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DATA DESCRIPTOR

The FunAqua dataset of global fungal biodiversity in aquatic ecosystems

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Advances in high-throughput DNA sequencing technologies have accelerated research on aquatic microorganisms, their diversity, and ecological functions. This has also significantly advanced research on fungi that shape aquatic ecosystems and food webs. Here, we present the FunAqua dataset, which describes global fungal biodiversity in aquatic ecosystems based on eukaryotic long-read metabarcoding data from 1,076 sediment and 1,299 filtered water samples collected across 1,452 sampling sites in 87 countries. We targeted the internal transcribed spacer (ITS) region. In total, 50,152 fungal OTUs were recorded. The sequencing data is accompanied by comprehensive sample metadata, including physicochemical data of water and sediments. The FunAqua dataset provides a new resource for analysing and better understanding the biodiversity of fungi in different types of aquatic habitats across biogeographical regions. The dataset described in this data paper will be periodically updated with additional metabarcoding data.

Background & Summary

Fungi are ubiquitous in aquatic environments, thriving in both marine and freshwater habitats¹. They are important contributors to the functioning of aquatic ecosystems, primarily as parasites, decomposers, or mutualists. Parasitic fungi can regulate toxic algal blooms and, in the process, produce zoospores edible to zooplankton, promoting trophic transfer via the mycoloop². In freshwater streams, the decomposition of plant material, including leaves, twigs and pollen, is primarily driven by fungi³. Lastly, previous research indicates that these fungi establish various types of mutualistic relationships with different organisms. For example, arbuscular mycorrhizal fungi colonise the roots⁴ of aquatic plants. There are also semiaquatic lichens and corresponding lichenicolous⁵ fungi.

Although fungi in aquatic ecosystems play diverse and ecologically significant roles, they are understudied and their global diversity is poorly documented compared to other aquatic microorganisms and terrestrial fungi⁶. Furthermore, metabarcoding has revealed an abundance of aquatic dark matter fungi, which lack ecological, taxonomic, and morphological descriptions, highlighting the need for further research⁷. A more comprehensive understanding of aquatic fungal communities is essential for defining their ecological roles, including broader impacts that they have on ecosystem functioning as well as the environmental factors shaping their diversity and distribution.

Advances in high-throughput sequencing technologies, particularly metabarcoding, have revealed an unexpectedly high aquatic fungal diversity that has remained undetected by microscopy and cultivation-based methods⁸. However, current knowledge about global aquatic fungal biodiversity and distribution still remains limited, and fungal sequences isolated from aquatic samples are underrepresented in reference databases⁹. Two online resources, <https://marinefungi.org>¹⁰ and <https://freshwaterfungi.org>¹¹ document and classify marine and freshwater fungi, respectively. Additionally, a database named ParAquaSeq contains rRNA sequences of zoosporic parasites, including zoosporic parasitic fungi¹². Nevertheless, comprehensive, publicly available metabarcoding datasets with a global scope and uniform methodology are still lacking for the vast majority of fungi in aquatic ecosystems.

Assembling datasets from published sequencing data is challenging due to variations in methodology and insufficient metadata¹³. These challenges can significantly impact research outcomes and limit the ability to

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conduct comparative analyses. To advance our understanding of the diversity of fungi in aquatic ecosystems, we initiated the collaborative project “FunAqua”. This project aims to explore and document the diversity of fungi in aquatic ecosystems and the environmental factors that shape their biodiversity, contributing to a deeper understanding of their ecological roles and distribution patterns in both freshwater and marine ecosystems. Here we provide and describe the FunAqua dataset which comprises eukaryotic sequences obtained from 1,299 filtered water and 1,076 sediment samples collected from 1,452 sampling sites in 87 countries across all continents (Fig. 1a,b). Metabarcoding was based on amplification and sequencing of the entire internal transcribed spacers (ITS) region¹⁴. The ITS region is a universal fungal barcode¹⁵ and has the most extensive reference database coverage among the rRNA operon barcodes¹⁶. In addition to the raw and processed sequencing data, the FunAqua dataset is accompanied by detailed environmental metadata, providing valuable context for interpreting community composition and ecological patterns.

Methods

Organization. The FunAqua project was established as a global, collaborative, voluntary initiative to advance the understanding of fungal biodiversity across diverse freshwater and marine ecosystems in countries around the world (Fig. 1). To ensure the robustness and comparability of data across a range of different aquatic environments, protocols were developed for all key steps, including sample collection, filtration, preservation and transportation of water and sediment samples. Two protocol versions were provided to participants: (1) basic protocols for general sample collection, suitable for a wide range of interested users; and (2) advanced protocols for researchers with access to specific equipment to collect additional environmental data (e.g., depth, water temperature, pH, and dissolved oxygen). Sampling and sample processing protocols are published and described in detail in Panksep *et al.* (2023¹⁷, 2025¹⁸).

While some variation was inevitable due to local and environmental constraints (e.g., the type of filters and the preservatives used), all participating teams adhered to the core guidelines to ensure data consistency. Any deviations were transparently documented in the metadata report sheet (Supplementary Item 1) to support accurate interpretation and data standardisation.

After sampling, collaborators sent their samples to a central laboratory at the Estonian University of Life Sciences (EMU), where all samples were registered and stored until further processing. All molecular analyses, including DNA extraction, PCR amplification, and library preparation were conducted at the Mycology and Microbiology Center, University of Tartu (UT), ensuring centralised quality control and consistency across laboratory workflows.

Sampling. Between March 2018 and September 2024, a total of 1,126 sediment samples, 1,351 filtered water samples and 558 unfiltered water samples, collected by the consortium, were analysed. Filtered water samples and sediment samples were collected for metabarcoding, and unfiltered water for chemical analyses. Of the processed samples, 1,076 sediment and 1,299 filtered water samples were successfully sequenced and are included in the current version of the dataset. Metabarcoding data from the remaining samples will be incorporated with future dataset updates.

Water for eDNA metabarcoding was filtered using 12 different types of filters, including SterivexTM units (n = 754; Millipore, USA), cellulose-based (n = 313), glass-filled polytetrafluoroethylene (n = 2), polyvinylidene fluoride (n = 12), nylon (n = 11), polycarbonate (n = 69), polyethylene (n = 8) and polyethersulfone (n = 49) filters. The filters used for 80 of the samples were not specified by the sample providers. The majority of the filters (n = 1,285) had a pore size of 0.2–0.22 µm as predefined in the protocol. A small subset (n = 14) of samples were filtered with filters that had a pore size of 0.1 µm. Water collected for filtration was filtered immediately after sampling on-site or within the day of sample collection. Both sediment and filtered water samples were immediately preserved in ethanol and stored at -20 °C until further processing. In locations where maintaining a cold chain for sample preservation and transportation was not feasible, samples (n = 397) were preserved using Longmire’s buffer¹⁹, which is widely employed in eDNA workflows for its ability to preserve nucleic acids at ambient temperature and inhibit nuclease activity²⁰. Samples preserved in ethanol were shipped frozen on dry ice or ice packs in insulated shipping containers for subsequent molecular and chemical analyses.

Molecular analysis. *DNA extraction.* Sediment samples were placed in envelopes and dried overnight in an air-drying cabinet at 35 °C. The dried material was then transferred to 2-mL tubes and homogenised using an MM 200 Mixer Mill (RETSCH, Germany) with three 2-mm metal beads at 30 Hz for 10 mins. DNA was extracted from 0.2 g of homogenised sediment samples using the MagAttract PowerSoil DNA Kit (Qiagen, Germany) and a KingFisherTM Flex machine (Thermo Fisher Scientific), according to the manufacturer’s protocol. Additional DNA extractions were conducted on sediment samples (n = 367) that failed to amplify during the initial PCR run. The re-extractions followed the same protocol without modifications, as the aim was to verify that the absence or the low number of sequences was not due to issues in the original DNA extraction process.

DNA from filtered water samples was extracted using the NucleoMag[®] DNA/RNA Water kit (Macherey-Nagel, Germany) with a modified protocol performed on the KingFisherTM Flex machine. Additional steps were incorporated to extract the DNA from the preservation buffer in addition to the filter. The buffer was transferred from the filters to 2-mL tubes and centrifuged for 5 mins at 11,000 g. The supernatant was removed, and the remaining pellet was dried at 56 °C. SterivexTM filter units were dried at 56 °C to evaporate any remaining buffer. Other types of filters were removed from their tubes in a laminar flow hood, air-dried on sterile petri dishes, and transferred into 5-mL type A bead tubes. Then, 900 µL of C1 and MWA1 lysis solutions were added to the dried pellets from SterivexTM tubes and non-SterivexTM tubes, respectively. The pellets were resuspended and mixed with the lysis solution by pipetting 10 times, and the solution was transferred back onto the original respective filters. If no buffer remained in the Sterivex units, buffer C1 was added directly to the filter. The rest

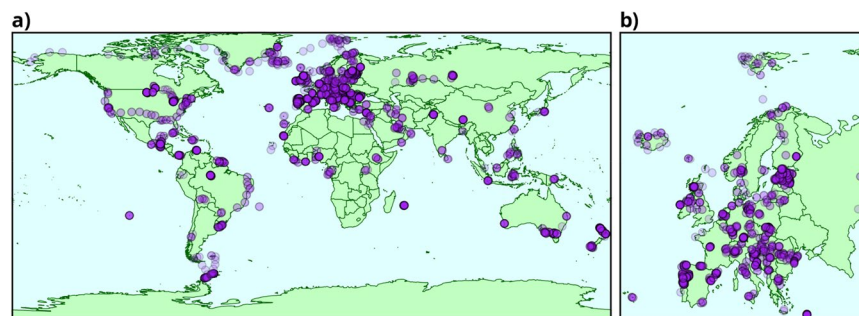


Fig. 1 (a) Global distribution of sampling sites for the FunAqua dataset. (b) Zoomed in view of the European sampling plots.

of the extraction process largely followed the manufacturer's instructions with two exceptions. The incubation step at 70 °C was extended from 5 mins to 10 mins to enhance the lysis of fungal cell walls. Secondly, the binding, washing and elution steps were performed according to the manufacturer's protocol, but on a KingFisher™ Flex machine.

PCR amplification and preparation for sequencing. PCR amplification of the rRNA operon full ITS region (average amplicon length 866 bp) was performed using the ITS9mun/ITS4ngsuni primer pair¹⁴. Both primers were indexed with one of 115 12-base indices that differed from each other by at least four nucleotides²¹. The PCR reactions were carried out in two 25-μL replicates, which were pooled thereafter. The PCR reaction mix contained 5 μL of 5 × HOT FIREPol Master Mix (Solis BioDyne, Estonia), 0.5 μL of both reverse and forward primers (20 μM), 1 μL of template DNA, and 18 μL of ddH₂O. Thermal cycling included initial denaturation at 95 °C (15 min), followed by 30 cycles of denaturation at 95 °C (30 seconds), primer annealing at 55 °C (30 seconds) and extension at 72 °C (1 min). The program ended with a final extension step at 72 °C (1 min). PCR products were visualised on a 1% agarose gel under UV illumination at 280 nm. Products with a weak or no visible band were reamplified using 33–38 cycles with an increased (2–3 μL) amount of DNA. If increasing the amount of template DNA did not yield visible bands on an agarose gel, the amount of template DNA was decreased to 0.5 μL to account for potential PCR inhibition due to co-extracted chemicals. Each PCR reaction included a negative control to check for cross-contamination and a positive control (UniSpike, an artificial DNA molecule) to ensure that any amplification failures were not due to systemic issues.

PCR products of various amplicons were pooled based on band intensity observed on agarose gels. Pooled libraries were purified using the FavorPrep™ GEL/PCR Purification kit (FAVORGEN BIOTECH CORP, Taiwan) following a modified protocol. Specifically, pooled libraries were mixed with 3 volumes of FADF buffer (instead of the standard 5 volumes), and purification was performed in duplicates using two separate columns. Each column was eluted in 60 μL of the elution buffer, and the resulting eluates were combined to yield a final volume of 120 μL.

The pooled libraries were sent to the Norwegian Sequencing Centre (University of Oslo, Norway) for library preparation and sequencing. Library preparation and DNA sequencing were performed using the PacBio Sequel II System (Pacific Biosciences, Palo Alto, USA) as described previously²¹.

Bioinformatics. Bioinformatic processing followed the open-source NextITS workflow v1.0.0²², orchestrated with Nextflow v25.04.6²³ and containerised with Singularity²⁴. Demultiplexing of PacBio HiFi reads (circular consensus sequences, CCS) was performed with LIMA v2.12.0 (Pacific Biosciences). For primer trimming, cutadapt v5.0²⁵ was used with two errors allowed and a minimum overlap of 10 nucleotides for both forward and reverse primers. Reads lacking both primer sites in the correct orientations were discarded. Sequences shorter than 200 nucleotides were removed. During quality filtering, we excluded reads with more than 0.6 expected errors per 100 bp (computed from Phred quality scores on a per-read, length-normalized basis), homopolymers >25 nucleotides, and >4 ambiguous bases. Full-length ITS regions were retrieved with ITSx v1.1.3²⁶. Chimeric sequences were removed using a two-step scheme by first applying *de novo* (with a maximum chimera score of 0.6)²⁷ and then reference-based filtering (using the EUKARYOME v1.9.3 database)¹⁶ algorithms within VSEARCH v2.29.4²⁸.

Tag-jump (index cross-talk) removal was performed using the UNCROSS2 algorithm²⁹. Dereplicated, abundance-sorted reads were denoised using UNOISE3³⁰, with parameters alpha = 6 and minsize = 1, and clustered into OTUs at 98% pairwise sequence similarity using VSEARCH v2.29.4²⁸. Taxonomic annotation of representative sequences was performed using BLASTn v2.16.0+, where EUKARYOME v1.9.3¹⁶ served as a reference database.

Chemical analysis. *Unfiltered water samples.* Chemical analysis was conducted on a subset of 558 unfiltered water samples, which were sent frozen to EMU. The analyses included measurements of electrical conductivity, pH, total phosphorus (TP), and total nitrogen (TN). Conductivity and pH were measured with XS COND 8 (XS Instruments, Italy) and inoLab® pH 7110 (WTW) benchtop meters (Xylem Analytics, Germany), respectively. TP concentrations were determined using the molybdenum blue spectrophotometric method³¹ after oxidation to phosphate (PO₄³⁻) with potassium persulfate (K₂S₂O₈). This method conforms to the international

File name	File function	Description	File format
FunAqua_metadata.tsv	Sample data	Metadata corresponding to each sample	Tab-delimited text file (.tsv)
FunAqua_fungi_otu.csv	OTU abundance data	Table containing sequence counts of each fungal OTU in each sample	Comma-separated text file (.csv)
FunAqua_euk_otu.csv	OTU abundance data	Table containing sequence counts of each non-fungal OTU in each sample	Comma-separated text file (.csv)
FunAqua_fungi_tax.csv	Taxonomy table	Table with taxonomic classification data for fungal OTUs	Comma-separated text file (.csv)
FunAqua_euk_tax.csv	Taxonomy table	Table with taxonomic classification data for non-fungal OTUs	Comma-separated text file (.csv)
FunAqua_Water chemistry_Data.csv	Water chemistry data	Table containing results of water chemistry analyses	Comma-separated text file (.tsv)
FunAqua_sediment_chemistry.csv	Sediment elemental analysis data	Table containing results of sediment elemental analyses	Comma-separated text file (.csv)
FunAqua_sediment_chemistry_references.csv	Sediment elemental analysis reference data	Includes analyses of calibration ranges for reference data	Comma-separated text file (.csv)

Table 1. FunAqua dataset composition.

standard for the determination of phosphorus in ground, surface, and waste waters³². TN concentrations were determined using the ultraviolet spectrophotometric screening method³³ after oxidation to nitrate (NO_3^-) with $\text{K}_2\text{S}_2\text{O}_8$. This method conforms to the international standard for determination of nitrogen using oxidative digestion with peroxodisulfate³⁴.

Sediment samples. Nutrient analysis was performed for a subset of 463 sediment samples collected from 382 sampling sites. Dried sediment samples (0.25 g) were transferred into 50-mL PTFM vessels and dissolved in HNO_3 , using the Multiwave Pro (Anton Paar, Austria) system equipped with a 24HVT50 rotor (Anton Paar). The digestion step followed the EPA 3051 A standard method³⁵. The Montana soil sample NIST® 2711a (National Institute of Standards and Technology, USA) was included in each digestion sequence as a quality control.

Digests were analysed using the 4210 MP-AES (Agilent, USA) instrument equipped with CETAC™ U5000AT +Ultrasonic Nebulizer (ThermoFisher Scientific) to enhance analytical signal strength. Calibration ranges of elements and analytical wavelengths for each element are provided in the dataset (see the Data Records chapter). Sediment samples from batches 1, 2 and 4 were diluted 10-fold with sterile deionized water prior to the analysis. Samples that were fully dissolved during digestion were analysed separately in batch 3 after a 30-fold dilution due to the high element concentrations in the digest. NIST® 2711a control samples were also diluted 30-fold for consistency.

A secondary calibration standard was run for every 15–20 samples throughout the analytical sequence. A third-order polynomial fit of the secondary calibration data was used to correct for a time-dependent instrumental drift. For quality control, a 10-fold dilution of NIST® 1643 F (Trace Elements in Natural Water, National Institute of Standards and Technology), spiked with 1 mg/L phosphorus using a single-element P standard, was included in each analytical run alongside the secondary calibration standards. Average recoveries from the NIST® 1643 F and NIST® 2711a are presented in the dataset (see the Data Records chapter). Duplicate measurements of the same sample aliquots within a single analytical run showed <5% deviation in elemental concentrations.

Data Records

The dataset is accessible via [Zenodo](#)³⁶. The dataset is organized into 8 parts (Table 1): the metadata table, the water chemistry dataset, the sediment elemental analysis data, and the sediment elemental analysis reference data; the fungal and non-fungal OTU tables; and the fungal and non-fungal taxonomy tables. A data dictionary file describing all fields, including units of measurement wherever applicable, is also included in the dataset.

Raw sequencing data can be obtained at the European Nucleotide Archive under bioproject PRJEB104083³⁷ (biosamples SAMEA120633854 - SAMEA120634929; SAMEA120637523 - SAMEA120638821) in FASTQ format. The demultiplexed sequences have been trimmed of primers and indices. OTU representative sequences (FASTA files), OTU tables and associated taxonomic assignments will also be published through the PlutoF platform and made available through GBIF as DNA-derived biodiversity occurrence data.

The FunAqua dataset is published under Creative Commons BY 4.0 license and is freely available to use.

Metadata. To ensure consistency in data reporting and facilitate downstream analyses, a unified metadata template was developed, which standardised the recording of sample-specific information, environmental variables, and any protocol deviations, enabling the integration of data into a comprehensive, harmonised dataset.

The metadata and biological data structure are detailed in the following tables: Table 2 presents the fields used for FunAqua sample metadata. Table 3 defines the fields of the FunAqua OTU abundance data, where the OTU field serves as the primary key variable linked to the taxonomic table, and the remaining fields represent

Column name	Dataset	Description
sample_code	General metadata	Identifier for samples
plot_name	General metadata	Name or identifier of the sampling plot or site
sample_tue	General metadata	Accession IDs assigned by the University of Tartu repository
sample_source	General metadata	Environmental matrix from which the sample was collected
sample_source_description	General metadata	Additional details about the sample source
sampling_point_depth	General metadata	Depth at which the sample was collected
date	General metadata	Date of sample collection in ISO format
country	General metadata	Country of sample collection
continent	General metadata	Continent of sample collection
location_name	General metadata	Specific location name of the sample collection
lat	General metadata	Latitudinal coordinate of the sampling location
long	General metadata	Longitudinal coordinate of the sampling location
biome	General metadata	Ecological biome classification of the sampling site (e.g., freshwater lake, river, wetland)
elevation	General metadata	Elevation of the waterbody surface during observation relative to the sea level
amount_filtered	General metadata	Volume of material filtered during sample processing
filter_type	General metadata	Type of filter used for sample processing
filter_pore_size	General metadata	The size of the filter pores
plot_name	Water chemistry data	Name or identifier of the sampling plot or site
Conductivity	Water chemistry data	Electrical conductivity of the sample
pH	Water chemistry data	Measure of the acidity or alkalinity of the sample
TP	Water chemistry data	Total phosphorus concentration in the sample
TN	Water chemistry data	Total nitrogen concentration in the sample
plot_name	Sediment chemistry data	Name or identifier of the sampling plot or site
sample_weight	Sediment chemistry data	Mass of the sediment sample used for analysis
P_mg_per_kg	Sediment chemistry data	Phosphorus concentration
P_SD	Sediment chemistry data	Standard deviation of phosphorus concentration
Fe_mg_per_kg	Sediment chemistry data	Iron concentration
Fe_SD	Sediment chemistry data	Standard deviation of iron concentration
Mg_mg_per_kg	Sediment chemistry data	Magnesium concentration
Mg_SD	Sediment chemistry data	Standard deviation of magnesium concentration
Na_mg_per_kg	Sediment chemistry data	Sodium concentration
Na_SD	Sediment chemistry data	Standard deviation of sodium concentration
Ca_mg_per_kg	Sediment chemistry data	Calcium concentration
Ca_SD	Sediment chemistry data	Standard deviation of calcium concentration
K_mg_per_kg	Sediment chemistry data	Potassium concentration
K_SD	Sediment chemistry data	Standard deviation of potassium concentration
batch	Sediment chemistry data	Name of the measurement batch
sample	Sediment chemistry data	Name of the reference material used
P_mg_per_kg	Sediment chemistry data	Phosphorus concentration
P_SD	Sediment chemistry data	Standard deviation of phosphorus concentration
Fe_mg_per_kg	Sediment chemistry reference data	Iron concentration
Fe_SD	Sediment chemistry reference data	Standard deviation of iron concentration
Mg_mg_per_kg	Sediment chemistry reference data	Magnesium concentration
Mg_SD	Sediment chemistry reference data	Standard deviation of magnesium concentration
Na_mg_per_kg	Sediment chemistry reference data	Sodium concentration
Na_SD	Sediment chemistry reference data	Standard deviation of sodium concentration
Ca_mg_per_kg	Sediment chemistry reference data	Calcium concentration
Ca_SD	Sediment chemistry reference data	Standard deviation of calcium concentration
K_mg_per_kg	Sediment chemistry reference data	Potassium concentration
Continued		

Column name	Dataset	Description
K_SD	Sediment chemistry reference data	Standard deviation of potassium concentration
Batch	Sediment chemistry reference data	Name of the measurement batch

Table 2. Fields for the FunAqua metadata.

Field	Description
OTU	OTU query ID. Key variable for this table and the taxonomic table (see Table 4).
Remaining fields	Field names are unique sample identifiers. Values are OTU abundance numbers of the OTU corresponding to the row.

Table 3. The fields of FunAqua OTU abundance data.

Field	Description
OTU	OTU query ID. Key variable for the OTU abundance table
Kingdom	Assigned kingdom
Phylum	Assigned phylum
Class	Assigned class
Order	Assigned order
Family	Assigned family
Genus	Assigned genus
Species	Assigned species

Table 4. The fields of FunAqua taxonomic classification data.

unique sample identifiers containing their respective OTU abundance values. Table 4 describes the fields of the FunAqua taxonomic classification data, providing the hierarchical lineage for each OTU from kingdom to species level, using the OTU query ID as the key variable for integration with the abundance data.

We used the terminology and standards of Darwin Core³⁸ and MIMARKS³⁹ to facilitate machine readability and communication.

Sample collection sites were assigned alphanumeric IDs in the format “W” followed by 4 digits (e.g., W0001). Samples collected from each site were given a suffix “_w1” or “_s1”, indicating type as filtered water or sediment samples, respectively. The samples for which metadata had already been uploaded to the PlutoF⁴⁰ cloud data manager by the sample collectors lack ID’s following the former system and use only the accession ID’s assigned by the repository of the University of Tartu (acronym TUE, with 6-digit accession numbers). All other physical samples and their respective DNA samples are also deposited in TUE.

We categorised sampling sites into 10 different biomes (Fig. 3). These categories were generalised for uniformity purposes. Aquatic habitats, which could not be categorised under the 10 biomes, are labelled as “aquatic: other”.

Technical Validation

Location data verification. Latitude and longitude records for each waterbody were manually verified using Google Maps (satellite and map views). A location was marked as verified if it met one of the following criteria:

- (1) corresponded to a waterbody with a matching *plot name*;
- (2) was in or close to a waterbody with no name or a different name, but the provided location name matched a nearby city or region
- (3) was near an unnamed waterbody in the correct country, with no other waterbodies nearby.

Locations that did not meet any of these criteria were considered unverified. In such cases, data collectors were contacted by the FunAqua coordinator to confirm or correct the coordinates (latitude and longitude in decimal degrees; WGS84).

Sampling coverage. Of the 1,452 sampling sites included in the dataset, 63% (920 sites) had both sediment and filtered water samples. The sampling sites were predominantly located in Europe, and the 1,327 European samples accounted for 56% of all samples (Fig. 2). Samples from North America accounted for 15% of the total samples and this was the continent that supplied the second highest number of samples.

The largest proportion of samples were collected from freshwater lakes (44%; 1047 samples), followed by freshwater rivers (32%; 768), saltwater marine (14%; 337) and brackish marine (7%; 157) environments (Fig. 3).

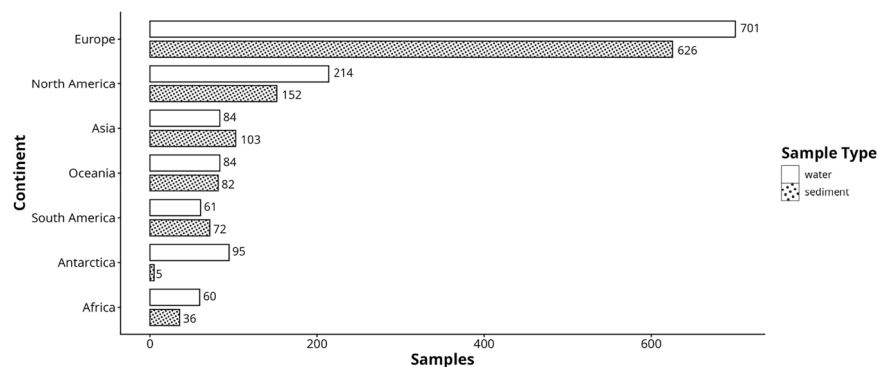


Fig. 2 Number of sediment and filtered water samples collected from each continent in the FunAqua dataset.

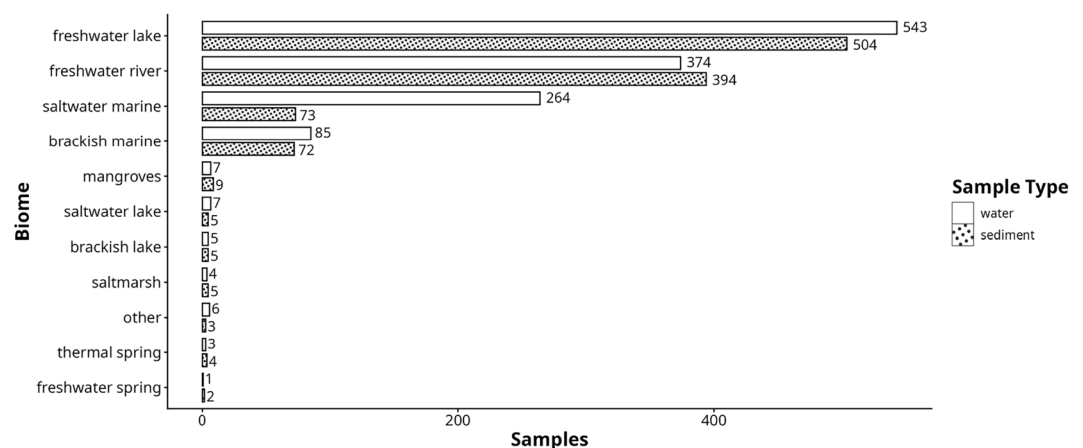


Fig. 3 Number of sediment and filtered water samples collected from each biome in the FunAqua dataset.

Mangroves (16 samples), saltwater lakes (12), brackish lakes (10), salt marshes (9), thermal springs (7) and freshwater springs (3) were much less represented in sampling (Fig. 3).

Freshwater lake, freshwater river, and saltwater marine biomes were sampled on all continents. Brackish marine samples were absent from the Antarctic and South American sample sets. Sampling campaigns conducted in Europe and Asia covered the greatest number of biome types (8 biomes each), followed by North America (7) (Fig. 4). Samples from Antarctica were predominantly of saltwater marine origin, comprising 60% of sediment samples and 83% of filtered water samples.

Contamination control. Each sequencing library and PCR reaction included both a positive and a negative control. Fungal OTUs were detected in 19 out of 28 negative controls. The majority of these OTUs were either low quality or showed signs of contamination, likely originating from the positive control. High-quality fungal OTUs detected in the negative controls were more abundant and common in other samples within each sequencing library, indicating minor cross-contamination. There were no signs of library-wide contamination, as there were no OTUs which were present in the majority of samples in a library and in the negative control. OTUs deemed to be caused by contamination were removed from the dataset.

Bioinformatic validation. Amplicon integrity and marker correctness were validated through stringent sequence- and structure-based filtering. Only reads containing the complete set of expected index combinations and both primers in correct orientation were retained. Sequences with missing primers, inverted primer orientations, or multiple primer occurrences were discarded to remove non-specific products and reads misassembled during the generation of circular consensus PacBio sequences. To confirm that reads represented genuine full-length ITS amplicons, we processed all sequences with ITSx, requiring detection of rRNA regions (SSU, ITS1, 5.8S, ITS2, and LSU) in the correct order without overlap. Using the ITSx HMM profiles, we further required that all detected subregions within a sequence matched the same major eukaryotic lineage, enabling removal of cross-lineage chimeras and ensuring that only structurally consistent ITS sequences were retained for downstream analysis.

To minimise spurious diversity, we applied per-sample homopolymer correction to reduce systematic indel errors characteristic of long-read ITS sequencing, followed by global denoising with the UNOISE algorithm to collapse residual sequencing noise and artefactual sequence variants. Prior to clustering, reads were sorted

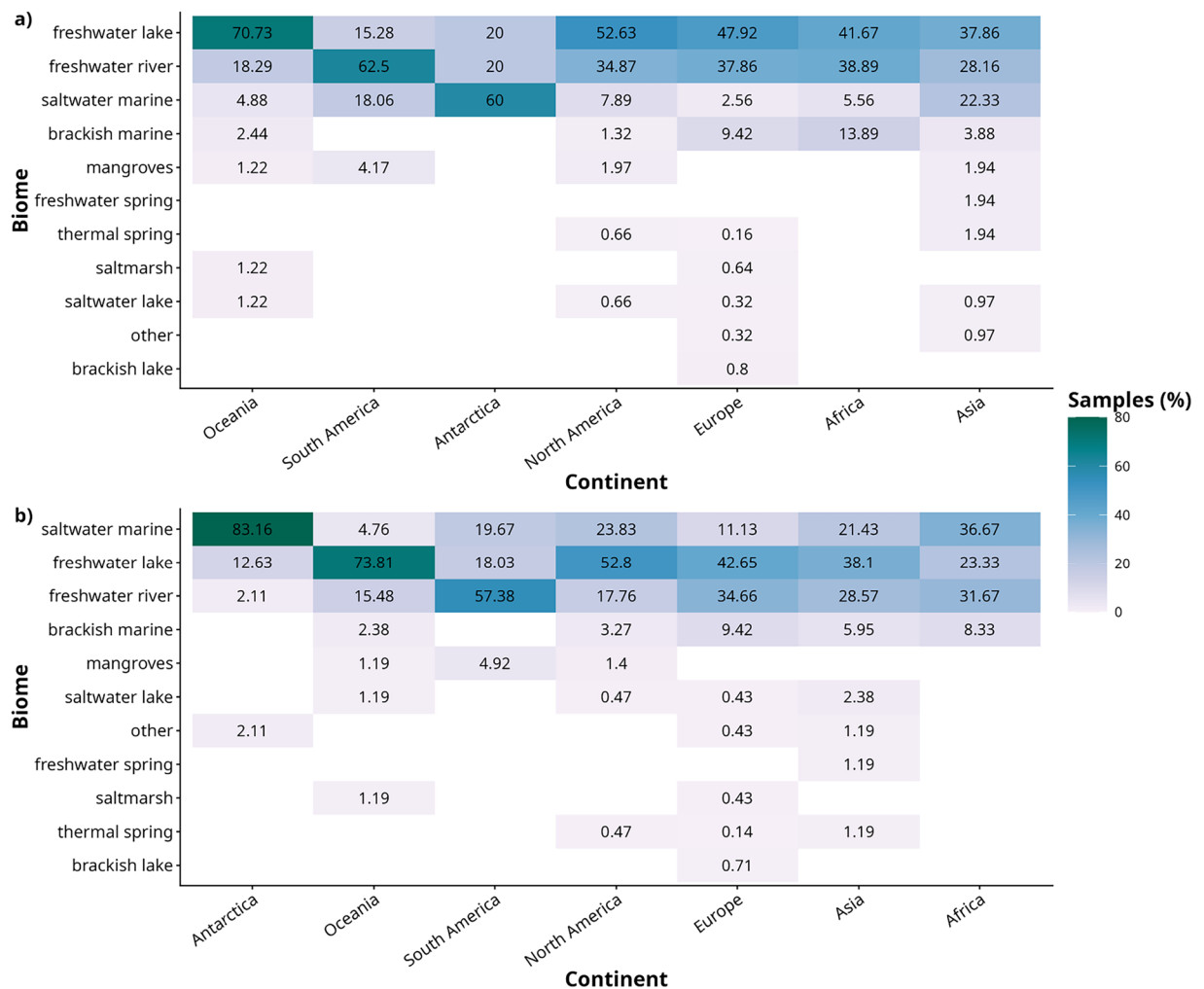


Fig. 4 Heat map charts depicting the proportion (%) of samples collected from each biome on each continent in the FunAqua dataset. **(a)** Proportions of sediment samples from each biome on each continent. **(b)** Proportions of filtered water samples from each biome on each continent.

jointly by abundance and average Phred score, ensuring that high-quality, well-supported sequences served as candidate OTU representatives.

Sequence processing followed the open-source NextITS workflow, orchestrated with Nextflow and containerised using Singularity, which ensures the full reproducibility of software versions and parameters across different computing environments.

Taxonomic validation. To ensure the reliability of taxonomic assignments, we applied a multi-step quality control procedure. First, all OTUs were assigned taxonomy using BLASTn against the EUKARYOME reference database, which currently provides the most comprehensive coverage of aquatic fungal lineages as well as other eukaryotes commonly misidentified as fungi (e.g., Amoebozoa, Nucleariidae, Choanoflagellata). Assignments were retained only when they met minimum thresholds for sequence identity (65%), alignment coverage (40%), and E-value (e^{-50}). OTUs failing these criteria were assigned to the last reliable taxonomic rank or marked as unclassified at lower ranks.

Second, we manually inspected taxonomic assignments for known problematic groups, including environmental clades with uncertain phylogenetic placement and taxa prone to misidentification due to incomplete reference sequences. Conflicting assignments (where multiple high-scoring BLAST hits suggested different taxa) were resolved by examining the consensus across top hits and, where necessary, truncating assignments to higher taxonomic ranks to avoid overclassification. Reliable taxonomic ranks were determined using e-value and sequence similarity thresholds for species- to phylum-level assignments following Tedersoo *et al.* (2021²¹). Third, we flagged low-quality OTUs based on multiple criteria: (1) singletons and doubletons with PHRED quality scores <30; and (2) taxa with overall abundance <10 exhibiting three or more indel openings compared with the number of substitutions.

Sequencing depth and taxonomic coverage. Overall, we analysed 1,126 sediment samples and 1,351 filtered water samples. No high-quality eukaryotic sequences could be detected in 50 sediment and 52 filtered water samples, likely due to the presence of PCR inhibitors. Additionally, the amount of DNA present on a filter depends on the volume of water filtered; thus, insufficient filtration volumes could yield suboptimal results. Samples with no remaining high-quality eukaryotic sequences were removed from the dataset and underwent further quality assessment processes. A subset ($n = 367$) of sediment samples were re-extracted and re-sequenced due to subpar results in the first round of sequencing. For these samples, a sequencing depth threshold of 350 reads was set. The copy meeting this threshold was retained in the dataset, while the other was discarded. If neither copy met this criterion, the OTU count data were merged. This process was conducted separately for non-fungal and fungal OTUs to ensure optimal coverage for both groups. The selection of the minimal sequencing depth threshold did not result in a significant loss of sequences or OTUs, as most discarded samples had fewer than 100 reads.

The total number of reads recovered was 13,064,293 (fungal: 2,888,694; non-fungal: 10,175,599). The OTU clustering resulted in a total of 496,061 eukaryotic OTUs, of which 132,417 (27%) represented fungi. After additional quality control using the methods described in taxonomic validation, 72% (358,253) of the detected OTUs were considered to be of low-quality. Of the remaining 137,808 high-quality OTUs (11,822,454 reads), the fungal kingdom comprised the largest subset of eukaryotic OTUs at 36.4% (50,152 OTUs; 2,065,407 reads), followed by Alveolata (16.6%; 22,936 OTUs; 3,618,896 reads) and Straminipila (13.6%; 18,744 OTUs; 1,256,493 reads). Kingdom-level classification could not be assigned to 650 OTUs (0.47%). The low-quality OTUs were excluded from the final dataset.

Sediments had a greater number of eukaryotic OTUs (90,865 OTUs; 6,048,432 reads) than water samples (74,110 OTUs; 5,774,022 reads), with 27,167 OTUs overlapping between the two sample types. Fungal OTUs were detected in 98% of the sediment samples ($n = 1,056$) and 93% of the filtered water samples ($n = 1,212$), from which 34,145 (1,335,319 reads) and 26,296 (730,088) fungal OTUs were recovered, respectively. Across all samples, fungi remained the most OTU-rich kingdom in both sediments (37.6% of OTUs) and water (35.5%).

The type of filter used for sample filtration had a significant effect on the number of log₁₀-transformed fungal sequences detected (Kruskal-Wallis chi-squared = 98.802, $df = 11$, p -value = 3.082×10^{-16}); however, these results should be interpreted with caution. Among the 66 pairwise comparisons of filter types, 20 showed clear differences in fungal sequence numbers (pairwise Wilcoxon test $p < 0.05$). Cellulose acetate filters differed significantly from nine other filter types in the number of detected log₁₀-transformed fungal sequences. However, the sample size for that specific type of filter was low ($n = 7$) and all these samples originated from the same general location.

The majority of OTUs in the dataset had a sequence count greater than one (82% of OTUs; 112,456 OTUs), while 25,352 OTUs (18%) were singletons. There was a slightly higher proportion of singletons among the fungal OTUs (21% of fungal OTUs; 10,714 OTUs) compared to other eukaryotes (17% of non-fungal OTUs; 14,638 OTUs). Based on the high-quality sequences, the overall median sequencing depth was 2,080 (IQR: 406–5,529.5). The median sequencing depth was greater in filtered water samples (2,330; IQR: 559.5–5072.5) than in sediments (1,658; 255.5–6345.5). For fungal sequences, the median sequencing depth was 125 (20–590), with sediments showing a higher median sequencing depth (196; 26–907.3) than filtered water samples (94; 15–381).

Rarefaction curves for most samples reached saturation or near-saturation when considering all high-quality eukaryotic OTUs (Fig. 5a). In contrast, rarefaction curves for fungal OTU accumulation suggested that sequencing depth was insufficient to reach saturation across all samples (Fig. 5b,c). The OTU accumulation curves (Fig. 5d) demonstrated a steady increase in the number of fungal OTUs detected across samples, with accumulation progressing more slowly in filtered water samples.

The lack of sufficient sequencing depth has been documented in previous studies that have used metabarcoding for the identification of fungi in aquatic ecosystems^{41–43}. This is probably related to the small size of unpooled samples, and the widespread presence of air-borne spores from terrestrial fungal species, which can number in the millions⁴⁴.

Based on the Good's coverage index, 79% ($n = 1,874$) of samples achieved sufficient sequencing depth ($C > 0.89$), indicating that non-singleton OTUs accounted for at least 90% of the observed sequences. The median Good's coverage index value across was 0.97 (IQR: 0.91–0.99). In contrast, 6% of samples ($n = 141$) had a high proportion of sequences belonging to singletons ($\geq 50\%$), corresponding to low coverage values ($C < 0.51$). Analysis of fungal OTUs showed that only 57% of samples ($n = 1,296$) met the coverage threshold ($C > 0.89$), with a median index of 0.90 (IQR: 0.70–0.98). Notably, 20% of fungal samples ($n = 454$) exhibited $\geq 50\%$ singleton OTUs ($C < 0.51$).

Continent-wise, fungal sequences were recovered from 1,295 European, 350 North American, 185 Asian, 162 Oceanian, 128 South American, 90 African, and 58 Antarctic samples. European samples had the highest number of high-quality fungal sequences (1,371,636 reads; 35,523 unique OTUs), followed by North America (310,230; 10,959) and Asia (145,444; 6,686) (Fig. 6a). Sediments had a higher number of detected OTUs on all continents, except for Oceania (water: 2,616 OTUs; sediments: 2,404) and Antarctica (494; 14) (Fig. 6b).

Among the sampled biomes, fungal sequences were detected in 1,026 freshwater lakes, 752 freshwater rivers, 276 saltwater marine, 153 brackish marine, 10 saltwater lakes, 9 brackish lakes, and 8 saltmarsh samples. All samples from mangroves, thermal springs, and freshwater springs contained fungal OTUs. Freshwater rivers contained the most fungal reads (983,828 reads; 31,267 unique OTUs), followed by freshwater lakes (879,797; 27,677), saltwater marine biome (108,936; 4,106), and brackish marine biome (70,039; 4,553) (Fig. 7a). From other biomes, a total of 22,807 fungal sequences (2,224 OTUs) were detected. In 7 biomes the number of fungal OTUs was larger in sediments than in water, while for the others, the OTUs detected from water outnumbered the sedimentary OTUs (Fig. 7b).

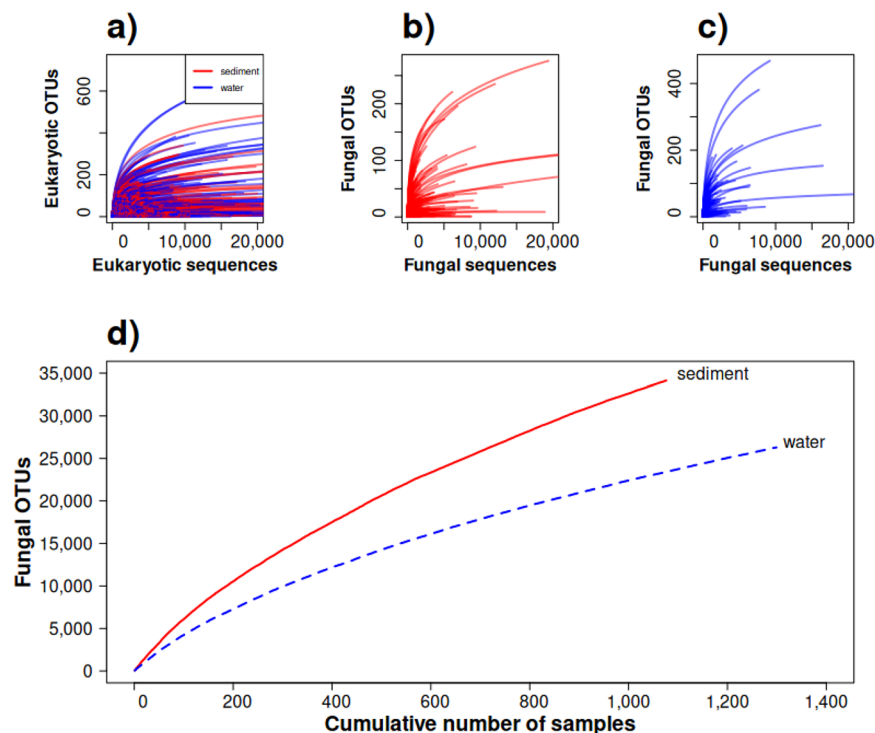


Fig. 5 (a) Rarefaction curves of all eukaryotes in all samples in the FunAqua dataset. (b) Rarefaction curves of fungal OTUs in sediment samples. (c) Rarefaction curves of fungal OTUs in filtered water samples. (d) OTU accumulation curve of fungal OTUs.

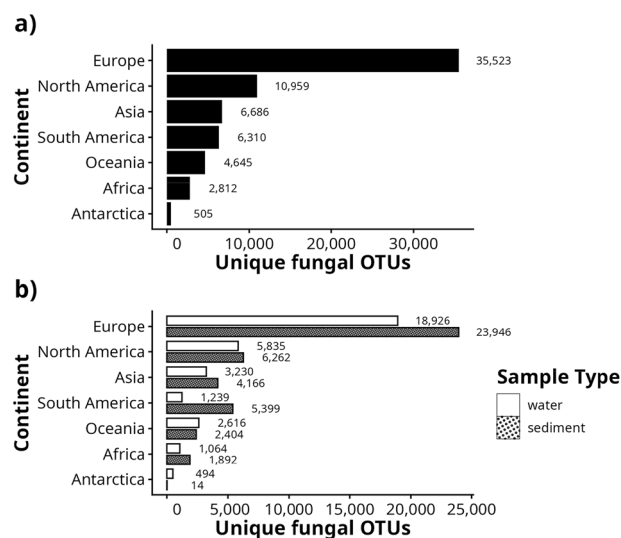


Fig. 6 (a) number of unique fungal OTUs detected in all samples of each continent combined; (b) number of unique fungal OTUs detected in sediment and filtered water samples from each continent.

Fungal taxonomic coverage. Among the high-quality fungal OTUs detected, the phylum Ascomycota was the most abundant, comprising 35.6% (17,858 OTUs) of all fungal OTUs across combined samples (Fig. 8). This was followed by Rozellomycota (21.6%; 10,823 OTUs), Chytridiomycota (14.7%; 7,374 OTUs), and Basidiomycota (13.6%; 6,821 OTUs) (Fig. 8).

Clear differences in phylum-level composition were observed between sediment and water samples. In both environments, Ascomycota and Rozellomycota were dominant, however, their relative abundances differed. In sediments, Ascomycota accounted for 39.0% (13,300 OTUs) and Rozellomycota for 23.6% (8,046 OTUs), whereas in water samples these values were lower, at 31.5% (8,294 OTUs) and 19.7% (5,168 OTUs), respectively (Fig. 8). In sediment samples, Rozellomycota was followed by Basidiomycota (12.8%; 4,363 OTUs) and

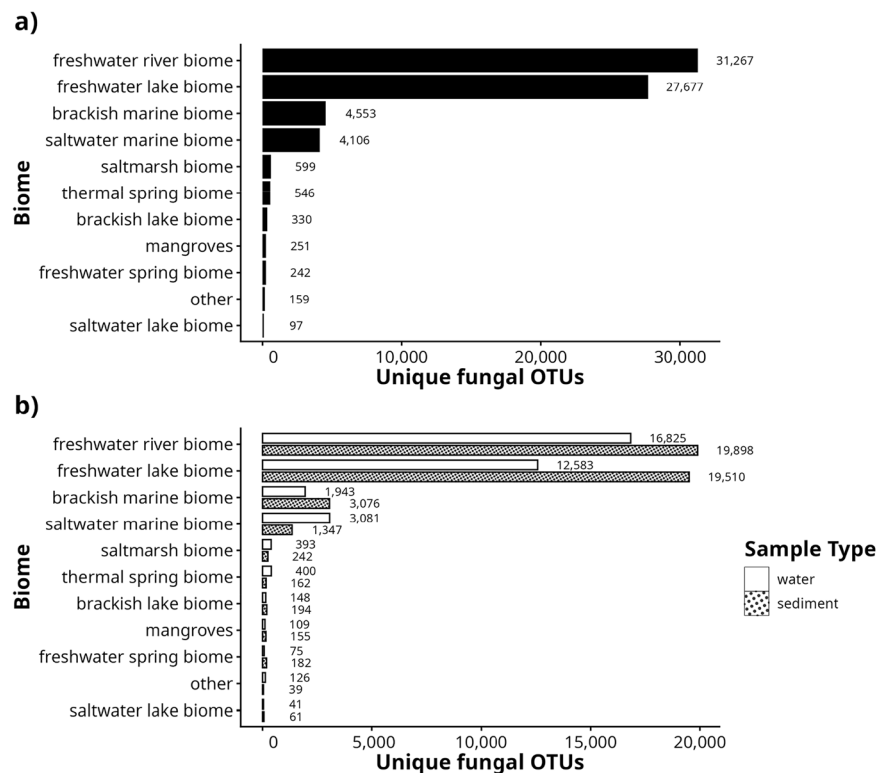


Fig. 7 (a) number of unique fungal OTUs detected in all samples of each biome combined; (b) number of unique fungal OTUs detected in sediment and filtered water samples from each biome.

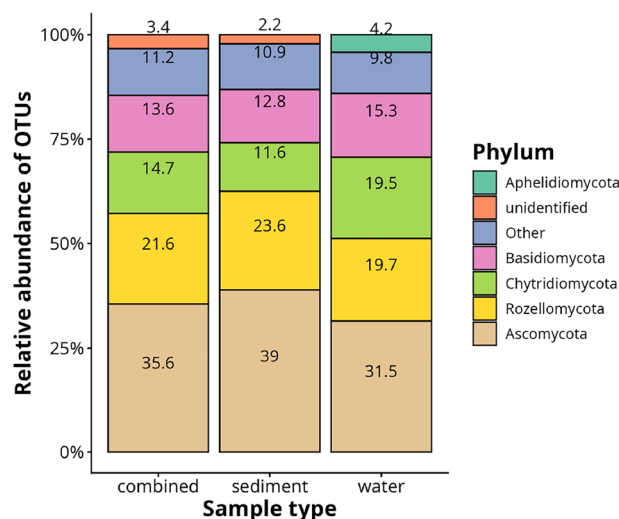


Fig. 8 Stacked bar chart presenting the relative abundance of the 5 most abundant phyla's OTUs given in percentages.

Chytridiomycota (11.6%; 3,962 OTUs) (Fig. 8). In contrast, in filtered water samples, Chytridiomycota (19.5%; 5,120 OTUs) closely followed Rozellomycota, with Basidiomycota ranking next (15.3%; 4,017 OTUs) (Fig. 8).

At both the class and order levels, unidentified Rozellomycota taxa represented the most abundant groups. When all samples were combined, these accounted for 20.7% (10,381 OTUs), with higher proportions in sediments (23.1%; 7,883 OTUs) than in water (18.2%; 4,796 OTUs). Among identified classes, Sordariomycetes was the most abundant overall (10.3%; 5,164 OTUs) and in sediment samples (11.8%; 4,043 OTUs), followed by Agaricomycetes (9.3%; 3,169) in sediments. Agaricomycetes (9.3%; 2,437) was the most abundant identified class in water, followed by Dothideomycetes (9.2%; 2,426) in water samples (Fig. 9).

At the order level, unidentified Rozellomycota orders remained the most abundant across all samples (17.7%; 8,882 OTUs), dominating both sediments (19.2%; 6,571 OTUs) and filtered water samples (15.8%; 4,143 OTUs)

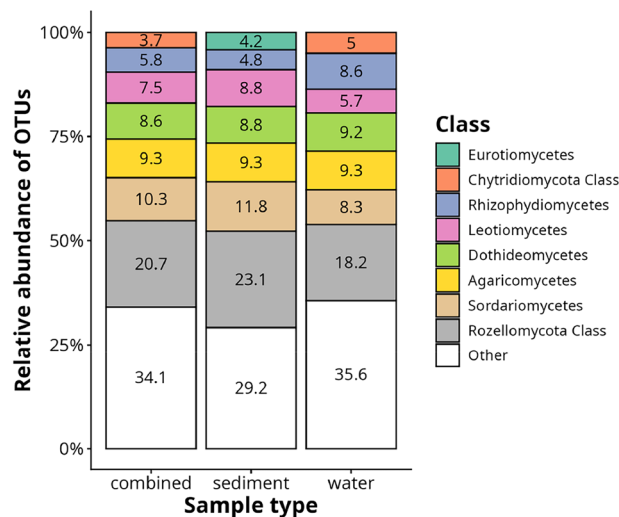
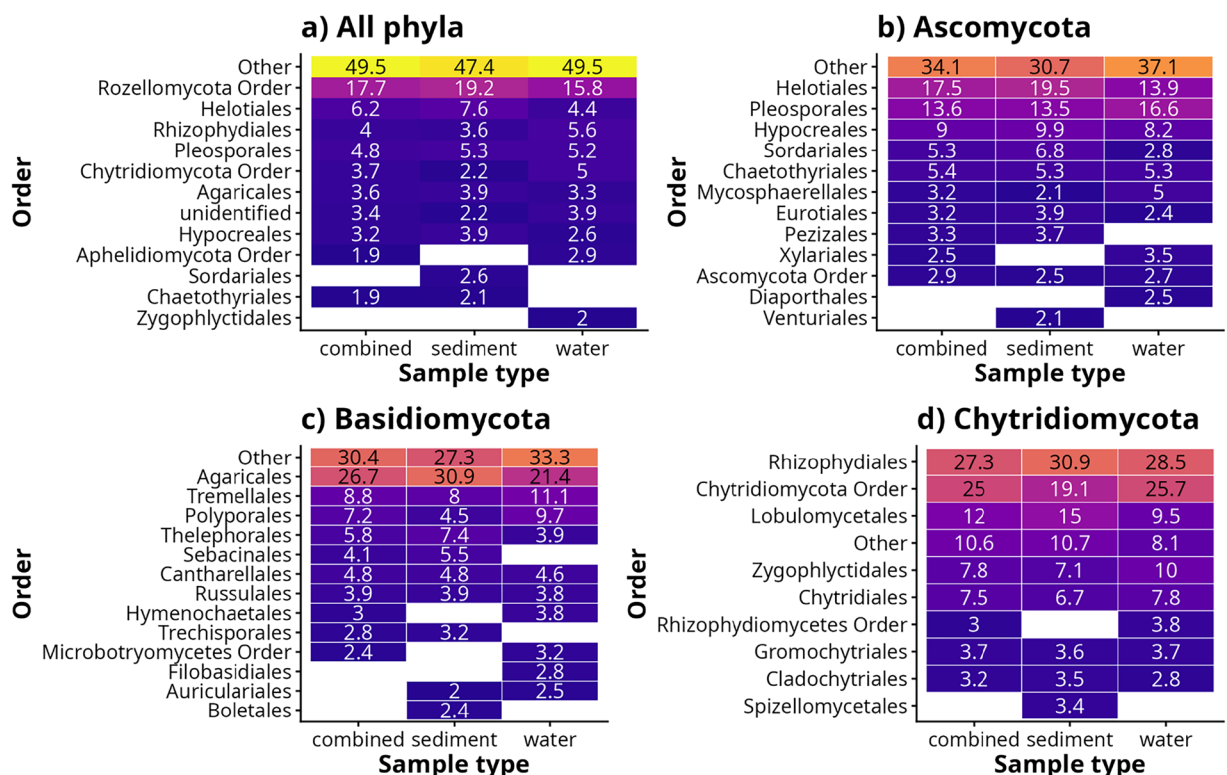


Fig. 9 Stacked barchart presenting the relative abundance of the 7 most abundant classes' OTUs given in percentages.



Relative abundance (%) of OTUs

0 10 20 30 40 50

Fig. 10 Heatmaps depicting the most OTU-abundant orders' relative abundance in both sample types combined and separately. (a) Heatmap depicting the relative abundance of the most OTU-abundant orders overall given in percentages of all fungal OTUs; (b) heatmap depicting the relative abundance of Ascomycota orders given in percentages of Ascomycota OTUs; (c) heatmap depicting the relative abundance of Basidiomycota orders given in percentages of Basidiomycota OTUs; (d) heatmap depicting the relative abundance of Chytridiomycota orders given in percentages of Chytridiomycota OTUs. Names on the Y-axis ending with "Order" are unidentified orders of that specific taxon.

(Fig. 10a). Helotiales was the most abundant order overall (6.2%; 3,132 OTUs) and in sediments (7.6%; 2,591 OTUs) (Fig. 10a). In contrast, in water samples, Rhizophydiales was the most abundant order (5.6%; 1,461

OTUs), exceeding Helotiales (4.4%; 1,151) in relative abundance (Fig. 10a). Helotiales comprised the largest subset of Ascomycota OTUs (17.5% of Ascomycota OTUs), and was subsequently the most abundant Ascomycota order in sediments (19.5%) (Fig. 10b). The most abundant Ascomycota order in water samples was Pleosporales (16.6%) (Fig. 10b). Agaricales accounted for 26.7% of Basidiomycota OTUs, making it the most abundant Basidiomycota order in both sediment (30.9%) and water (21.4%) samples (Fig. 10c). Among the chytrid OTUs, Rhizophydiales dominated, making it the most abundant in all samples combined (27.3%), sediments (30.9%) and water (28.5%) (Fig. 10d).

Usage Notes

The FunAqua dataset is designed for broad reuse in fungal ecology, biogeography, and comparative biodiversity studies. Users should be aware of the following considerations when working with the data:

1. While most samples achieved adequate coverage for community-level analyses, samples with low sequencing depth may underestimate fungal OTU richness. We recommend filtering or rarefying samples or accounting for the sequencing depth in a way appropriate to the research question.
2. OTUs were clustered at 98% similarity, which approximates species-level resolution for ITS sequences across various fungal groups. However, it may merge closely related taxa, e.g., in some groups of filamentous Ascomycota, or split variable species, for example, in chytrids.
3. Our dataset does not distinguish between actively growing aquatic fungi, organisms inhabiting particles fallen into water or deposited spores. Therefore, caution is needed when drawing conclusions regarding the activity, lifestyle, traits or ecological functions of these fungi in water and sediments.
4. While the vast majority of the fungal kingdom is surveyed mainly based on the ITS marker, chytrids, apothelids, rozellids, and microsporidians mainly rely on SSU and/or LSU markers. Consequently, the diversity in these groups may be underestimated in ITS-based surveys. Although much of the taxonomic information from SSU and LSU markers has been transferred to long-read datasets through phylogenetic analyses in EUKARYOME, taxa within these early-diverging fungal lineages remain associated with greater taxonomic uncertainty.
5. The sampling locations are biased towards Europe. This is largely due to the project's voluntary nature. At the time of publishing, additional sampling efforts are in process to cover underrepresented regions, and the dataset will be updated after the new samples are analysed.

We encourage researchers to contact the corresponding authors prior to large-scale reuse to facilitate collaboration and ensure the most up-to-date metadata are utilized. To promote transparent and fair usage, this dataset is tagged with Data Reuse Information (DRI)⁴⁵ as described in the metadata files.

Data availability

The eight main dataset files are accessible on Zenodo at <https://doi.org/10.5281/zenodo.20509884>³⁶, and the sequencing data can be obtained at the European Nucleotide Archive (ENA)³⁷ under BioProject PRJEB104083 (Run Accessions ERR15968726 – ERR15971436). All data are currently under embargo and will be publicly released upon publication of this manuscript.

Code availability

The R pipeline used to produce the majority of the technical validation steps covered under sections “Sampling coverage”, “Sequencing depth” and “Fungal Taxonomic coverage” is available on Zenodo together with the dataset. The code is accompanied by a RMarkdown document which produces a semi-interactive HTML output for data exploration.

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Additional information

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