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Environmental screening through nanopore native sequencing leads to the detection of *Batrachochytrium dendrobatidis* in La Mandria Regional Park, Italy

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ABSTRACT

Health surveillance of wildlife is crucial for the early detection of emerging pathogens. The One Health Integrated Wildlife Monitoring approach combines information from the biotic components of the ecosystem such as the abundance and diversity of wildlife with disease surveillance of animals (domestic and wild). Genomics-based detection of pathogens through environmental samples is a promising component of such programs. In this study, we analysed water samples from eight different irrigation channels in the La Mandria Regional Park, Italy, through PCR-free nanopore native sequencing to identify potential pathogens of importance for wildlife populations. Besides the pathogen *Fascioloides magna* that was previously detected in the park, we found evidence of other candidate pathogens including – for the first time in this area – the detection of the fungal pathogen *Batrachochytrium dendrobatidis*, and their possible host species. We confirmed the presence of *B. dendrobatidis* through ddPCR, and could demonstrate the detection of significant wildlife pathogens months before its first case was reported in wild animals of the park. Together with inferences on potential host species, these findings demonstrate the potential of genomics-based environmental monitoring through native nanopore sequencing in the context of One Health.

1. Introduction

Wildlife species are reservoirs of multi-host pathogens and may be a potential source of infection to both domestic animals and humans (Gortázar et al., 2016). Therefore, surveillance of wildlife populations for diseases is essential for the early detection of emerging diseases, safeguarding both animal conservation efforts and public health (Artois et al., 2009).

Within recent years wildlife disease surveillance concepts have evolved. In Europe, such efforts were traditionally focused to a large extent on passive and to a lesser extent on active surveillance of pathogens. The need for more harmonized pathogen (or disease) and wildlife population surveillance has led to the development of integrated wildlife monitoring programs where passive (scanning or general) and active (targeted) disease surveillance is combined with population monitoring (Cardoso et al., 2022). More recently the definition of integrated wildlife monitoring has evolved with the incorporation of data from all biotic components of the ecosystem (such as environmental samples) to data from disease surveillance and the monitoring of wild populations, resulting in One

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Health-integrated wildlife surveillance (EFSA, 2023). The environment is recognized as a container of all the biotic and abiotic components including the pathogens which act as a reservoir of infections and indicator of risk to humans and domestic animals. Environmentally mediated infectious agents (*i.e.,* pathogens with the potential to survive outside of a human or vertebrate host for more than one day) comprise 80% of World Health Organisation (WHO)-tracked pathogen species known to infect humans (455 of 560) (Hopkins et al., 2022; Sokolow et al., 2022). Therefore, in an integrated wildlife surveillance program, the environment needs to be considered as a frontline for infection or infestation monitoring with the overall aim of early detection of pathogens.

Environmental Nucleic Acids (eNA) are cellular or extracellular genetic material (DNA or RNA) released into the environmental samples (water, soil, air, sediment, etc.) from either micro- or macro-organisms. Such shedding depends on biomass, age, and feeding activity of the organism as well as physiology, life history, and spatiotemporal use of territory (Taberlet et al., 2012; Ficetola et al., 2008; Barnes and Turner, 2016; Goldberg et al., 2016; Hering et al., 2018). Despite being a relatively new method of surveying, eNA has already proven to have enormous potential in biological monitoring in particular for wildlife populations whether in terms of pathogen/invasive species detection or relative abundance estimation (Amarasiri et al., 2021). Another important aspect of eNA methods is their non-invasive and less cumbersome sampling efforts where direct sampling of hosts is challenging or may cause disturbance or destruction of habitat (Wheeler et al., 2004; Zemanova, 2021).

Environmental detection of pathogens through eNA methods may result in an essential surveillance component under the One Health integrated wildlife surveillance program mainly aimed at the early detection of pathogens (ENETWILD-consortium et al., 2023). However, it still lacks standardized and harmonized sampling strategies and analytical methods capable of maximizing biodiversity capture to detect more prioritized targets at once.

The majority of eNA studies are focused on water samples and metabarcoding is the most frequent multi-target analytical approach for analysis of such samples. However, the spectrum of biodiversity that can be captured is limited by choice of primers and amplification efficiency (Deiner et al., 2017; Ruppert et al., 2019). In the context of One Health integrated wildlife monitoring, PCR-free methods, such as native sequencing via metagenomic or metatranscriptomic approaches, appear particularly relevant. These methods have the potential to identify a broader range of taxa, including pathogens, their hosts (both novel and established), as well as invasive, threatened, endangered, or ecologically significant species, such as game species of interest to conservationists and wildlife managers (Bass et al., 2023). This is important, particularly for the detection of parasitic diseases where multiple host types, parasitic forms and environmental contexts complicate the pathobiology of the parasitic disease. Moreover, this holistic approach enables us to profile the existing species community, addressing information gaps in a given area. This, in turn, can help identify potential future invaders, emerging pathogens affecting animals or plants, and previously unknown but critically important species for public health, food security, and wildlife management (Barwell et al., 2023; Violle et al., 2007).

The early warning aspect of the One Health integrated wildlife surveillance program depends on the technical/analytical part of the early detection procedure. Oxford Nanopore Technologies' *MinION* is a portable and cost-efficient real-time genomic sequencing device (Quick et al., 2014; Ip et al., 2015; Struelens et al., 2024) which base-calls ionic current signals (squiggle) produced by nucleotide sequences passing through nanopores into sequence data in real time (Urban et al., 2023). Nanopore-based sequencing offers features such as real-time and native DNA/ RNA sequence generation (direct sequencing without the requirement for the prior amplification) from short to ultra-long fragments of several megabases. The cost per sample for nanopore sequencing ranges from 40 to 100 euros, depending on the sequencing kit used. Despite this, the relatively low barrier to entry makes the *MinION* sequencer an attractive option for new laboratories (Urban et al., 2023; Stevens et al., 2023). Nanopore sequencing technology, while continuously advancing, still exhibits a relatively higher error rate in raw sequences compared to other Next-Generation Sequencing (NGS) platforms. This necessitates the use of highly accurate basecalling models (e.g., the Super High-Accuracy model), particularly for native sequencing, de novo assembly projects, and low-frequency variant analysis (Delahaye and Nicolas, 2021; Doorenspleet et al., 2021). These features make nanopore sequencing a suitable method for native sequencing of environmental samples to enhance integrated wildlife monitoring programs and simultaneously accelerate early detection of pathogens.

The overall aim of this study was to investigate the potential of nanopore native sequencing for the detection of environmentally mediated pathogens with an emphasis on the detection of wildlife-associated pathogens and their hosts. We therefore obtained water samples from the La Mandria Regional Park (LMRP), Italy, which tested positive for the presence of *Fascioloides magna* (a trematode of wild and domestic ungulates and an invasive alien species in Europe (Bassi, 1875)) and of its intermediate lymnaeid snail host *Galba truncatula* using duplex quantitative PCR (qPCR) (Varzandi et al., 2024). We generated native nanopore sequencing data of these samples using the portable *MinION* platform, and analyzed the data to (i) prove the detection of known pathogens and their various hosts, and (ii) extend the pathogen and host spectrum detection to identify other pathogens and wildlife hosts as well as native and alien species, including the fungal pathogen *Batrachochytrium dendrobatidis*. Given the comprehensive nature of native sequencing, which enables an in-depth exploration of sequencing data, biodiversity richness and evenness were also analyzed across samples. This investigation sought to identify potential associations between sampling locations, whether inside or outside the fenced-off area, and the presence or absence of snails.

2. Materials and methods

2.1. Study area

La Mandria Regional Park (LMRP) is a protected area spanning approximately 6500 ha in northeastern Turin. The park features an internal area of about 3000 ha, separated by a 30 km-long wall from the external pre-park area. This internal fenced-off section is home to four ungulate species: wild boar, red deer, roe deer, and fallow deer. This area is registered as an Alpine site of community

importance and a Special Area of Conservation (SAC) in which measures of conservation for natural and semi-natural habitats and wildlife are applied to achieve the aims of Natura 2000 network in biodiversity safeguarding in Europe (European Commission, 2008). It is recognized as a primary hotspot of *F. magna*. LMRP encompasses diverse freshwater habitats, including artificial lakes, streams, and canals. The park's entire water network contributes to the Ceronda River in its southern region, which flows through the internal area and merges with the Stura di Lanzo River downstream before joining the Po River (Fig. 1).

2.2. eDNA sampling and extraction

Our retrospective analysis involved water samples collected from La Mandria Regional Park and extracted in September 2022 (DNA samples stored at −20 °C). The selection of sampling points, sample collection, and DNA extraction methods are detailed in our previous study, where the same samples were tested using a duplex qPCR assay for the simultaneous detection of *F. magna* and *G. truncatula* (Varzandi et al., 2024). Briefly, we selected eight sampling points in La Mandria Regional Park based on the presence or absence of lymnaeid snails (Fig. 1). At each point, between 7 and 20 liters of water were filtered using Waterra 0.45-micron filters (Douchet et al., 2009). Longmire buffer was added to the filter capsules, which were then preserved at room temperature until extraction. DNA was extracted from each capsule in two replicates, dividing the extracted liquid into two parts using DNeasy PowerSoil Pro Kits (Qiagen, Hilden, Germany).

2.3. Library preparation and nanopore sequencing

From each extraction replicate, approximately 90 ng of eDNA was pooled into a new microcentrifuge tube to achieve a final amount of approximately 180 ng eDNA in a final volume of 10 μL per each sequencing sample. Library preparation of the eight sequencing samples was performed using Oxford Nanopore Technologies' Native Barcoding Kit 24 V14 for genomic DNA (Oxford Nanopore Technologies, Oxford, UK). A negative control containing Ultra High Performance Liquid Chromatography level water was included in the sequencing experiment to control for sequencing contamination. The sequencing experiment was run on a R10.4.1 flowcell for 24 h using *MinION* device. We used Dorado v0.7.2 (dna_r10.4.1_e8.2_400bps_hac@v4.3.0 (GitHub, 2010)) for Super high-accuracy (SUP) basecalling of all environmental samples and the negative control. The DNA control strand was removed from raw fastq files using NanoLyse v1.2.1. Sequencing adapters and barcodes were removed using Porechop v0.2.4 (Wick, 2024), and the reads were filtered at a minimum quality score of 15 and minimum length of 100 bases using Nanofilt v2.7.1 (De Coster et al., 2018).

2.4. Taxonomy assignment and dataset curation

In order to increase the number of identified taxa, taxonomic classification of sequencing reads were performed using two methods: i) kraken2 and ii) Megablast, considering NCBI's *core-nt* as reference database (database downloaded 22.01.2025) (Altschul et al., 1990; Ciuffreda et al., 2021). The core nucleotide database (*core_nt*) is an alternative to the default nucleotide (*nt*) database, offering better-defined content while being less than half the size, making it more computationally efficient. Reads with an identity percentage of more than 95%, E-value less than 1e−10, alignment length of more than 50 bases, alignment length/query length (al/ql) ratio of

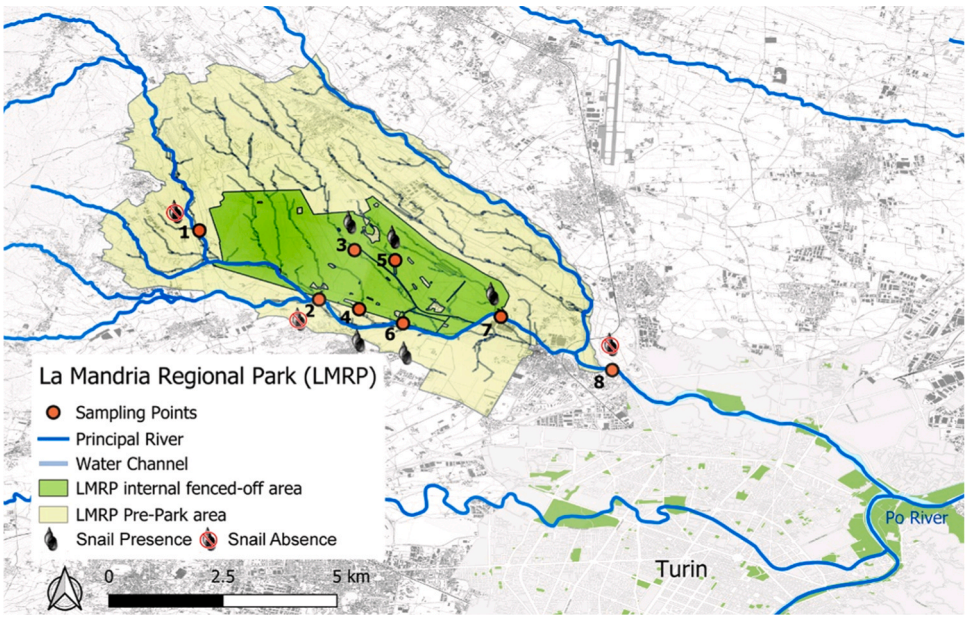

Fig. 1. Map of La Mandria Regional Park - Presence or Absence of lymnaeid snails was determined visionally.

more than 0.9 were included in the dataset. In the case of long reads with query length/subject length (ql/sl) ratio more than 1, al/ql ratio was set to 0.5 to avoid losing long reads hitting short available reference subjects. Finally, duplicated reads were removed by selecting the assignment with the highest percentage of identity among them.

In the case of Kraken2, there is no consensus on the confidence score threshold for taxonomy classification and most previous studies used default score of zero and some recent studies such as Ring and colleagues applied a more stringent score of 0.05 (Ring et al., 2023). Therefore we decided to consider 0.05 confidence score to increase the precision of classification by Kraken2. In both classification datasets, using TaxonKit (v0.18.01) (Shen and Ren, 2021), all assigned TaxIDs were reformatted to an eight-level list from Kingdom to Strain and were added to the datasets for downstream analyses.

2.5. Screening list preparation

A list of species names and TaxIDs was prepared using species present in The Eukaryotic Pathogen, Vector and Host Informatics Resource (VEuPathDB) (Amos et al., 2021), autochthonous (Park's Fauna) or allochthonous (Alien Species) species indicated in La Mandria site-specific measures report (DGR 24-4043, 2016), a DNA-based subset of the 50 zoonotic pathogens preselected for prioritization exercise by the One Health working group of EFSA for environmental detection and published by the ENETWILD consortium (ENETWILD-consortium et al., 2023) (Enetwild), closely-related, co-occurring and species involved in *F. magna* life-cycle (*F. magna*-related) and Gastropod species found in our study. The addition of gastropods to our screening list was due to the lack of information regarding the species presentin the park. This list (Supplementary file 1), including 752 species, was finally used to screen both datasets containing taxonomy-assigned reads from Megablast and kraken2.

2.6. Statistical analyses

On both datasets, the number of reads was down-sampled to the number of reads in the sample with the lowest read number. The Shannon and Simpson indices were calculated for each sampling point and tested with Kruskal-Wallis test statistics. The PCoA analysis

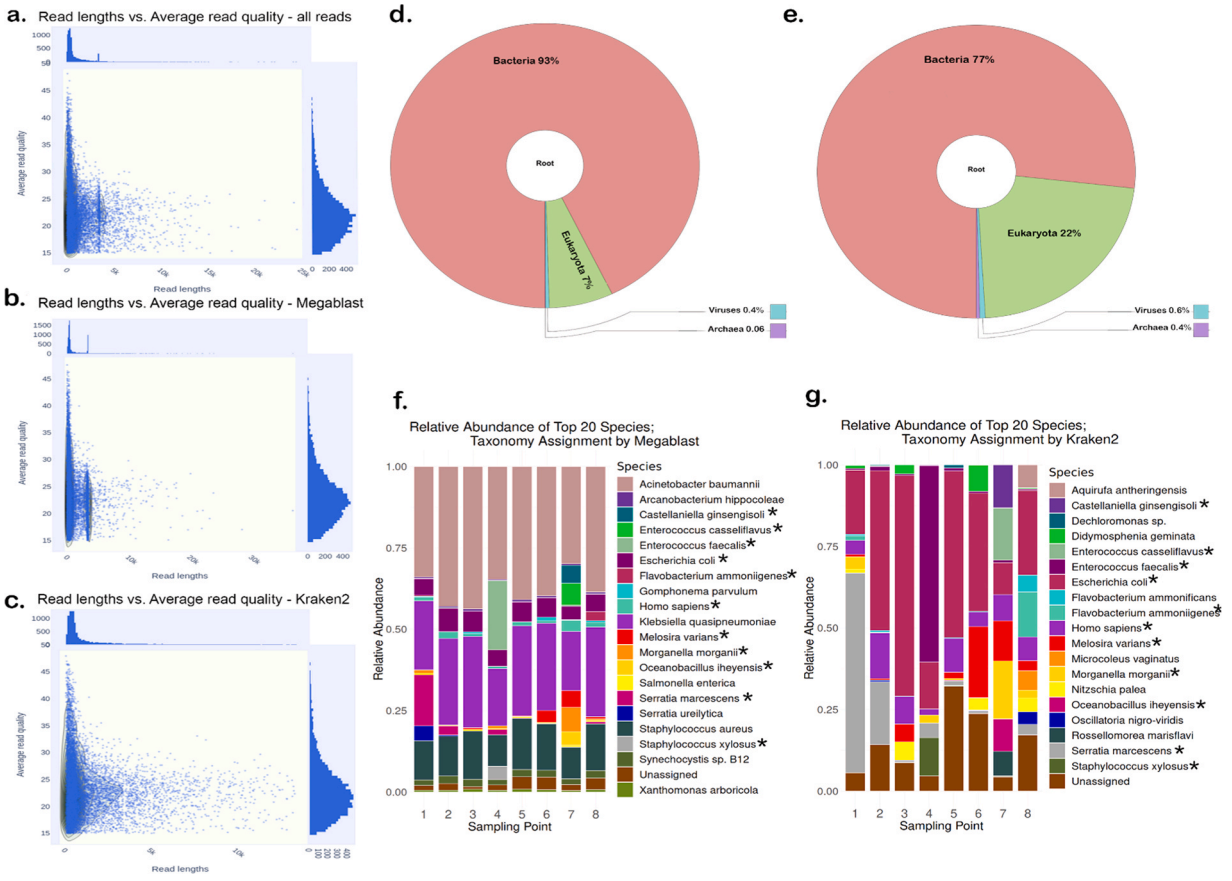

Fig. 2. Read length Vs. Average read quality for a. all unclassified reads, b. classified reads by Megablast (after filtering) c. classified reads by Kraken2 (plot is visualized using NanoPlot (De Coster et al., 2018)). d. and e. General distribution of identified taxa for Megablast and Kraken2. f. and g. Top 20 most abundant species identified by Megablast and Kraken2. (Asterisks indicate species identified in both classification methods.).

of the Bray-Curtis dissimilarity index between sampling points was also performed to investigate the beta diversity. We used the *vegan* package in R for alpha and beta diversity analyses.

2.7. ddPCR for B. dendrobatidis detection

A ddPCR assay for the detection of *B. dendrobatidis* was applied to the environmental samples using ddPCR Supermix for probes (Bio-Rad Laboratories CA, USA) and primers and probe previously described (with FAM and BHQ1 as fluorochromes and quencher respectively) (Boyle et al., 2004) following an optimized protocol for ddPCR (Porco et al., 2024). Briefly, ddPCR reaction had a final volume of 22 μL containing 11 μl ddPCR Supermix for Probes, primer and probe concentrations of 760 nM and 430 nM respectively and 1 μL of eDNA (0.5 μL from each extraction replicate). The PCR cycling program followed the manufacturer's instructions with an annealing temperature of 46°C for *B. dendrobatidis* and results were read on a Bio-Rad QX200 suite (Droplet Digital PCR Applications Guide, 2025). A double-strand synthetic DNA frgament (5'-CCTTGATATAATACAGTGTGCCATATGTCACGAGTCGAA-CAAAATTTATTTATTTTTTCGACAAATTAATTGGAAATTGAATAATTTAATTGAAAAAAATTGAAAATAAATATTAAAAACAACTTTTGA-CAACGGATCTCTTGGCT-3') was constructed (Macrogen Europe) to be used as the positive control for the *B. dendrobatidis* ddPCR assay.

3. Results

After initial filtration using NanoFilt (minimum read length of 100 bases and minimum quality score of 15), the total number of native nanopore sequencing reads was 692,180. The mean reads number per sample achieved 86,105 ranging from 46,741 to 162,870. The reads had a mean and median length of 1,289 bases and 582 bases, respectively, and mean and median Nanopore quality scores of 20 and 21.7 (Fig. 2a). Following taxonomic assignment via Megablast, 48,310 reads remained, with a mean and median read length of 1,352 bases and 562 bases, and Nanopore quality scores of 20.7 and 22.6 (Fig. 2b). Classification with Kraken2 yielded 113,353 reads, with mean and median read lengths of 1,277 and 582 bases, and Nanopore quality scores of 20.5 and 22.3, respectively (Fig. 2c). The Megablast-assigned reads exhibited a mean and median identity percentage of 98.75% and 99.11%, ranging from 95% to 100%.

Taxonomic classification of all environmental samples with Megablast identified 1466 species across 709 genera, whereas Kraken2 identified roughly twice as many species and genera, totaling 2562 and 1223 respectively. The identified taxa distribution revealed a prokaryotic dominance of approximately 93% and 77% with Megablast and Kraken2 methods respectively (Fig. 2d&e). Among the top 20 most abundant species identified, most were bacterial, with some freshwater diatoms, and human DNA in both classifications. Out of 20 top species, 11 species were shared and the rest were different between the results of the two classification methods (Fig. 2f&g). The top 20 species accounted for 88% and 9% of the classified reads for Megablast and Kraken2, respectively, indicating Kraken2's

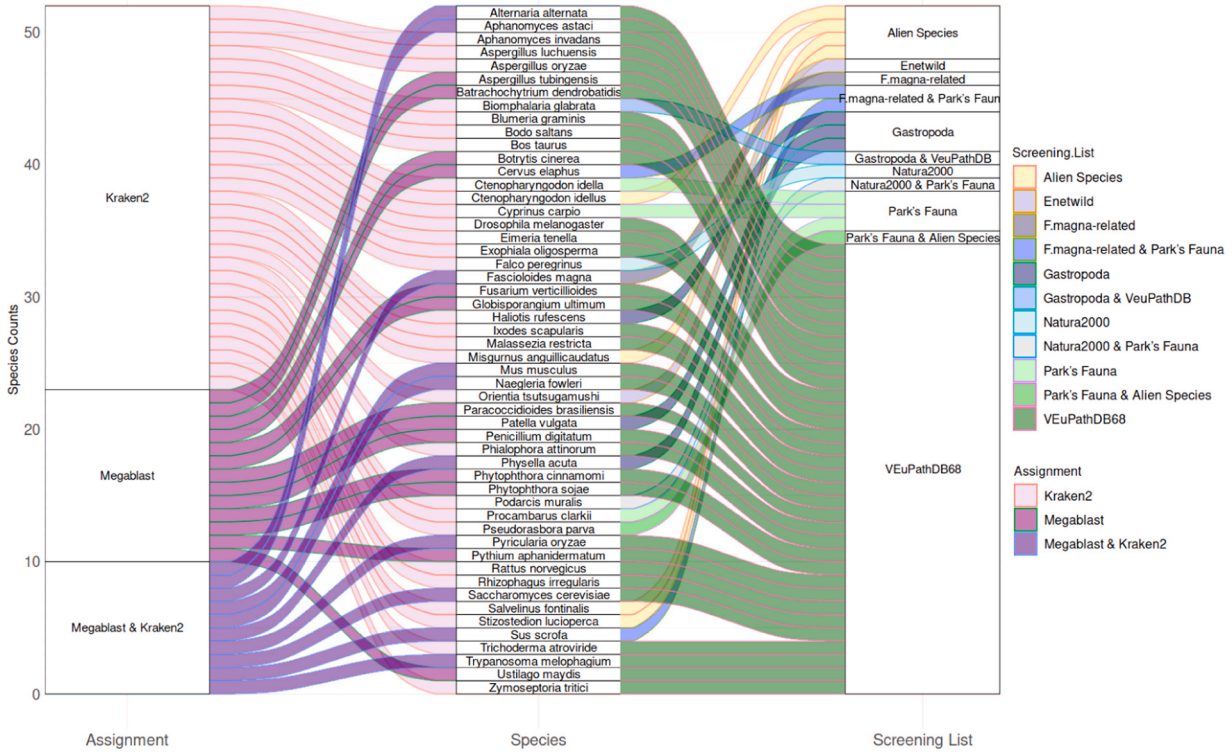

Fig. 3. Identified species by both taxonomy assignment tools and their relation to different subsets of the screening list.

broader identified taxonomic diversity. The majority of classified reads with blast were assigned to species level and to a lesser extent to genera while reads classified with Kraken2 were assigned to different taxonomic levels. The sequencing negative (blank) control contained less than 0.5% (less than 500 reads) of the total sequenced reads and few reads were assigned to *Eschrechia coli, Phenylobacterium zucineum, Morganella morgani and Homo sapiens*.

We evaluated differences in biodiversity richness and evenness across samples (down-sampled to 2994, and 8387 reads in Megablast and Kraken2 classified datasets respectively), finding no statistically significant differences in either dataset (Supplementary Figures a, b, c&d). However, principal component analysis of the Bray-Curtis dissimilarity matrix revealed sample compositions clustering into three distinct groups. No association was identified between these clusters and specific variables such as sampling location in the park or snail presence/absence, except for one consistent cluster: the point immediately downstream of the park (point 7) in both datasets (Supplementary Figures e&f).

We screened both taxonomy assignment datasets against a screening list that included species from various categories: eukaryotic pathogens, vectors, hosts, the park's fauna and flora species of conservation importance, alien species, gastropods identified in this study, *F. magna*, and species involved in its lifecycle. This screening identified 53 unique species, with 13 species identified only by Megablast, 30 only by Kraken2, and 10 overlapping species identified by both tools (Fig. 3). The identified species spanned all categories of the screening list including *F. magna* and its various hosts such as *Cervus elaphus* (definitive host), *Bos taurus* (aberrant host) and *Sus scrofa* (dead-end host). In the previous study, a duplex qPCR test conducted on four replicates of each sample detected *F. magna* at sampling locations 1, 4, and 7, with average copy numbers of 5, 18, and 10, respectively. *G. truncatula* was detected at all locations, with copy numbers ranging from an average of 12–111 (Varzandi et al., 2024). However, nanopore sequencing detected *F. magna* only at sampling location 4 and did not detect *G. truncatula*.

Following screening, most identified species were assigned to the VEuPathDB subset, including *Trypanosoma melophagium* (a sheep parasite transmitted by sheep keds), *B. dendrobatidis* (an amphibian chytrid fungal pathogen), *Pyricularia oryzae* (a fungal plant pathogen), and *Naegleria fowleri* (an ameba causing primary amebic meningoencephalitis in humans). Additionally, species were identified across all subsets of the screening list, including *Physella acuta* (Gastropoda), *Salvelinus fontinalis* (an alien species), *Cyprinus carpio* (part of the park's fauna), *Falco peregrinus* (a Natura 2000 species), and *Orientia tsutsugamushi* (Enetwild).

After screening, the identified species exhibited read counts ranging from one to 12, with most pathogens, including *F. magna* and *B. dendrobatidis*, detected by single reads, showing 98% and 97% identity, respectively, in Megablast results. Given previous amplification-based detection reports, we anticipated the presence of *F. magna* in the environmental samples (Varzandi et al., 2024). However, considering reports of a chytridiomycosis outbreak in the park, we specifically tested for *B. dendrobatidis* and confirmed its presence using a ddPCR assay. The assay detected absolute quantifications of 11, 2, and 8 copies in samples 2, 5, and 7, respectively, across three of our eight sampling locations (Fig. 4) (Meletiadis et al., 2025).

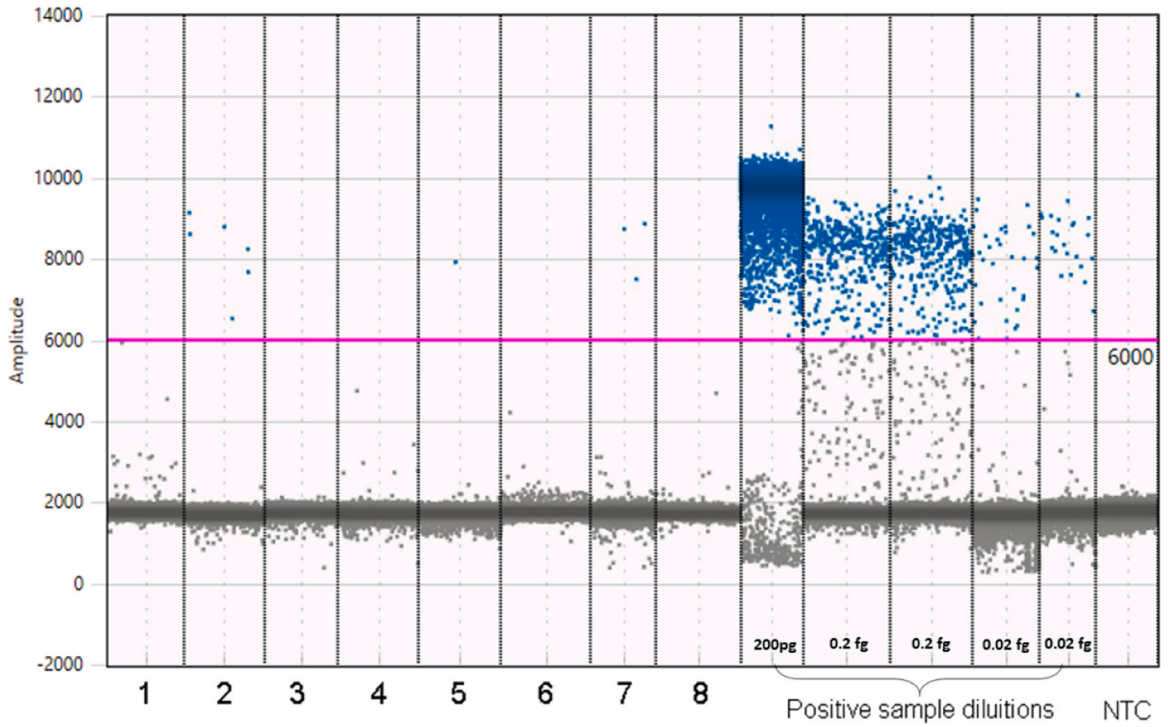

Fig. 4. 1–8 are ddPCR results for pooled sample. The positive samples' concentration range was from 200 picograms to 0.02 femtograms. The amplitude threshold to call positive droplets was considered at 6000, based on positive sample results and in agreement with Bio-Rad's specialists consults.

4. Discussion

In this study, we investigated the biodiversity and pathogen load of environmental samples that could be detected by nanopore native sequencing. Our results demonstrate that nanopore native sequencing not only captures overall biodiversity but also identifies low-abundant, ecologically significant species among them eukaryotic pathogens, parasites, and their potential hosts.

Native nanopore sequencing of samples which were previously tested for the presence of *F. magna* and *G. truncatula*, reaffirmed the previously detected presence of *F. magna*, and furthermore detected several potential hosts along the parasite's lifecycle (Fig. 3). However, native nanopore sequencing detected *F. magna* DNA at only one sampling location and did not detect *G. truncatula*. It is important to acknowledge that native nanopore sequencing is generally less sensitive than amplicon-based methods like qPCR. Nevertheless, this limitation in sensitivity could be further influenced by the presence of a higher abundance of other closely-related species, particularly gastropods such as *Physella acuta*, an invasive species commonly found in freshwater habitats near the study LMRP area (PIANO, 2026). This species originally native to southern Africa is known to be the most prevalent freshwater snail around the world due to its remarkable invasive capability in habitat expansion (Ebbs et al., 2018; Dumidae et al., 2024; Griffiths et al., 2015; Lawton et al., 2018). *P. acuta* may also serve as an intermediate host or transitional carrier for *F. magna*, as recent studies have suggested its involvement in several human trematode infections, including echinostomiasis and fascioliasis, which share similar intermediate hosts within their parasitic lifecycle and biopathogenesis (Kanev, 1994; Dreyfuss et al., 2002). Another contributing factor could be the limited availability of complete genomic references in the NCBI's *core-nt* database, a common challenge in identifying species from PCR-free and native sequencing reads. However, we suspect that the abundance and distribution of *P. acuta* were more influential in its detection than the availability of complete reference genomes, as both species have mitochondrial genomes available in such database.

Our results confirmed the presence of *B. dendrobatidis* inside and downstream area of the park. The detection of *B. dendrobatidis* is significant given its global impact on wildlife health, and given an outbreak in the La Mandria park that was only discovered many months later (Meletiadis et al., 2025). Pathogenic chytrids can infect a broad range of hosts, including other fungi, algae, plants, and amphibians (Taylor et al., 2015). Notably, *B. dendrobatidis* is linked to high mortality rates in amphibians, contributing to global declines and extinctions (Fisher et al., 2009). We could, however, not identify any known amphibian hosts in the park's fauna subset of species for this pathogen within both datasets (Fig. 3); nevertheless, other amphibian species (from genera *Hyla* and *Xenopus*) were detected only by Kraken2. To further verify our findings, we utilized ddPCR, which corroborated our nanopore sequencing results and additionally detected *B. dendrobatidis*'s at other sampling locations, including within the park (Figs. 1&4). Chytridiomycota (true chytrids) are fungi of an aquatic origin, displaying morphological adaptations in the form of zoospores or amoeboid spores that are adapted to dispersal in aquatic habitats (Grossart et al., 2019). They play various ecological roles as bioeroders, decomposers, parasites, and mutualists in freshwater, brackish, and marine habitats, and are also prevalent in soil. In addition to amphibians, *B. dendrobatidis* has been detected in non-amphibian vertebrates and invertebrates; however, their roles as true reservoirs or vectors remain uncertain (Carvalho et al., 2024). *B. dendrobatidis* is a generalist pathogen that can potentially bring a host species into extinction while persisting in other hosts or remaining in environmental stages in lakes, streams, and even in the rain where it has been readily detected (Prado et al., 2023). Importantly, the first case of *B. dendrobatidis* infection and mortality in La Mandria Park was reported in June 2023 (Federici et al., 2008), while our samples had already been collected in the first two weeks of September 2022. Nanopore sequencing and follow-up ddPCR could have therefore detected the presence of *B. dendrobatidis* both inside and outside the park months before the observation of clinical manifestations and mortality cases. This underscores the importance of holistic continuous monitoring in protected or natural parks, representing a significant opportunity for early pathogen detection that can enhance wildlife monitoring programs.

The species identified in our study, particularly pathogens, exhibit a bias toward aquatic species (*B. dendrobatidis*) or pathogens requiring an aquatic environment for parts of their lifecycle (*F. magna*). Therefore, we recommend incorporating soil and water sampling to enhance biodiversity capture. Current literature indicates that most studies employing eNA methods are based on ad-hoc sampling site selection. While eDNA sampling has proven effective for aquatic microorganisms (Cordier et al., 2009; Mansfeldt et al., 2020), detecting terrestrial vertebrates via stream networks is possible with sensitive eDNA protocols, more sampling replicates, and higher filtered water volumes (Lyet et al., 2021). For eNA methods to effectively complement integrated wildlife management, it is advisable to establish a harmonized sampling site selection strategy (e.g., based on water basins and flow segments) to ensure unbiased biodiversity detection across various study areas through carefully planned eDNA sampling events (Altermatt et al., 2023).

The presence of human DNA among the most abundant identified species in all samples, along with the negative (blank) control for sequencing, which showed a very low number of sequenced reads, mostly belonging to human DNA, suggests that the samples contained a high amount of human DNA. This contamination likely originated from the collected samples rather than field or laboratory procedures; however, such a possibility cannot be ruled out. The results obtained from the two taxonomy classifiers differed in both the identified taxa and the number of reads assigned. Megablast produced fewer identified reads, with most classified at the species level, whereas Kraken2 yielded a higher number of identified reads across various taxonomic levels. This variation stems from differences in their classification algorithms. Megablast, a local sequence alignment tool, is optimized for comparing queries to closely related sequences, making it highly effective for identifying sequences with 95% or greater similarity (e.g., within the same species or closely related species). It is also known for its speed. In contrast, Kraken2 is a taxonomic sequence classifier that assigns taxonomic labels by analyzing k-mers within a query sequence. It queries a database that maps k-mers to the lowest common ancestor (LCA) of all genomes containing a given k-mer, allowing for broader taxonomic classification (Altschul et al., 1990; Wood et al., 2019).

Here, we demonstrate that native nanopore sequencing of environmental samples can significantly improve our understanding of wildlife health within the One Health framework. By capturing data across the human-animal-ecosystem health continuum, this

approach has the potential to enable early detection of emerging diseases and biological invasions. Notably, we identified the pathogenic *F. magna* along with its various host species, detected *B. dendrobatidis* months before the first observed mortalities in the park, and detected DNA from diverse array of pathogens posing potential zoonotic or direct public health risks in water samples. While these findings further validate the potential of portable nanopore sequencing as an effective tool for environmental screening (Urban et al., 2023), further optimization and validation regarding the impact of sampling, taxonomy-agnostic nucleic acid extraction, and sequencing depth on taxon detection are necessary before this approach can be widely adopted for environmental screening. Nanopore's direct RNA sequencing approaches might further facilitate the detection of RNA viruses by allowing for the extraction of total or viral RNA using commercially available kits, thus broadening detection capabilities across all three kingdoms of the tree of life (Xue et al., 2024; Nema et al., 2024; Keller et al., 2018; Perlas et al., 2024).The adaptive sampling feature of nanopore sequencing could further enhance monitoring efforts by enabling *in silico* enrichment of prioritized species while concurrently sequencing all available reads in the prepared sequencing library. This would improve the cost-effectiveness of sequencing experiments and streamline downstream data analyses.

5. Conclusions

In conclusion, our study demonstrates the potential of nanopore native sequencing as a tool for environmental biodiversity and pathogen detection with minimal effort, offering valuable insights into wildlife health and ecosystem dynamics. We successfully identified key pathogens, such as *B. dendrobatidis* and *F. magna*, along with their host species, and detected *B. dendrobatidis* months before clinical manifestations, underscoring the value of continuous monitoring for early warning systems in wildlife management. While the approach shows promise, further optimization of sampling strategies, sequencing protocols, and data analysis workflows are needed to maximize its accuracy and applicability for large-scale environmental screening. The integration of nanopore sequencing into wildlife health monitoring programs, particularly through its potential for real-time, portable sequencing, could significantly enhance our ability to manage ecosystems and mitigate the spread of emerging pathogens.

Ethics and consent

No approval of research ethics committees was required to accomplish the goals of this study.

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Competing interests

The authors have no competing interests to declare that are relevant to the content of this article.

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Authors' contributions statement

A.R.V., and E.F. contributed to conceptualization and methodology. A.R.V., contributed to investigation and field samplings. L.U., and E.F. contributed to the supervision of the study. A.R.V., and T.R., conducted the sequencing analyses. A.R.V. contributed to writing the manuscript's original draft. L.U, A.R.V., and E.F. contributed to the critical review and editing of the manuscript. E.F., and S.Z., contributed to Funding acquisition. All authors reviewed the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.gecco.2025.e03517.

Data availability

Data will be made available on request.

References

Altermatt, F., et al., 2023. Quantifying biodiversity using eDNA from water bodies: general principles and recommendations for sampling designs. Environ. DNA 5, 671–682.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Amarasiri, M., Furukawa, T., Nakajima, F., Sei, K., 2021. Pathogens and disease vectors/hosts monitoring in aquatic environments: potential of using eDNA/eRNA based approach. Sci. Total Environ. 796, 148810.

Amos, B., et al., 2021. VEuPathDB: the eukaryotic pathogen, vector and host bioinformatics resource center. Nucleic Acids Res. 50.

Artois, M., et al., 2009. Wildlife disease surveillance and monitoring. Manag. Dis. Wild Mamm. 187–213. https://doi.org/10.1007/978-4-431-77134-0_10.

Barnes, M.A., Turner, C.R., 2016. The ecology of environmental DNA and implications for conservation genetics. Conserv. Genet. 17, 1–17.

Barwell, L., Purse, B., Hesketh, H., 2023. Invasive parasite bio-characteristics. Parasites Biol. Invasions 58–76. https://doi.org/10.1079/9781789248135.0005.

Bass, D., Christison, K.W., Stentiford, G.D., Cook, L.S.J., Hartikainen, H., 2023. Environmental DNA/RNA for pathogen and parasite detection, surveillance, and ecology. Trends Parasitol. 39, 285–304.

Bassi, R., 1875. Sulla Cachessia Ittero-Verminosa, o Marciaia Dei Cervi, Causata Dal Distomum Magnum. (Tip. Il Conte Cavour).

Boyle, D.G., Boyle, D.B., Olsen, V., Morgan, J. a T., Hyatt, A.D., 2004. Rapid quantitative detection of chytridiomycosis (Batrachochytrium dendrobatidis) in amphibian samples using real-time Taqman PCR assay. Dis. Aquat. Organ 60, 141–148.

Cardoso, B., et al., 2022. Stepping up from wildlife disease surveillance to integrated wildlife monitoring in Europe. Res. Vet. Sci. 144, 149–156.

Carvalho, T., Belasen, A.M., Toledo, L.F., James, T.Y., 2024. Coevolution of a generalist pathogen with many hosts: the case of the amphibian chytrid *Batrachochytrium dendrobatidis*. Curr. Opin. Microbiol. 78, 102435.

Ciuffreda, L., Rodríguez-Pérez, H., Flores, C., 2021. Nanopore sequencing and its application to the study of microbial communities. Comput. Struct. Biotechnol. J. 19, 1497.

Cordier, T. et al. Patterns of eukaryotic diversity from the surface to the deep-ocean sediment. Sci. Adv., 8, eabj9309.

De Coster, W., D'Hert, S., Schultz, D.T., Cruts, M., Van Broeckhoven, C., 2018. NanoPack: visualizing and processing long-read sequencing data. Bioinformatics 34, 2666–2669.

Deiner, K., et al., 2017. Environmental DNA metabarcoding: transforming how we survey animal and plant communities. Mol. Ecol. 26, 5872–5895.

Delahaye, C., Nicolas, J., 2021. Sequencing DNA with nanopores: troubles and biases. PLoS One 16, e0257521.

DGR 24-4043_2016_All G MdC_IT1110079_MANDRIA.pdf. ⟨https://download.mase.gov.it/Natura2000/Materiale%20Designazione%20ZSC/Piemonte/04_Misure%20di%20Conservazione/DGR%2024-4043_2016_All%20G%20MdC_IT1110079_MANDRIA.pdf⟩.

Doorenspleet, K., 2023. et al. The long and the short of it: Nanopore based eDNA metabarcoding of marine vertebrates works; sensitivity and specificity depend on amplicon lengths. 2011.11.26.470087 Preprint at https://doi.org/10.1101/2021.11.26.470087.

Douchet, P. et al. Make visible the invisible: Optimized development of an environmental DNA metabarcoding tool for the characterization of trematode parasitic communities. Environmental DNA n/a,.

Dreyfuss, G., Vignoles, P., Abrous, M., Rondelaud, D., 2002. Unusual snail species involved in the transmission of Fasciola hepatica in watercress beds in central France. Parasite 9, 113–120.

Droplet Digital PCR Applications Guide. ⟨https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf⟩.

Dumidae, A., et al., 2024. Genetic diversity and population structure of Physella acuta (Gastropoda: Physidae) in Thailand using mitochondrial gene markers: COI and 16S rDNA. Sci. Rep. 14, 13161.

Ebbs, E.T., Loker, E.S., Brant, S.V., 2018. Phylogeography and genetics of the globally invasive snail Physa acuta Draparnaud 1805, and its potential to serve as an intermediate host to larval digenetic trematodes. BMC Evolut. Biol. 18, 103.

EFSA. 2023⟨https://www.efsa.europa.eu/en/supporting/pub/en-8241⟩.

ENETWILD-consortium, et al., 2023. A pilot on integrated wildlife monitoring at European scale: environmental detection of selected pathogens in the European Observatory of Wildlife. EFSA Support. Publ. 20.

European Commission: Directorate-General for Environment, Mézard, N., Sundseth, K. & Wegefelt, S. Natura 2000 – Protecting Europe's Biodiversity. (European Commission, 2008). doi:10.2779/45963.

Federici, S., et al., 2008. Identification of the pathogen Batrachochytrium dendrobatidis in amphibian populations of a plain area in the Northwest of Italy. Herpetol. Notes 1, 33–37.

Ficetola, G.F., Miaud, C., Pompanon, F., Taberlet, P., 2008. Species detection using environmental DNA from water samples. Biol. Lett. 4, 423–425.

Fisher, M.C., Garner, T.W.J., Walker, S.F., 2009. Global emergence of Batrachochytrium dendrobatidis and amphibian chytridiomycosis in space, time, and host. Annu. Rev. Microbiol. 63, 291–310.

GitHub – nanoporetech/dorado: Oxford Nanopore's Basecaller. ⟨https://github.com/nanoporetech/dorado⟩.

Goldberg, C.S., et al., 2016. Critical considerations for the application of environmental DNA methods to detect aquatic species. Methods Ecol. Evol. 7, 1299–1307.

Gortázar, C., Ruiz-Fons, J.F., Höfle, U., 2016. Infections shared with wildlife: an updated perspective. Eur. J. Wildl. Res. 62, 511–525.

Griffiths, C.L., Day, J.A. & Picker, M., 2015. Freshwater Life: A Field Guide to the Plants and Animals of Southern Africa. (Struik Nature).

Grossart, H.-P., et al., 2019. Fungi in aquatic ecosystems. Nat. Rev. Microbiol. 17, 339–354.

Hering, D., et al., 2018. Implementation options for DNA-based identification into ecological status assessment under the European Water Framework Directive. Water Res. 138, 192–205.

Hopkins, S.R., et al., 2022. Environmental persistence of the world's most burdensome infectious and parasitic diseases. Front. Public Health 10, 892366.

Ip, C.L.C., et al., 2015. MinION analysis and reference consortium: phase 1 data release and analysis. F1000Res 4, 1075.

Kanev, I., 1994. Life-cycle, delimitation and redescription of Echinostoma revolutum (Froelich, 1802) (Trematoda: Echinostomatidae). Syst. Parasitol. 28, 125–144.

Keller, M.W., et al., 2018. Direct RNA sequencing of the coding complete influenza A virus genome. Sci. Rep. 8, 14408.

Lawton, S.P., Allan, F., Hayes, P.M., Smit, N.J., 2018. DNA barcoding of the medically important freshwater snail Physa acuta reveals multiple invasion events into Africa. Acta Trop. 188, 86–92.

Lyet, A., et al., 2021. eDNA sampled from stream networks correlates with camera trap detection rates of terrestrial mammals. Sci. Rep. 11, 11362.

Mansfeldt, C., et al., 2020. Microbial community shifts in streams receiving treated wastewater effluent. Sci. Total Environ. 709, 135727.

Meletiadis, A., et al., 2025. Prevalence of batrachochytrium dendrobatidis in amphibians in Northwestern Italy's Protected Areas. Animals 15, 157.

Nema, R.K., et al., 2024. Protocol for Detection of pathogenic enteric RNA viruses by regular monitoring of environmental samples from wastewater treatment plants using Droplet Digital PCR. Sci. One Health, 100080. https://doi.org/10.1016/j.soh.2024.100080.

Perlas, A. et al., 2024. Latest RNA and DNA nanopore sequencing allows for rapid avian influenza profiling, 2024.02.28.582540 Preprint at https://doi.org/10.1101/2024.02.28.582540.

PIANODI GESTIONE - Zona Speciale di Conservazione Monte Musine' e laghi di Caselette.

Porco, D., et al., 2024. eDNA-based monitoring of Batrachochytrium dendrobatidis and Batrachochytrium salamandrivorans with ddPCR in Luxembourg ponds: taking signals below the Limit of Detection (LOD) into account. BMC Ecol. Evol. 24, 4.

Prado, J.S., Ernetti, J.R., Pontes, M.R., Toledo, L.F., 2023. Chytrid in the clouds: an alternative passive transport of a lethal pathogen for amphibians. Hydrobiologia 850, 2061–2073.

Quick, J., Quinlan, A.R., Loman, N.J., 2014. A reference bacterial genome dataset generated on the MinION™ portable single-molecule nanopore sequencer. Gigascience 3, 22.

Ring, N., et al., 2023. Rapid metagenomic sequencing for diagnosis and antimicrobial sensitivity prediction of canine bacterial infections. Micro Genom. 9.

Ruppert, K.M., Kline, R.J., Rahman, M.S., 2019. Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: a systematic review in methods, monitoring, and applications of global eDNA. Glob. Ecol. Conserv. 17, e00547.

Shen, W., Ren, H., 2021. TaxonKit: a practical and efficient NCBI taxonomy toolkit. J. Genet. Genom. 48, 844–850.
Sokolow, S.H., et al., 2022. Ecological and socioeconomic factors associated with the human burden of environmentally mediated pathogens: a global analysis. Lancet Planet. Health 6, e870–e879.
Stevens, B.M., Creed, T.B., Reardon, C.L., Manter, D.K., 2023. Comparison of Oxford Nanopore Technologies and Illumina MiSeq sequencing with mock communities and agricultural soil. Sci. Rep. 13, 9323.
Struelens, M.J., et al., 2024. Real-time genomic surveillance for enhanced control of infectious diseases and antimicrobial resistance. Front. Sci. 2.
Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., Willerslev, E., 2012. Towards next-generation biodiversity assessment using DNA metabarcoding. Mol. Ecol. 21, 2045–2050.
Taylor, T.N., Krings, M., Taylor, E.L., 2015. 4 - Chytridiomycota. In: Taylor, T.N., Krings, M., Taylor, E.L. (Eds.), Fossil Fungi. Academic Press, San Diego, pp. 41–67. https://doi.org/10.1016/B978-0-12-387731-4.00004-9.
Urban, L., et al., 2023. Real-time genomics for one health. Mol. Syst. Biol. 19, e11686.
Varzandi, A.R., et al., 2024. Development of a qPCR Duplex Assay for simultaneous detection of Fascioloides magna and Galba truncatula in eDNA samples: monitoring beyond boundaries. Sci. Total Environ. 916, 170338.
Violle, C., et al., 2007. Let the concept of trait be functional! Oikos 116, 882–892.
Wheeler, Q.D., Raven, P.H., Wilson, E.O., 2004. Taxonomy: impediment or expedient? Science 303, 285–285.
Wick, R. , 2024. rrwick/Porechop.
Wood, D.E., Lu, J., Langmead, B., 2019. Improved metagenomic analysis with Kraken 2. Genome Biol. 20, 257.
Xue, Z., et al., 2024. Environmental RNA metabarcoding for ballast water microbial diversity: minimizing false positives. Sci. Total Environ. 955, 176902.
Zemanova, M.A., 2021. Noninvasive genetic assessment is an effective wildlife research tool when compared with other approaches. Genes 12, 1672.