive (Deiner, Bik, et al., 2017; Ruppert et al., 2019). A global lack of taxonomic expertise and the need to increase throughput has led to the development of DNA-based methods for biodiversity assessment over the last 10-15 years (Kelly et al., 2014; Thomsen et al., 2012). These methods enable novel, non-invasive, and cost-effective biomonitoring solutions which can be performed at a larger scale and even by non-specialised personnel (Taberlet et al., 2012). This DNA revolution has been built on metabarcoding, which refers to high throughput sequencing of PCR amplified gene fragments of DNA derived from environmental (eDNA) or community samples (Alberdi et al., 2017; Deiner, Bik, et al., 2017). The most commonly used sequencing type for metabarcoding is short-read sequencing like Illumina (NovaSeq or MiSeq). This platform has been the golden standard due to its high efficiency, low error rate, and cost effectiveness, though it is limited to a maximum amplicon length of ~550 bp (Deiner, Bik, et al., 2017). Targeting short regions provides insufficient information content for taxon identification, especially for closely related species, while also reducing the number of available primer binding sites hence making primer selection more difficult (Egeter et al., 2022).

Increasingly more studies use long-read sequencing as an alternative approach for classic metabarcoding, through Oxford Nanopore Technologies (ONT) or Pacific Biosciences (PacBio) sequencers. These two sequencing platforms are able to perform single-molecule real-time sequencing (MacKenzie & Argyropoulos, 2023; Rhoads & Au, 2015). They also provide options for longer insert sequences compared to Illumina as they are not limited to specific read lengths, or could be used in the field as is the case for the instrument MinION from ONT. Published literature of ONT or PacBio based biodiversity analyses, including applications and tools, has been rapidly growing especially over the last two-three years (e.g. (Doorenspleet, Jansen, et al., 2025; Jamy et al., 2022; Maggini et al., 2024; Truelove et al., 2019)) (Table 1). Although long-read sequencing is not yet routinely used in biodiversity analysis, it is becoming more common and it has already been tested in different settings such as aquatic ecosystems, soil or air samples, fungal profiling and more (e.g. (Doorenspleet, Jansen, et al., 2025; Heeger et al., 2018; Truelove et al., 2019; Urban et al., 2023)).

DNA barcoding was introduced in the early 2000's, promoting sequencing of standardized DNA regions from individual specimens which could be used as species identifiers (Hebert et al., 2003). Initial target of DNA Barcoding was the Cytochrome C oxidase subunit I (COI) mtDNA marker (~650 bp). Other markers were added later on to improve detection of different target groups (Deagle et al., 2014), including other mitochondrial and ribosomal genes such as the 12S, 16S, and ITS regions (Andújar et al., 2018; Miya et al., 2015; Tedersoo et al., 2022). eDNA metabarcoding enables the simultaneous sequencing of DNA from many species present in environmental samples such as water or soil, and can provide a broader understanding of the composition of whole communities, or specific taxa (detection of invasive or rare species) (Deiner, Bik, et al., 2017; Ruppert et al., 2019; Skelton et al., 2022). Metabarcoding has relied heavily on DNA barcoding which has been the backbone of further advances in environmental sequencing by initiating the movement of large scale sequencing for biodiversity studies and also populating barcode reference databases. Similarly, the marker genes

most commonly used for metabarcoding follow previous developments in DNA barcoding (Andújar et al., 2018).

The use of longer and more informative metabarcoding amplicons with ONT and PacBio may enhance the taxonomic resolution by covering larger fragments or multiple marker genes. This could help improve phylogenetic resolution and characterisation of unknown species in complex communities (Doorenspleet, Jansen, et al., 2025). A known drawback of ONT and a potential reason why it has not been so widely applied yet, is its high error rate (95% MinION). However, recent improvements of ONT's sequencing kits and new bioinformatic tools have significantly enhanced accuracy. Additionally, sequencing costs can be equivalent or cheaper with MinION though other issues need to be taken into consideration (flow cell type, kit type, and run time). The pricing per sample may vary for other instruments and also the number of samples that can be multiplexed (Baloğlu et al., 2021; Egeter et al., 2022). Furthermore, ONT offers the MinION, a small and portable device which can be used in the field, supporting real time data generation for biodiversity monitoring studies (Krehenwinkel, Pomerantz, & Prost, 2019; Maestri et al., 2019). PacBio amplicon sequencing, which has also been more popular for whole genome sequencing, is also implemented for amplicon analysis of environmental samples and for DNA barcoding. Most applications of amplicon PacBio to date are targeting fungi or microbial eukaryotes (e.g.(Gueidan & Li, 2022; Jamy et al., 2022; Runnel et al., 2022). In contrast to ONT, the PacBio platform offers highly accurate reads with very low error rates, while also enabling long amplicon sizes (Kucuk et al., 2023).

Here we provide a complete overview of the current applications of long-read sequencing in biodiversity studies for both ONT and PacBio sequencing technologies. We discuss the areas of DNA barcoding of individual specimens, PCR-based (metabarcoding) and PCR-free (shotgun) sequencing applications from eDNA and community samples for analysis of eukaryotes. We give an in-depth overview of existing literature and also provide a comprehensive guide for sample preparation from DNA extraction and library preparation, to sequencing and bioinformatic analysis for long-read biodiversity analyses. With an interest in promoting standardisation of methodology and reproducibility for future studies we also discuss open data and best practices and provide future perspectives on the use of long-read technologies for biodiversity analyses. Ultimately, this review aims to facilitate new users entering the field, and experienced users to fine-tune their work and encourage further development in this field.

2. Nanopore sequencing

The concept of nanopore sequencing was developed in the 1990s, but it became generally available as a sequencing instrument after 2014, with the release of the portable MinION by Oxford Nanopore Technologies (ONT) (Branton et al., 2008; MacKenzie & Argyropoulos, 2023). ONT allows real-time single-molecule sequencing of short to ultra-long reads, ranging from 50 bp to >4 Mb. The platform uses a nanoscale protein pore known as the "nanopore" (MacKenzie & Argyropoulos, 2023), which enables the recognition of individual nucleotides through the detection of electrical signals which are emitted as the molecule moves through the nanopore (Fig. 1) (Wang et al., 2021). As the DNA molecule passes through the pore, each nucleotide base causes a characteristic current disruption which is computationally translated by a basecalling algorithm (Jain et al., 2016). The translocation movement of the DNA molecule and its speed are controlled by a motor protein which is attached at

the 5' end of the template strand during library preparation (Deamer et al., 2016; Wang et al., 2021) (Fig. 1). Initial ONT releases suffered very low accuracy levels, with the first R6 nanopores performing at an accuracy level of 60% (Rang et al., 2018). Newer releases have upgraded chemistries, different types of nanopores and motor proteins, and improved accuracy (Rang et al., 2018), which for the latest R10 nanopores reach 99% accuracy and 400-450 bases/sec translocation rate.

Existing ONT instruments include the MinION, GridION, and PromethION (Fig. 1), which vary in capabilities and throughput. The MinION is the standard ONT flow cell (2,048 nanopores), which can be used individually with the portable MinION sequencing device. The GridION is a benchtop device suitable for medium-scale projects that can accommodate up to 5 parallel runs with MinION flow cells. The PromethION flow cells (10,700 nanopores) can be run in parallel on the same device in 24 or 48 modules, making this instrument suitable for larger projects. Finally, the Flongle is a smaller flow cell (126 nanopores), which can be adapted to use with either the MinION or the GridION, providing a faster and cost-efficient solution for a low number of samples. The amount of data generated by a flow cell during a run depends on the number of active pores, and duration of the run, while the translocation rate can also affect the overall yield. The maximum estimated output of any ONT platform is 100-200 Gb (PromethION) (https://nanoporetech.com/).

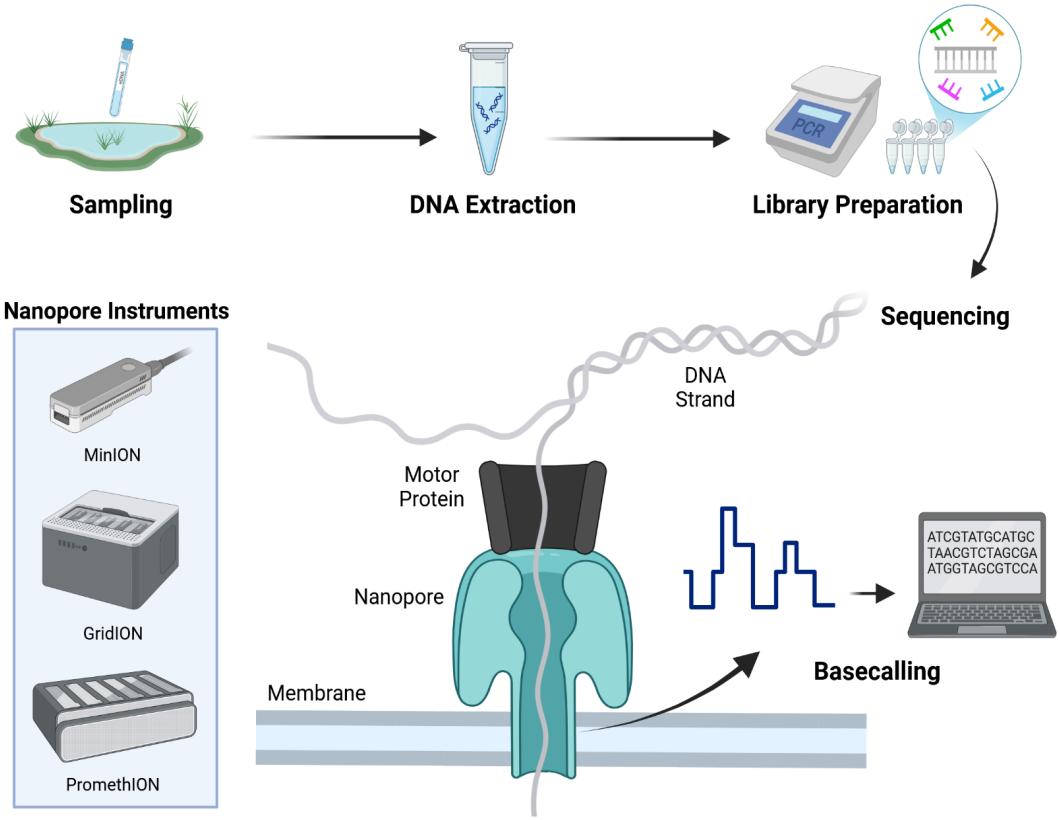

Figure 1
Sample collection and processing, followed by sequencing with ONT. Bottom center: structure of the nanopore and movement of the DNA strand through the pore leading to data generation. Box: currently available ONT instruments (Image generated with BioRender).

3. Applications of ONT for biodiversity analysis

Studies utilising ONT sequencing for analysis of eukaryotic biodiversity have been rapidly increasing over the last few years. Here we provide an overview of studies using ONT sequencing to perform DNA barcoding of individual specimens, analysis of eDNA or communities of eukaryotes, and some examples of microbial studies with biodiversity implications.

a. DNA Barcoding

ONT sequencing for biodiversity analysis was first used for DNA barcoding of individual specimens (e.g.(Menegon et al., 2017; Pomerantz et al., 2018)), especially taking advantage of the portability of the MinION, but quickly expanded to other applications. Sanger-based barcoding is costly per sample and individualized specimen treatment makes sequencing preparation time consuming (Hebert et al., 2018; Srivathsan & Meier, 2024). ONT can be used to increase the throughput of DNA barcoding pipelines and dramatically accelerate the population of reference databases, which is a long-wanted resolution for the barcoding community. Furthermore, high multiplexing capacity means reduced cost per barcode (Hebert et al., 2025; Maestri et al., 2019; Pomerantz et al., 2018). Benchmarking of ONT vs Sanger generated barcodes demonstrated the same levels of accuracy (Koblmüller et al., 2024), while library preparation for ONT (vs Sanger and PacBio) was also shown to be less time consuming overall (Cuber et al., 2023).

Due to Illumina read-length limitations, direct comparisons between ONT and Illumina obtained barcodes are not very common. However, Srivathsan et al. (2019) sequenced ONT barcodes for more than 7,000 Dipteran specimens yielding 99.99% accuracy compared to the Illumina data. In this case, comparison was only possible for a 313 bp subset of the full-length barcode that corresponded to the available length of the Illumina amplicon. A further advantage of using ONT barcoding is its high multiplexing capacity, which was recently demonstrated with the generation of barcodes for >100,000 insect specimens in a single run (Hebert et al., 2025).

Considerable development has been made to enable ONT field-based applications. Seah et al. (2020) tested field-friendly DNA barcoding methods in different preserved and non-invasive wildlife samples (scat, hair, feathers). Also Vasilita et al. (2024), evaluated express protocols for species-level sorting of insects, obtaining 250 barcodes in ~6 hours, with minimal laboratory equipment. Studies which performed ONT sequencing in the field include work on rainforest and national parks. For example Pomerantz et al. (2018) tested the feasibility of a portable laboratory for real-time barcoding of reptiles in the Ecuadorian rainforest, whilst Maestri et al. (2019) implemented in situ DNA extraction and barcoding for frog and beetle species in the Temburong National Park, in Borneo.

Long ONT barcode protocols have been developed aiming to improve downstream phylogenetic analysis. For example, sequencing of the full length ribosomal cluster (~4,000 bp) enabled the identification of arthropods with high phylogenetic resolution (Krehenwinkel, Pomerantz, Henderson, et al., 2019). Similarly, Gajski et al. (2024) sequenced large fractions of mtDNA and nuclear genes (COI, Cytb, 18S-28S) of arthropods with a multiplex PCR barcoding approach, which corresponded to more than 6,000 bp of data per specimen. This approach was considerably more efficient, producing much more sequencing data per specimen than combining individual Sanger-sequenced barcodes.

b. PCR-based eDNA and community sample sequencing

Classic metabarcoding analysis focuses on short fragments of DNA, restricted by read length limitations of Illumina platforms (e.g. <500 bp), while with eDNA samples there is the additional issue of DNA fragmentation due to degradation (Deiner, Bik, et al., 2017). Nevertheless, sequencing of longer than the typical short fragments and full fish mitogenomes with long-range PCR have been done from aquatic eDNA (Bista et al., 2017; Deiner, Renshaw, et al., 2017). Furthermore, longer fragments are highly abundant in community samples (Baloğlu et al., 2021). Targeting longer fragments with long-read technologies could increase information content of the data and improve taxonomic and phylogenetic resolution.

Community sample sequencing with ONT has been performed in various settings from either mock (e.g. for validation purposes (Maggini et al., 2024)) or natural communities. In Srivathsan et al. (2021) arthropod communities sequenced through amplicon pools were used to demonstrate ONT scalability and cost-efficiency, while Toxqui Rodríguez et al. (2023) used artificial mixtures of seafood samples to evaluate the efficiency of ONT for detection of seafood fraud. Similarly, Voorhuijzen-Harink et al. (2019) and Ho et al. (2020) sequenced fish mixtures or highly processed seafood samples respectively for food authentication purposes. Additionally, Voorhuijzen-Harink et al. (2019) also demonstrated that the accuracy levels of ONT vs Illumina data from the fish mixtures were comparable. This was also shown for bulk zooplankton (Chang et al., 2024; Semmouri et al., 2021) and macrobenthos (Doorenspleet, Mailli, et al., 2025) that were sequenced with both platforms.

ONT sequencing for characterisation of eukaryotic communities from eDNA samples, extracted from water samples, is currently most commonly used for fish diversity monitoring and fisheries management (Table 1). In two cases where short and long amplicons were sequenced in parallel (170 & 2,000/2,400 bp), it was shown that higher detection rate was actually achieved with the shorter amplicons (Doorenspleet, Jansen, et al., 2025; Maggini et al., 2024). This could be due to lower availability of longer fragments in eDNA samples due to degradation (Doorenspleet, Jansen, et al., 2025). Nevertheless, longer fragments improved species assignment in closely related species, while overall detection rates were correlated to low species abundance and low genetic variance (Maggini et al., 2024). Finally, short amplicon ONT eDNA metabarcoding (170-200 bp) (Kasmi et al., 2024; Munian et al., 2024) was shown to outperform conventional methods of fish monitoring both in the overall diversity detected and in terms of cost efficiency.

Besides fish, aquatic eDNA metabarcoding with ONT has been used for detection of invasive bivalves (Egeter et al., 2022), where a comparison between a typical Illumina workflow vs ONT amplicons demonstrated comparable results. Furthermore, MinION was used for white shark detection in the open ocean, performing all steps of wet-lab and bioinformatic analysis on board ship within ~48 hours, thus highlighting ONT's applicability for real-time evaluation of biodiversity (Truelove et al., 2019). ONT has also been used for pollinator and pathogen monitoring in plants. Here, eDNA collected from flowers was used to assess the diversity of pollinators, which detected a large part of the pollinator diversity, though lower than Illumina and traditional methods (Harris et al., 2023). Furthermore, Theologidis et al. (2023) used ONT for detection of fungi and endophytes infecting young olive tree shoots as a potential phyto-diagnostic method. Finally, Bludau et al. (2025) used amplicon ONT sequencing to characterise protist communities.

c. PCR-free or shotgun eDNA and community sample sequencing

PCR-free (shotgun) sequencing of eDNA or community samples offers an alternative as a non-targeted biodiversity monitoring tool. In this case sequencing is not limited to single gene markers but represents fragments of all nuclear and mitochondrial genomes present in the sample (Linard et al., 2015; Peel et al., 2019). This method bypasses potential primer bias, allowing a more universal view of the community composition of the sample (Bista et al., 2018). Few studies have applied this approach to eDNA samples so far. In Munian et al. (2024) PCR-free ONT sequencing was used for detection of freshwater fish from water samples, and similarly in Nousias et al. (2024) for assessment of riverine biodiversity. Furthermore, Koda et al. (2023) used shotgun sequencing of eDNA to detect the presence of bobcat DNA extracted from soil samples of pawprints, while Urban et al. (2023) performed genomic biomonitoring of the endangered kākāpō bird, by analysing soil samples collected from the areas that the birds frequented. Besides environmental samples, Shum et al. (2024) used shotgun ONT for fish and seafood traceability in fish markets including highly degraded samples, as a way of confirming identity and geographic origin with a more rapid turnaround.

d. Microbial community sequencing

Microbial community analysis is most commonly performed through 16S amplicon or shotgun (metagenomic) sequencing (Quince et al., 2017). Numerous studies have used ONT for microbial community sequencing focusing on clinical applications (for review see (Kim et al., 2024) and references therein). In fewer cases microbial analysis of environmental samples is used with biodiversity implications and here we review a few cases. For example, amplicon ONT sequencing (16S) was used to assess the impacts of agriculture or aquaculture practises in the environment through assessment of microbial communities (Stevens et al., 2023; Stoeck et al., 2024). Similarly, ONT was used to assess the potential health impact of dust microbiomes collected from air samples (Nygaard et al., 2020). In all three cases above, contrasting ONT and Illumina data showed comparable results (Nygaard et al., 2020; Stevens et al., 2023; Stoeck et al., 2024). Furthermore, the portable MinION was used to establish an atlas of bacterial diversity in the River Cam in Cambridge by sequencing along space and time, aiming to monitor water quality in relation to surrounding land use (Urban et al., 2021).

Additionally, shotgun metagenomic sequencing of microbial communities provides wider opportunities for community assessment, and the possibility of assembling whole microbial genomes (metagenome-assembled genomes, MAGs) (Quince et al., 2017). When shotgun sequencing is used, added value can also be gained from the long reads, beyond the original aim of the project. For example, Liu et al. (2024) used shotgun sequencing of water samples to detect a functional shift in microbial communities related to environment depth, while mainly aiming at investigating cichlid diversity in lake Masoko. In Clark et al. (2025) ONT metagenomics was used to assess levels of antimicrobial resistance (AMR) in nearshore sediments from Antarctica showing poor incorporation of enteric bacteria from wastewater in bacterial biofilms and sediments. Finally, Reska et al. (2024) characterised the microbiome of air samples, confirming the potential use of ONT metagenomics in air eDNA studies.

Table 1

Overview of taxonomic target, gene marker, and amplicon length for published ONT & PacBio studies.

Approach	Taxa targeted	Gene marker	Fragment size (bp)	Studies	Platform
DNA barcoding	Invertebrates Insects Amphibians Reptiles Metazoa Arthropoda	COI 16S Cytb rDNA	313-500	Pomerantz et al., (2018); Seah et al., (2020); Srivathsan et al., (2021); Vasilita et al., (2024)	ONT
			600-4,500	Menegon et al., (2017); Srivathsan et al., (2018, 2019); Krehenwinkel, Pomerantz, Henderson, et al., (2019); Cuber et al., (2023); Gajski et al., (2024); Koblmüller et al., (2024); Maestri et al., (2019); Vasilita et al., (2024); Hebert et al., (2025)	ONT
	Arthropoda Invertebrates Fungi	COI ITS LSU	650-1,000	Hebert et al., (2018); Runnel et al., (2022); Cuber et al., (2023)	PacBio
Metabarcoding	**Sample Type**	COI 12S 16S 18S Cytb ITS LSU	313-425	Voorhuijzen-Harink et al., (2019); Ho et al., (2020); Srivathsan et al., (2021); van der Reis et al., (2022); Chang et al., (2024); Doorenspleet, Mailli, et al., (2025)	ONT
	Community		650-2,758	Ho et al., (2020); Semmouri et al., (2021); Baloğlu et al., (2021); Srivathsan et al., (2021); Toxqui Rodríguez et al., (2023)	ONT
			585 - 6,300	Heeger et al., (2018); Orr et al., (2018); Gueidan et al., (2019); Kolaříková et al., (2021); Jamy et al., (2020, 2022); Gueidan & Li, (2022);	PacBio
	eDNA		130-550	Truelove et al., (2019); Egeter et al., (2022); Harris et al., (2023); Theologidis et al., (2023); Munian et al., (2024); Kasmi et al., (2024); Maggini et al., (2024); Doorenspleet, Jansen, et al., (2025)	ONT
			2,000-2,400	Maggini et al., (2024); Doorenspleet, Jansen, et al., (2025)	ONT
Shotgun	**eDNA**	n/a	n/a	Urban et al., (2023); Koda et al., (2023); Liu et al., 2024; Nousias et al., (2024); Doorenspleet, Mailli, et al., (2025)	ONT

4. Laboratory steps for ONT sequencing

Similar to other sequencing workflows, several standard steps are needed for the preparation of samples for ONT sequencing, including DNA extraction, PCR amplification (if appropriate), labelling, and adapter tagging (Bruce et al., 2021). Other important considerations include the amount of starting DNA, and the type of library prep strategy selected. Library prep for ONT can vary depending on the application such as using one or two PCR steps to amplify DNA before sequencing (Fig. 2), or attaching barcoded tags used for multiplexing many samples in a single run (Bohmann et al., 2022; Bruce et al., 2021).

a. DNA extraction

The DNA extraction method used varies based on the type of sample and the application. Ultimately what is important is that the extracted DNA meets the quality and quantity requirements needed for library preparation. When extracting tissue samples for DNA barcoding or community samples, DNA concentration is expected to be higher and less degraded compared to most eDNA samples (Bruce et al., 2021). eDNA samples may also contain impurities requiring purification steps for inhibitor removal to assure good results in downstream applications. Some of the most commonly used kits for extraction of environmental samples from soil or water are the DNeasy Blood and Tissue kit as well as the DNeasy PowerSoil and the DNeasy PowerWater kit from Qiagen (see Supplementary Table 1 for DNA extraction methods used per study). For field applications with MinION, the ease of DNA extraction should also be considered so that it can be performed in a non-lab setting. Such extraction methods which are fast and require minimal equipment, are the QuickExtract™ solution (Srivathsan et al., 2019) and the Chelex® 100 resin (Koblmüller et al., 2024; Seah et al., 2020). However, it is likely that these methods might not always support the best DNA quality and quantity, so it is important to also assess the suitability of the DNA extract for downstream applications. If a large field collection is planned it is also advisable to perform trials before field deployment (e.g.(Seah et al., 2020; Vasilita et al., 2024)). Suitability assessment of samples for sequencing includes concentration and purity checks (e.g. Qubit or Nanodrop), and fragment length verification (e.g. agarose gels, Tapestation, or Bioanalyzer)(Bruce et al., 2021).

b. Library prep for PCR-based ONT sequencing

Following DNA extraction, the first step is amplification, labelling with different barcodes, and finally ligation of an ONT sequencing adapter to each barcoded amplicon (Fig. 2). For library preparation, three different strategies are commonly used in ONT amplicon studies (Figs 2. a, c).

1) Single-step PCR: All the samples are labelled directly during the first amplification with the use of primers which already include unique barcodes attached to the 5’ end (barcode length 9 - 15 bp). This strategy has been used mostly in DNA barcoding studies (Gajski et al., 2024; Harris et al., 2023; Hebert et al., 2025; Ho et al., 2020; Koblmüller et al., 2024; Krehenwinkel, Pomerantz, Henderson, et al., 2019; Srivathsan et al., 2018, 2019, 2021; Vasilita et al., 2024), and in analysis of bacterial DNA from water samples (Urban et al., 2021) and zooplankton communities (Chang et al., 2024).

2) Two-step PCR with “tails”: Target specific primers complemented with specific ONT tails are used for the first amplification. These tails will allow the binding of the different barcodes in a follow-up PCR reaction, commonly using only a low number of cycles. This strategy has been used in several DNA

barcoding (Maestri et al., 2019; Pomerantz et al., 2018; Seah et al., 2020) and eDNA metabarcoding studies (Egeter et al., 2022; Huggins et al., 2024; Kasmi et al., 2024; Maestri et al., 2019; Pomerantz et al., 2018; Seah et al., 2020; Stevens et al., 2023; Theologidis et al., 2023).

3) Two-step PCR (without "tails"): This strategy also uses the target specific primers but without additional tails in the first amplification. To label each sample an extra reaction is needed to modify the amplicons' extremities and add barcode adapters (end-prep and barcode adapter ligation). Only after this reaction it is possible to perform the second PCR where the different barcodes are added. Fewer studies have used this strategy for DNA metabarcoding (Toxqui Rodríguez et al., 2023; van der Reis et al., 2022).

When extensive multiplexing is required, it is also possible to perform dual barcoding by using inner and outer barcodes. In this case the PCR Barcoding Expansion Kit is used (96 inner barcodes), followed by the Native Barcode kit (24 outer barcodes), which allows multiplexing up to 2,034 samples in the same flow cell (Supplementary Table 2). Performing clean-up steps between PCR reactions is required to remove non-target fragments, and primers from the previous reaction, and to ensure that only the intended amplicons are present in the final library. (Fig. 2.a). Usually magnetic beads (Ampure) are used for clean-up, which can discard byproducts based on size and according to beads-to-sample ratio.

In all strategies, after labelling, the samples are pooled equimolarly, and the final library is end-prepped, in a reaction that repairs and prepares the DNA ends for the sequencing adapter attachment (5' phosphorylated, 3' dA-tailed ends). Then a ligation reaction will attach the ONT sequencing adapters, consisting of an oligonucleotide and motor protein (Fig. 2.b), which interact with the nanopores during sequencing to allow the translocation of the DNA strands through the nanopore (Fig. 1). Finally, another clean-up step is used, either with Long Fragment Buffer (LFB) to enrich DNA fragments >3 kb, or with Short Fragment Buffer (SFB) to retain DNA fragments of all sizes. The finalised library is loaded and the run, which can be monitored in real time, can go on for up to 72 hours, or until the required number of reads has been achieved.

c. Library prep for PCR-free ONT sequencing

For the PCR-free shotgun approach, the PCR amplification steps are omitted, and the end prep and ONT sequencer adapter ligation can be done directly after DNA extraction (Koda et al., 2023). However, when multiplexing is used, a labelling step for the addition of individual barcodes needs to be included. This can be done with an ONT Rapid Barcoding kit that uses a transposase barcode complex to cleave gDNA and attach barcodes (Peel et al., 2019; Reska et al., 2024), or with a Ligation Sequencing kit with Native Barcodes that will attach barcodes to fragmented gDNA after an initial end-prep reaction (Munian et al., 2024; Reska et al., 2024).

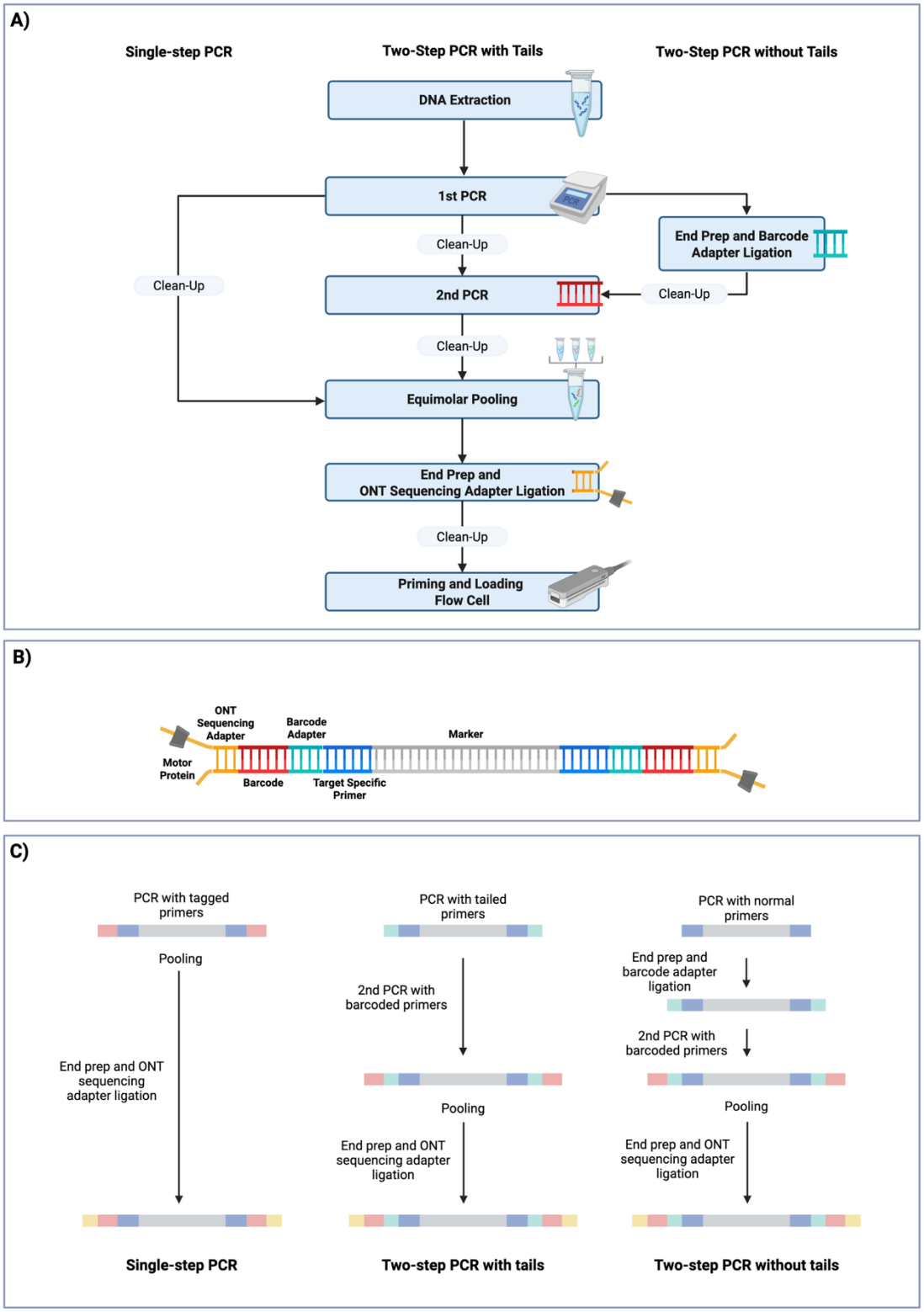

Figure 2

Strategies for library prep for ONT amplicon sequencing. A) library prep strategies: Single-step PCR, two-step PCR with tails, and two-Step PCR without tails B) structure of the amplicon with all types of adapters and barcodes, C) amplicon structure for each library preparation strategy. For PCR-free (shotgun) sequencing, the end prep and adapter ligation are performed without intermediate amplification steps (Image generated with BioRender).

d. Sample requirements for ONT sequencing

To ensure a successful ONT run and maximize sequencing efficiency, aspects that need to be considered are the quality and quantity of the sample, the type of flow cell and its chemistry, and the respective kits. To optimise sequencing yield, it is important that the activity of the nanopores is kept high. The occupancy of the nanopores is related to the amount of input DNA, more particularly the number of molecules available and their size. The loading amount of the final library depends both on the fragment size, and the type of flow cell used. Shorter fragments will require larger quantities because they are sequenced faster, while longer fragments keep the nanopores occupied for longer, meaning that the required amount is lower. However, there is not a linear relationship between the amount of DNA loaded into the flow cell and the sequencing yield, only a likely reduced output when these are underloaded.

With the latest chemistry flow cells R10.4.1 (early 2025), loading approximately 35-50 fmol of good quality library is recommended to achieve high pore occupancy (e.g.(Doorenspleet, Mailli, et al., 2025; Srivathsan et al., 2024). As these values also depend on the amount of DNA molecules and their size, it is advised that specific ONT guidelines are considered based on the library fragment length (Table 2, Supplementary Table 2). Nevertheless, lower DNA amounts may also be able to achieve good quality reads. Simon et al. (2023) provide a useful example based on testing several input quantities of mock microbial communities (1-1,000 ng), showing that lower DNA amounts than those recommended by ONT guidelines could be successful when applied to metagenomics. This aspect of library prep for ONT metabarcoding has not been systematically explored to date, and comparison between studies is challenging due to different flow cell types and fragment size used, but also due to incomplete reporting. For future work it is important that information such as DNA input is included to enable further standardization and inter-study comparisons.

As multiplexing is normally used for biodiversity studies, the most commonly used ONT kit for library prep is the Ligation Sequencing Kit (SQK-LSK114). For this kit "barcoding" needs to be performed beforehand either with ONT PCR Barcoding Expansion kits (Doorenspleet, Jansen, et al., 2025; Egeter et al., 2022; Pomerantz et al., 2018) or with customized/tagged primers (Hebert et al., 2025; Koblmüller et al., 2024; Krehenwinkel, Pomerantz, Henderson, et al., 2019). Other less commonly used kits are the Native Barcoding and the Rapid Barcoding kits, which already include the "barcoding" of the samples and do not require a PCR reaction. For the SQK-LSK114 kit 100-200 fmol of amplified DNA is recommended as the optimal input (Doorenspleet, Jansen, et al., 2025; Srivathsan & Meier, 2024). The Native Barcoding kits have a long library preparation time (~140 min) and are usually recommended for gDNA samples (Munian et al., 2024), but can also be used with amplicons (Bludau et al., 2025; Stoeck et al., 2024). The Rapid Barcoding kit has a shorter prep time (60min) because it performs fragmentation and barcode attachment in the same step, though this might reduce yield and read quality. This kit has been used in microbial shotgun studies (Clark et al., 2025; Reska et al., 2024). See Table 2 for input requirements for the Ligation Sequencing Kit, and Supplementary Table 2 for other kits.

Table 2

Input DNA amount for library prep and flow cell loading for ONT, according to fragment length and type of flow cell (Ligation Sequencing kit SQK-LSK114 kit). For other kits see Supplementary Table 2.

Fragment Length	Starting Input		Loading Amount	
	Flongle	MinION and PromethION	Flongle	MinION and PromethION
<1 kb	50 fmol	200 fmol	5-10 fmol	100 fmol
1-10 kb	50-100 fmol	100-200 fmol	5-10 fmol	35-50 fmol
>10 kb	500 ng	1 ug		300 ng

5. Bioinformatic analysis of ONT data

Current bioinformatics pipelines used for metabarcoding are based on Illumina data, which are characterised by set read length. In contrast ONT (and PacBio) data can generate both long and short fragments with varying error profiles (van der Vorst et al., 2024), hence bioinformatic tools need to be able to accommodate these differences. During ONT sequencing, reads are generated through the conversion of raw data collected in the form of electric signals into nucleotide sequences, which is called basecalling (Wang et al., 2021). After basecalling, quality filtering and processing steps (Fig. 3) are performed to obtain the final dataset according to each application.

a. DNA barcoding

In DNA barcoding each "barcode" corresponds to one species, which differs from environmental samples, and the objective is to generate a final consensus sequence for each sample/specimen. After demultiplexing, the adapter and "barcode" sequences are trimmed, and the reads are filtered based on quality scores and size. A clustering step is usually performed at a lower percentage of identity, since the variation in the reads from a single specimen is often due to sequencing error or contamination (Hebert et al., 2025; Maestri et al., 2019). Alternatively clustering is performed for each "barcode" only keeping the reads from the most abundant cluster or from clusters above a certain threshold (Maestri et al., 2019; Seah et al., 2020). After this step, a draft consensus sequence is generated for each sample (specimen). This draft consensus will be polished, usually by mapping all the reads back to the draft consensus (Krehenwinkel, Pomerantz, Henderson, et al., 2019; Maestri et al., 2019; Pomerantz et al., 2018) or by re-clustering the sequences at a higher identity percentage and doing a new consensus calling (Hebert et al., 2025). The final consensus sequence can be trimmed to the exact size to obtain the final DNA barcode sequence and then aligned to reference sequences in databases (Hebert et al., 2025; Maestri et al., 2019). The order of the processing steps may differ between studies. The ONTbarcoder pipeline, and more recent update v2 (Srivathsan et al., 2021, 2024) is one of the most commonly used to date for ONT barcoding data analysis (e.g. (Cuber et al., 2023; Harris et al., 2023; Koblmüller et al., 2024; Vasilita et al., 2024)).

b. eDNA and community sample metabarcoding

eDNA or community samples contain many different sequences from multiple species, so in contrast to DNA barcoding of individual specimens the sequences need to be assigned taxonomy computationally. Initial preprocessing steps include demultiplexing, adapter trimming, general quality control and size filtering, followed by clustering of Operational Taxonomic Units (OTU) or Amplicon Sequencing Variant (ASV) processing, and taxonomic assignment (for general overview of metabarcoding tools see (Hakimzadeh et al., 2024)) (Fig. 3). The exact order with which some of these steps are performed may differ between studies. After demultiplexing, the barcode and adapter sequences are trimmed and reads that do not have the correct primer sequence are discarded (Maggini et al., 2024). Quality assessment and filtering of the reads is performed according to quality scores and expected fragment length, removing low-quality reads and improving accuracy. OTU clustering is performed according to a similarity threshold, which could vary based on the level of intraspecific diversity of the target groups (Alberdi et al., 2017), the ONT kit/chemistry, basecalling algorithm, or fragment length (Doorenspleet, Jansen, et al., 2025). Due to ONT's error profile, clusters with a low number of reads are often eliminated in order to reduce noise. However, this step needs to be adjusted to each study depending on the target taxa and expected presence of rare/low-abundance species. Subsequently, the reads in each cluster are aligned and a draft consensus sequence is generated for each OTU. Further polishing can be done to improve the accuracy of the consensus sequences (Doorenspleet, Jansen, et al., 2025). After obtaining the final sequences and counting the reads assigned to each one, an OTU table can be built summarizing the abundance of each OTU across all samples. ASV analysis is performed by identifying sequence variations directly at the single-nucleotide level without clustering, which retains more resolution (Overgaard et al., 2024). Extra processing steps like dereplication and removal of chimeras can also be performed (Munian et al., 2024; Semmouri et al., 2021; van der Reis et al., 2022). Taxonomic assignment of the identified OTUs or ASVs is performed usually by BLAST (BLASTn, MegaBLAST) against a reference database containing barcode sequences (Doorenspleet, Mailli, et al., 2025; van der Vorst et al., 2024). Accurate taxonomic assignment for metabarcoding datasets is linked to completeness of reference databases (McGee et al., 2019; Zinger et al., 2019).

Few bioinformatic pipelines for ONT metabarcoding data analysis are currently available (Supplementary Table 3). Some of the most complete and commonly used pipelines for metabarcoding are DECONA (Doorenspleet, Jansen, et al., 2025), PIMENTA (van der Vorst et al., 2024), and MSI (Egeter et al., 2022), which perform similar overall analysis, but with some variations. The DECONA pipeline is a simple command line tool which supports use by researchers without extensive bioinformatics background (Doorenspleet, Jansen, et al., 2025). DECONA starts by read clustering, followed by alignment and assembly of clusters. After this, the consensus sequences obtained are re-clustered and polished, and finally the taxonomic assignment is done (Doorenspleet, Jansen, et al., 2025). Similarly, PIMENTA also follows these steps, with the difference that the re-clustering step is done with all samples together instead of individually and there is no polishing of the consensus sequences (van der Vorst et al., 2024). On the other hand, MSI performs polishing before clustering (Egeter et al., 2022). Other introduced pipelines, are eNano, which is designed as a "simple-to-understand" tool (Dierickx et al., 2024), and the ASHURE pipeline which is a Python based pipeline performing multiple processing steps of metabarcoding analysis (Baloğlu et al., 2021).

Method standardization for ONT data analysis is still lacking, which complicates the validation and comparison of results between different studies (van der Vorst et al., 2024). Further development of standardized bioinformatic pipelines, streamlining their use, and clear reporting of each step undertaken will support ease of analysis and reproducibility of biodiversity studies in the future (Harris et al., 2023; Kasmi et al., 2024; Truelove et al., 2019).

c. PCR-Free (shotgun)

In shotgun sequencing, data originates from across the nuclear or organelle genome instead of a specific gene marker, and accurate assembly or species assignment can be a bioinformatic challenge. The initial preprocessing steps are as previously described (basecalling, demultiplexing, quality control). Since this is a non-targeted approach, there is no specific size filtering, because the length of the random sequenced fragments is variable. To taxonomically assign reads, mapping against reference genomes or organelles is performed (Bista et al., 2018; Linard et al., 2015; Quince et al., 2017). Most studies to date using PCR-free ONT sequencing target prokaryotes (e.g. (Clark et al., 2025; Liu et al., 2024; Reska et al., 2024)). Limited resources of eukaryotic reference genomes also make it difficult to apply this technique across many species. In a study by Peel et al. (2019) a shotgun approach was used to analyse mixed pollen samples with a more cost effective and likely quantitative aspect than PCR-based. A pipeline (RevMet) was also developed which does not require assembly of a reference genome, but instead performs a genome skim, a low-coverage whole genome sequencing, which is used for identification (Peel et al., 2019).

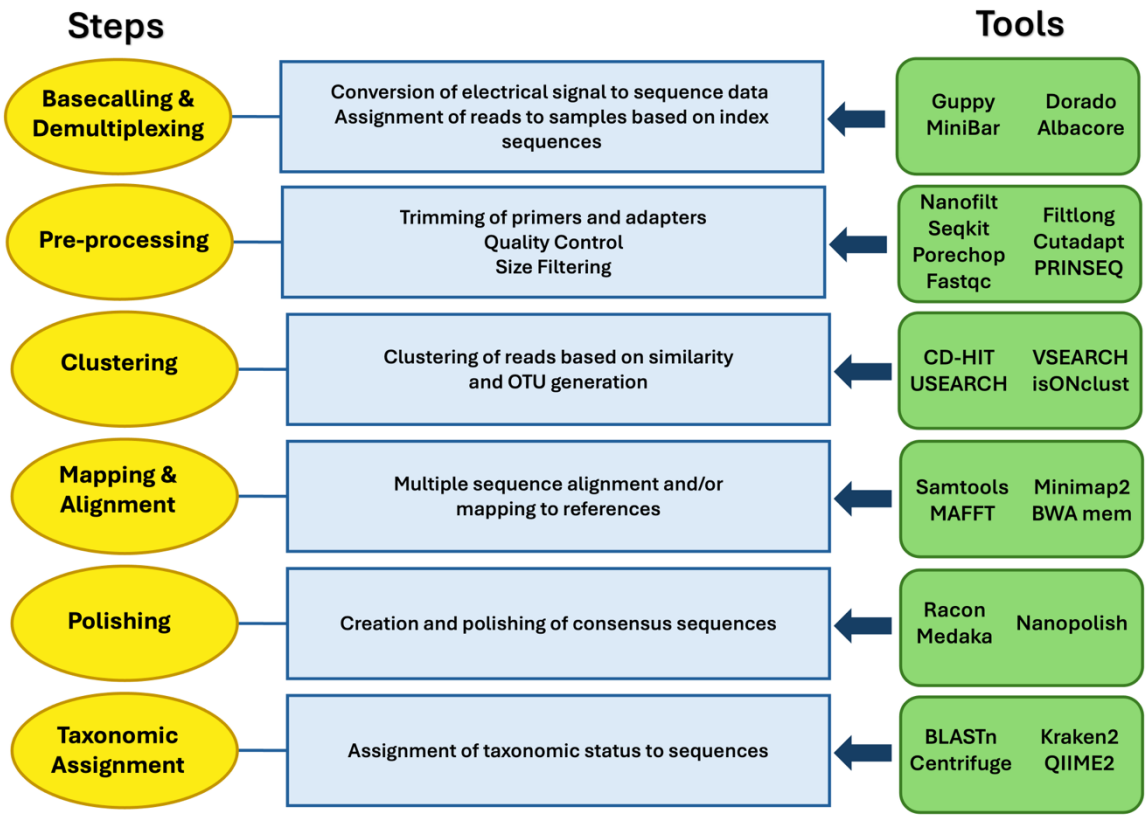

Figure 3

Bioinformatics tools. Typical processing steps used for data analysis of long-read biodiversity datasets, and suggested tools for each step. The order of processing steps may vary (sources for each tool are listed in Supplementary Table 3).

6. PacBio sequencing in biodiversity studies

PacBio is a long-read sequencing technology developed by Pacific Biosciences and is also known as "SMRT" sequencing due to performing Single-Molecule Real-Time sequencing (Rhoads & Au, 2015). The company became active in the early 2000's and was soon established in the area of long-read sequencing, marketing initially the PacBio RSI and II systems, and subsequently releasing the PacBio Sequel (I, II, and IIe) from 2015 onwards. Current instruments are the Revio and a bench top version, the Vega, as well as various Sequel instruments still in use. Sequencing is performed in SMRT cells, in Continuous Long Reads (CLR), and with the use of Circular Consensus Sequencing (CCS), which massively improves the final error rates. With CCS a consensus sequence is derived after multiple passes of each individual molecule, which makes it possible to convert noisy individual subreads into a highly accurate final read (Wenger et al., 2019). With the most recent introduction of High Fidelity (HiFi) reads and using CCS, the final accuracy exceeds 99% (Phred score 20), with a 10-30 Kb read length capability (Kucuk et al., 2023). The throughput of PacBio platforms increased largely with the introduction of the Revio which can yield up to 120 Gb per SMRT cell, compared to 30 Gb max throughput for the Sequel lle, while the Vega can yield approximately 60 Gb per SMRT cell.

PacBio Sequel for DNA barcoding was used by Hebert et al. (2018) to test its multiplexing and read length capabilities, by sequencing >5,000 taxa in a single run, targeting the COI barcoding region. Furthermore, PacBio was used for barcoding fungal voucher specimens using multiplexed amplicons of ITS and LSU resulting in higher success rates compared to Sanger (Runnel et al., 2022). The higher success rate was especially related to cases where polymorphic alleles were present, or when there was presence of contamination. In a comparison of barcoding performance between all three platforms Sanger, ONT, and PacBio, it was shown that both ONT and PacBio were suitable for DNA barcoding and biodiversity monitoring, while also suggesting that library preparation is less time consuming when using ONT (Cuber et al., 2023). Furthermore, high multiplexing and throughput can lower cost per barcode in comparison to Sanger sequencing (Hebert et al., 2018). The PacBio instruments are not currently widely used by individual labs due to their overall high annual service costs, as well as the cost of SMRT cells, which limits their presence mostly in core facilities (Hebert et al., 2018, 2025). Nevertheless, PacBio services are becoming more commonly available through sequencing service providers.

Implementation of PacBio metabarcoding has been particularly popular for analysis of fungi, which represent a good case for long-read sequencing applications due to the known size variability and complexity of markers used for analysis of fungal diversity such as ITS rRNA loci (Tedersoo et al., 2022). Initially, fungal metabarcoding pipelines were developed using PacBio RSII (CCS) (Heeger et al., 2018) or both RSII and Sequel (Kolaříková et al., 2021; Tedersoo et al., 2018) generally aiming at generation of long amplicons (>2,500 bp) of the eukaryotic rRNA operon containing multiple commonly targeted regions (SSU, LSU, ITS). Generation of a combination of amplicons based on specialised primers can further aid in the overall accuracy of species identification. This approach can be particularly beneficial for use in phylogenetics, as well as to improve taxon recovery in cases of groups with low genetic diversity where extending the length of the target region can be beneficial (Tedersoo et al., 2018). Lichenized fungi in particular are interesting because individual specimens may harbour other fungi or mixed fungal communities making them difficult to sequence with Sanger sequencing, while similar to other fungi the use of the ITS marker does not avail to short-read metabarcoding analysis either. For

these reasons Gueidan & Li (2022) and Gueidan et al. (2019) developed PacBio amplicon-based protocols for sequencing of lichen specimens from herbaria collections. Furthermore, few studies to date have used PacBio to characterise microbial eukaryote communities from environmental or cultured samples (Jamy et al., 2020, 2022; Orr et al., 2018). For small unicellular organisms like protists, using PacBio can help bypass the need for cloning and Sanger sequencing which can be very laborious and costly (Orr et al., 2018). Furthermore, beyond detection and characterization of community diversity, long amplicon data can be used to investigate evolutionary level questions, as was demonstrated by Jamy et al. (2022), where long-read amplicons aided in understanding habitat transitions across the eukaryote tree of life.

7. Sample prep and analysis of PacBio data

Extracting DNA for PacBio amplicon sequencing follows the same general considerations as with ONT. Methods vary based on application and as PacBio has been used mostly for fungal or microbial eukaryote sequencing the extraction types are more specific to these groups (see Supplementary Table 1). In general, to take advantage of long-read capabilities, it is beneficial to also consider using methods preserving long DNA fragments during extraction.

Sample preparation for PacBio-based DNA barcoding and metabarcoding involves initial PCR steps to generate target amplicons as described previously, with additional steps in order to attach barcodes for multiplexing (Fig. 2). For PacBio sequencing the double stranded DNA molecules are circularised after ligation with hairpin adaptors (Quail et al., 2012). This circularisation process enables repeated sequencing, generating multiple subreads as part of the CCS mode and HiFi sequencing (Cuber et al., 2023; Wenger et al., 2019). For amplicon library preparation a SMRTbell library prep kit is used (current version 3.0) (e.g. (Gueidan & Li, 2022)). The amount of input DNA required for library preparation is related to the mean size of the target amplicons and varies for different instruments and chemistries. For example, the input DNA could be as little as 50 ng for the latest Revio chemistry for amplicons shorter than 3 kb, and increase to >300 ng for amplicons larger than 10 kb. For optimal results it is recommended to make calculations based on the specific approach and application of each study. Barcoding for multiplexing can be performed during the initial PCR following one or two-step protocols, with pooling prior to subsequent library prep steps. For the SMRTbell library preparation end-repair and A-tailing steps are also included and performed on the "barcoded" samples prior to adapter ligation. As with other library prep protocols (Fig. 2), clean-up steps are performed at intervals and for the final library in accordance with the specific protocol.

Analysis of PacBio amplicon data includes steps and preprocessing similar to other sequencing technologies (Fig. 3). In several cases individual studies have developed pipelines to address various steps of the process that need optimization for PacBio data. These include a denoising pipeline for allele phasing of PacBio data (Gueidan et al., 2019), or a phylogeny-aware taxonomic assignment method taking advantage of phylogenetic information available from long amplicons used to improve taxonomic classification (Jamy et al., 2020). Another important issue during amplicon analysis is identification and removal of chimeric reads which are generated during PCR, which need to be addressed with appropriate bioinformatic steps. A workflow developed by Karst et al. (2021) uses unique molecular identifiers (UMIs) to reduce error rates and chimeras in long-read data (PacBio and ONT). UMIs, which are short sequences of 10-20 bases, are tagged to each template molecule enabling

sorting and analysis of individual reads. This method has been developed for long-read sequencing of microbial communities but could potentially be used for other amplicon applications (Overgaard et al., 2024). To support analyses of PacBio CCS amplicon data a pipeline was also developed based on the DADA2 algorithm and software, which was designed to determine ASVs with a high level resolution (single-nucleotide level) (Callahan et al., 2019).

8. Open data and reporting considerations

The principles of FAIR data reporting, Findability, Accessibility, Interoperability, and Reusability should be guiding all data driven scientific projects (Wilkinson et al., 2016). Along with reporting conventional data (e.g. DNA sequences) these principles also apply to tools, protocols, and workflows, which should also be reported diligently. During the development of the DNA barcoding and eDNA metabarcoding fields, data reporting has been evolving to reach community acceptable standards (Klymus et al., 2024). This reporting needs to be further extended to include details specific to long-read sequencing technologies. Hence it is suggested that for ONT and PacBio platforms this should always include the following. For sequencing: the specific type of flow cell or SMRT cells and version of sequencing chemistry, the kit used for library preparation, adapter type (according to technology), and amount of input DNA and loaded library for sequencing. For data reporting: the read length profiles achieved, read coverage per sample, error rates, computational steps (and scripts) used for data analyses.

Most researchers submit raw data in public repositories (e.g. INSDC which incorporates ENA and NCBI (Arita et al., 2021)), nevertheless in that form they are difficult to process as multiple intermediate steps are required to get a usable form, such as OTUs or ASVs (Fig. 3). Submission of processed data from metabarcoding studies in public databases needs to be promoted by the community and is an important step for advancing reproducibility and data reuse (Berry et al., 2020). Ongoing initiatives for integration of eDNA datasets in public repositories include plans for integration in databases, such as GBIF (Frøslev et al., 2023). It is important to consider though that such datasets need to be filtered and monitored before release to ensure removal of inappropriate reads, like contaminants.

Open data platforms and projects can have a considerable impact on the advancement of open science. Reference databases in particular, which are imperative for taxonomic assignment, are largely supported by public repositories. The Barcode of Life Database (BOLD) database, is a staple for the development of barcoding and metabarcoding research, acting not only as an open data repository but also supporting storage, analysis, and coordination (BOLD.v4 (Ratnasingham et al., 2024)). Large barcoding projects on wide geographic (BIOSCAN) or regional levels (e.g. ARISE, Netherlands), also promote open data generation for DNA barcoding resources. Initiatives like the Darwin Tree of Life (DToL), which focus on long-read whole genome sequencing, also integrate DNA barcoding in their pipeline. Here, barcoding supports taxonomic identification of species for genome sequencing, and at the same time populates barcode reference databases (Twyford et al., 2024). Furthermore, sequencing of whole genomes and organelles is necessary for future development, especially with the potential of long-read based biodiversity analysis. Such examples include taxon-specific organelle databases like MitoFish (fish mitogenomes) (Zhu et al., 2023), and ChloroplastDB (chloroplast genomes) (Cui et al., 2006). Additionally, initiatives such as the Earth BioGenome Project (EBP)(Lewin et al., 2018), DToL, and the European Reference Genomes Atlas (ERGA)(McCartney et al., 2024) are generating whole genome assemblies across the tree of life using long-read data. These initiatives which make their data immediately and openly available for use, constitute valuable resources for

potential future genome-wide applications for biodiversity monitoring.

9. Limitations and future perspectives

The low concentration and degraded nature of eDNA (Goldberg et al., 2016; Thomsen et al., 2012), could hinder the use of long-read platforms, while also limiting certain types of applications such as shotgun sequencing. Environmental samples are known to contain inhibitors or contaminants which could damage ONT nanopores causing sequencing failure or reduced yield. PacBio platforms are less sensitive to such inhibitors due to different chemistry used though this has not been systematically explored for environmental samples. Careful optimization of DNA extraction and library prep protocols is needed to boost sequencing efficiency for long-read applications, along with eDNA grade quality control and clean-lab procedures (Alberdi et al., 2017; Bruce et al., 2021).

The high error rate of ONT data has been a known concern likely hindering advances using this technology, however new developments in chemistry and bioinformatics solutions have mostly dealt with this issue. Improved basecalling algorithms, implementation of polishing steps, and improved workflows for clustering or classification have recently raised ONT data reliability to Illumina-like levels (Bludau et al., 2025; Chang et al., 2024; Sahlin et al., 2021). Similarly DNA barcodes from ONT and PacBio have been shown to present similar accuracy levels as Sanger and Illumina (Cuber et al., 2023; Srivathsan & Meier, 2024). Streamlining analysis and development of standardized bioinformatic pipelines for barcoding and metabarcoding will not only improve reliability of these applications but will also support future interstudy comparisons and reproducibility (van der Vorst et al., 2024).

Comparison of Illumina and ONT amplicon data demonstrated similar results for short amplicons (e.g. (Egeter et al., 2022; Kasmi et al., 2024; Munian et al., 2024)), but longer amplicons showed lower overall detection efficiency (Doorenspleet, Jansen, et al., 2025; Maggini et al., 2024). This issue could be related to lower availability of long fragments in eDNA samples and a possible solution to improve long fragment detection could be to increase overall sequencing depth. On the whole, using longer fragments provides added value to the analysis by improving taxonomic assignment for closely related species or low intraspecific divergence (Doorenspleet, Jansen, et al., 2025; Tedersoo et al., 2018).

An overall comparison of PacBio vs ONT platforms reveals a number of interesting points. PacBio instruments require a higher financial investment for instrument purchase compared to the minimal setup required by ONT, which also offers faster turnaround times since individual labs can set-up and run ONT independently (Seah et al., 2020). Nevertheless, the new desktop Vega instrument from PacBio might be more accessible to individual labs, hence potentially also reducing turnaround times. A unique feature of the ONT MinION, its portability, allows sequencing in the field with a host of real-time sequencing applications, which cannot currently be matched by other technologies.

Despite improvements to the data quality of ONT platforms, its accuracy is still something that needs to be managed, while PacBio read quality is very high, even more so since the introduction of HiFi reads (Chang et al., 2024; Kucuk et al., 2023). Throughput-wise the Revio presents a significant increase in output over previous instruments, reaching 120 Gb per SMRT cell, while a regular MinION flow cell outputs a maximum of 48 Gb. Both ONT and PacBio offer high multiplexing capabilities (up to 10,000 samples) (Cuber et al., 2023) providing good value for money for large projects, especially for DNA barcoding and standardised amplicon sequencing. ONT platforms offer more flexibility for small and medium sized projects with the Flongle and the MinION instruments, while the GridION and PromethION are more suitable for larger projects. In addition, the possibility of washing and reusing

ONT flow cells (as long as a suitable amount of nanopores remains active) can further reduce costs. On the other side, PacBio's Vega, has approximately half the sequencing capacity of the Revio, making it more suitable for smaller projects. Though Vega has not been used in published biodiversity work yet, it could provide a potential solution for future biodiversity projects. Selecting the most appropriate platform for a given project should take into consideration all the above factors as per the type of application, the nature and number of samples, and cost benefits for multiplexing.

Completeness of reference databases is an ongoing issue for existing DNA-based biodiversity monitoring applications (McGee et al., 2019; Zinger et al., 2019). Using short DNA barcode reference databases for taxonomic assignment of long-read data reduces the power of the application. In the future, reference databases need to expand to host longer fragments, and also make use of outputs from whole genome and organelle sequencing efforts (Lewin et al., 2018; Theissinger et al., 2023; Zhu et al., 2023).

Despite eDNA's mostly degraded state, it has been shown that sequencing whole mitogenomes could be achieved through long range amplification (Deiner, Renshaw, et al., 2017). Expanding eDNA sequencing to whole mitogenomes using the capabilities of long-read sequencers would represent a significant increase in data content provided from environmental samples. Sequencing of whole mitogenomes has been implemented mostly for community samples so far, with the use of shotgun sequencing, especially for arthropod communities (mito-metagenomics). This experience could be further utilised for eDNA mitogenome sequencing.

Expanding the use of shotgun sequencing could also resolve existing problems and offer exciting new opportunities for analysis. Specifically, using shotgun sequencing has been shown to reduce taxonomic (primer bias) and abundance estimation biases (Alberdi et al., 2017; Bista et al., 2018). Omitting the amplification step could potentially improve estimates of both diversity and abundance. Furthermore, shotgun sequencing could expand the scope of the data to wider areas of the nuclear and mitochondrial genome, moving beyond limited marker gene regions. This might make it possible to finally address population level questions. Currently population level work from eDNA has been limited mostly to species-specific assays (e.g. (Parsons et al., 2018; Sigsgaard et al., 2016)). In very few cases Illumina shotgun sequencing data have been used for population level analyses, such as for population inferences in cichlid fish from Lake Masoko (Liu et al., 2024), or for turtle population monitoring (Farrell et al., 2022). Utilization of whole genome data from consortia could contribute to further advancement of shotgun-based applications for biodiversity monitoring.

Finally, pairing long-read sequencing with other advancing technologies could be used to accelerate sample processing and data analysis. Employing robotic platforms for sample processing can boost operational speed and enable full use of the high multiplexing capabilities of long-read sequencing platforms. Such examples include development of high-throughput DNA extraction methods (e.g. (Hebert et al., 2018)), or metabarcoding sample processing workflows through liquid handling systems (Buchner et al., 2021). Furthermore, using Artificial Intelligence (AI) and cloud computing could dramatically improve analysis speed for large datasets, and enhance accuracy levels. AI-based analysis has been applied to large human genomic datasets (e.g. variant calling, error correction, gene expression) (GATK). For biodiversity monitoring applications, AI has been used for ecosystem status predictions through eDNA by training machine learning algorithms (Keck et al., 2023), and for improving annotation of eDNA reads (Flück et al., 2022), or DNA barcodes (Nanni et al., 2024).

Overall, large potential exists for further development and use of AI solutions for DNA based biodiversity monitoring coupled with long-read sequencing technologies.

10. Conclusions

The increasing impact of anthropogenic disturbances causing rapid loss of biodiversity presses the need for continued development of DNA based applications for biodiversity monitoring. The recently developed field of long-read sequencing for biodiversity monitoring can offer improved capabilities and new solutions by introducing novel applications and complementing existing DNA-based monitoring tools. Both long-read sequencing platforms, ONT and PacBio, can offer solutions to different needs. Using long-read technologies could help push the limits of biodiversity detection to higher standards and address questions which were previously out of reach, such as population level assessments from environmental samples. Continuous introduction of new instruments and chemistries, along with improved workflows and bioinformatics tools keeps raising the bar of quantity and quality of the data offered. Nevertheless, standardisation of methodologies is still required, along with the united efforts from the research community in support of FAIR reporting, open data access and reproducibility, for future development.

11. Glossary

- **DNA Barcode**: A fragment of DNA from a standardized marker gene region that can be used as a species identifier
- **Marker gene**: genes commonly used for DNA barcoding or metabarcoding, such as COI, 16S, ITS
- **Environmental DNA (eDNA)**: A complex mixture of DNA, often degraded, left by organisms in their environment. It can be collected without collection of the organism
- **Community sample:** bulk sample containing a mix of many different organisms (e.g. arthropod communities) which are co-extracted
- **Metabarcoding**: PCR-based high throughput sequencing method used for analysis of mixed community samples or eDNA samples
- **Metagenomics:** Shotgun sequencing (PCR-free), commonly used for sequencing of microbial communities or other community samples
- **Input DNA** (for first PCR): amount of extracted DNA needed for the first PCR during library prep
- **Loading DNA**: amount of final DNA library needed for loading the flow cell for ONT sequencing
- **"barcode":** unique short synthetic sequence added to amplicons during library prep to enable multiplexing of several samples in the same run

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Author contributions

IB: conceived the study and led the design and writing, IB, AL: wrote the manuscript. All authors read and approved the final manuscript.

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