Received: 26 November 2023 | Revised: 19 April 2024 | Accepted: 3 June 2024

DOI: 10.1002/edn3.576

Environmental DNA
Open Access
Dedicated to the study and use of environmental DNA for basic and applied sciences

WILEY

ORIGINAL ARTICLE

Community structure and functional diversity of aquatic fungi are correlated with water quality: Insights from multi-marker analysis of environmental DNA in a coastal watershed

Lauren Cortez French[1] | Michelle A. Jusino[2] | Randolph M. Chambers[3] | James Skelton[1]

[1]Department of Biology, William & Mary, Williamsburg, Virginia, USA

[2]Center for Forest Mycology Research, Northern Research Station, USDA Forest Service, Madison, Wisconsin, USA

[3]Keck Environmental Field Laboratory, William & Mary, Williamsburg, Virginia, USA

Correspondence
James Skelton, Department of Biology, William & Mary, Williamsburg, VA, USA.
Email: jeskelton@wm.edu

Funding information
College of William and Mary; Mycological Society of America

Abstract

Aquatic fungi drive important ecosystem processes, which may be affected by anthropogenic stressors, including changes in water quality. However, due to the great diversity of aquatic fungi, our understanding of the relationships among water quality variables, fungal community composition, and ecosystem function remains incomplete. Here, we show that aquatic fungal community structure and functional diversity correlate strongly with water quality. We used a multi-marker survey of environmental DNA and long-term water quality data to survey 17 stream, pond, and tidal creek sites within a coastal drainage network in southeastern Virginia, USA, targeting both the ITS2 and LSU barcoding regions. The community composition of Chytridiomycota and Ascomycota (fungal phyla) and aquatic hyphomycetes (a functional group) were strongly correlated with habitat type and water quality variables. Functional diversity of aquatic fungi decreased with warmer water, due mainly to the reduced richness of saprotrophic groups, and increased with total particulate phosphorus, due to the richness of algal parasites. Community dissimilarity analyses of samples using both ITS2 and LSU yielded consistent results, but per-sample alpha diversity differed. This work reveals a surprising degree of local variation in aquatic fungal community composition and functional diversity within a coastal watershed, which was coupled with variation in water quality variables. Furthermore, eDNA sampling is an effective and efficient means to accurately characterize diverse aquatic fungal communities with exciting implications for addressing basic and applied research questions. Our work supports the prediction that community composition and functional diversity of aquatic fungi are impacted by impaired water quality and global change.

KEYWORDS
aquatic fungi, Chytridiomycota, ecosystem function, eDNA, fungal diversity, water quality

1 | INTRODUCTION

Aquatic ecosystems provide important ecosystem functions and services. However, human activities cause changes to the physical, chemical, and biological properties of aquatic ecosystems with negative impacts on ecosystem processes. Aquatic ecosystem processes are affected by changes to flow and channel modification (Elosegi & Sabater, 2013), sedimentation (Apitz, 2012), nutrient loads (Woodward et al., 2012), and novel compounds such as pharmaceuticals (Rosi-Marshall & Royer, 2012) and microplastics (Nava & Leoni, 2021). It is well established that microbial communities, including aquatic fungi among other microbes, drive many aquatic ecosystem processes (Finlay et al., 1997; Grossart et al., 2019). However, microbes and the processes they facilitate are sensitive to environmental perturbations (Paerl et al., 2003). Therefore, there is a general need to understand how anthropogenic stressors impact aquatic fungal diversity and function.

Aquatic fungi encompass a broad range of taxonomic groups with the unifying characteristic of dependency on aquatic habitats for all or part of the fungi's life cycle (Grossart et al., 2019). Aquatic fungi are functionally important in aquatic systems, with a wide range of positive and negative impacts. For instance, some aquatic fungi convert detritus and inedible algae to food for fish and invertebrates, whereas others release bioactive, and potentially toxic, compounds into the environment (Grossart et al., 2019; Jones et al., 2014). Several aquatic fungi are known to be causative agents of some of the most devastating outbreaks of wildlife diseases (Oidtmann et al., 2002; Rothermel et al., 2008). Consequently, understanding how changes in water quality may affect freshwater fungal communities is essential to predicting how aquatic ecosystems will respond in a changing world.

One important functional group of aquatic fungi are the aquatic, or "Ingoldian," hyphomycetes (Ingold, 1942). Aquatic hyphomycetes are a polyphyletic group composed of Ascomycota and Basidiomycota detritivores that largely specialize in colonizing leaf litter and other dead plant matter. These fungi form an essential link between terrestrially derived biomass and higher aquatic trophic levels, driving carbon and nutrient cycling in many aquatic habitats (Bärlocher, 1992). Allochthonous input of leaf litter is the largest source of organic carbon in forested temperate streams and rivers. Colonization by aquatic hyphomycetes increases the nutritive value of detritus to detritivores, forming the base of stream food webs (Gulis et al., 2006). Additionally, much of the detrital biomass consumed by these fungi is converted into spores that are ingested by filter-feeding animals (Findlay & Arsuffi, 1989). Consequently, environmental disturbances that affect aquatic hyphomycetes may impact stream processes, thus creating the need to prioritize aquatic hyphomycete diversity in aquatic conservation planning goals (Barros & Seena, 2022). Individual taxa and species richness of aquatic hyphomycetes respond to changes in water chemistry, nutrient availability, organic matter, temperature, toxic metals, and other pollutants (reviewed in Ferreira et al., 2014), but the importance of environmental gradients in determining the community composition of aquatic hyphomycetes and other aquatic fungi is less clear.

Another important group of aquatic fungi are the zoosporic Chytridiomycota. Chytrids are generally abundant in aquatic habitats and have strong effects on aquatic communities (Comeau et al., 2016; Jobard et al., 2012; Lefèvre et al., 2012; Monchy et al., 2011). Perhaps the most notorious aquatic fungal species is *Batrachochytrium dendrobatidis*, a Chytrid that causes a fatal infectious disease in amphibians and devastates populations of vulnerable species worldwide (Rothermel et al., 2008). Chytrids also transfer energy and nutrients to higher trophic levels by parasitizing inedible algae and decomposing pollen through multiple mechanisms collectively called the "mycoloop" (Kagami et al., 2014). Fungal infection of pollen and algae significantly increases the food quality of these substrates through sterol and fatty acid production (Gerphagnon et al., 2019) and fragmentation of long algal filaments (Frenken et al., 2020). Chytrids also release an abundance of zoospores, which can be eaten by zooplankton (Grossart et al., 2019; Kagami et al., 2017). Collectively, the contribution of the mycoloop to overall secondary production can be considerable with estimates as high as 20% of total primary production transferred to zooplankton, comprising up to 40% of zooplankton diet (Grami et al., 2011; Rasconi et al., 2014). Thus, knowledge of the ecological factors that drive chytrid community composition is needed to improve our understanding of freshwater pelagic food web dynamics (Frenken et al., 2017).

Because taxonomic and functional groups of aquatic fungi are typically studied independently, we lack a holistic understanding of how entire communities of aquatic fungi respond to variation in ecological factors and anthropogenic disturbance. The application of broadly targeted high-throughput amplicon sequencing (HTAS) of aquatic fungal environmental DNA (eDNA) to survey entire communities could greatly expand our knowledge of fungal diversity in freshwater habitats, relationships between fungal taxa and their environment, and the extent of fungal contributions to ecosystem processes. We define aquatic fungal eDNA as any source of fungal DNA that may be recovered from bulk filtration of water including spores, hyphal fragments, sporangia, and extracellular DNA. One challenge of applying eDNA to the study of aquatic fungi is choosing a suitable genetic marker for metabarcoding. Marker choice affects taxon detection and taxonomic resolution (Ceballos-Escalera et al., 2022; Tedersoo et al., 2021). Poor marker choice can also result in amplification biases that limit the detection of some groups; likewise, amplification of non-target organisms can reduce the detection of target organisms by consuming a large portion of sequence reads (Reynolds et al., 2022; Tedersoo et al., 2022; Wurzbacher et al., 2016). Primers targeting the nuclear ribosomal internal transcribed spacer (ITS) gene region have been most commonly used in fungal metabarcoding (Schoch et al., 2012; Toju et al., 2012). Fewer freshwater fungal studies have used primers targeting the nuclear large ribosomal subunit (LSU) gene region. This region is more conserved and therefore may provide lower taxonomic resolution than

ITS, though in some cases, LSU assays will recover many taxa that do not amplify and/or sequence reliably with ITS-targeting primers (Reynolds et al., 2022; Skelton et al., 2019). Applying HTAS to aquatic fungal eDNA may be a powerful tool to study the composition of entire fungal communities, but the quality of results can be affected by marker choice.

We hypothesized that aquatic fungal community composition reflects fine-scale variation in water quality and distinct habitat types because individual taxa respond uniquely to environmental gradients. To test the predictions of our hypothesis, we conducted eDNA metabarcode sequencing, targeting the ITS2 and LSU gene regions which work in complement to provide taxonomic breadth and depth to our community analysis. This approach also allowed us to provide a direct sample-for-sample comparison of the results obtained using the two different markers to understand how marker choice affects the aquatic fungal community captured by metabarcoding. We then assessed the responsiveness of the aquatic fungal community to freshwater habitat type and water chemistry. We sourced long-term water quality data collected at 3 month intervals at our sampling sites to understand whether community composition is more strongly explained by short-term, season-specific water quality or by consistent, long-term water quality. Combining an eDNA metabarcoding survey of fungal communities with site-specific water quality data allowed us to effectively evaluate distinct responses of particular taxonomic and functional groupings to fine-scale variation in aquatic ecosystems.

2 | MATERIALS AND METHODS

2.1 | Study sites and sampling

Environmental DNA samples were collected from 17 sites in a coastal drainage network of the James River located in southeastern Virginia, USA. All sites were located in the College Creek watershed near Williamsburg, Virginia, and included nine streams, five ponds, and three oligohaline tidal creeks (Figure 1; Table S1). Sites consisted of a mix of exposed ponds and streams in residential neighborhoods, oligohaline and mesohaline tidal creeks, and densely wooded freshwater streams. The order in which sites were sampled was determined prior to sampling using a random number generator to prevent biasing effects of time. The 17 sites coincide with locations for which temperature, conductivity, oxygen saturation, pH, total suspended solids (TSS), total phosphorous, dissolved inorganic phosphorous (DIP), ammonium (NH^4), and Secchi depth (a measure of water clarity) have been collected by the College Creek Alliance at 3-month intervals consecutively since 2005. This publicly available dataset was accessed online and used in all of our statistical analyses where metabarcoding results are correlated with water quality (William & Mary CCA, 2022).

Using the Smith-Root eDNA Sampler Backpack (Thomas et al., 2018), three replicate 2-liter samples were collected at each site between March and May of 2021, when dominant fungal groups are likely to be at their peak abundance and diversity (Gsell

FIGURE 1 Map of the 17 study sites sampled within the College Creek watershed near Williamsburg, VA, USA.

et al., 2022; Iqbal, 1997). The three samples were collected roughly three meters apart at each site. Water from approximately 10 cm below the surface was vacuum-filtered at a maximum pressure of 12 psi through a sterile polyethersulfone (PES) membrane filter with 5 μm pores using Smith-Root single-use eDNA filter packs. Immediately after filtering in the field, each filter was placed in a sterile 1.5 mL tube with 1.0 mL filter-sterilized cetyltrimethylammonium bromide (CTAB) cell lysis solution (CLS; Lindner & Banik, 2009) using sterile forceps. Samples were stored at −80°C prior to DNA extraction.

2.2 | Molecular analyses

We analyzed all samples using both the ITS2 and LSU markers to increase the taxonomic breadth of detected fungal taxa and to provide a useful comparison of the efficacies of each marker. DNA extraction followed the CTAB and glassmilk extraction protocol of Brazee and Lindner (2013) with the modifications of Jusino et al. (2014). Filter samples in CLS solution were incubated in a water bath at 65°C for 15 min, briefly agitated by vortexing, and returned to the water bath for 45 min. Samples were then centrifuged at 10,000 rcf (relative centrifugal force) for 1 min before 100 μL of supernatant CLS was removed from each sample and transferred to 0.2 mL strip tubes and extraction proceeded from there. Negative controls were included with each batch of extractions to identify any contamination during the extraction process. The negative extraction control samples were identical to actual samples except that the filters were not used to filter environmental water.

Initial PCR amplification was performed on all 51 samples and all negative extraction controls in preparation of both ITS2 and LSU amplicon libraries. Primers used for ITS2 barcoding targeted the fITS7 (Ihrmark et al., 2012) and ITS4 (White et al., 1990) priming sites and were modified for Illumina high-throughput sequencing. To prepare the ITS2 amplicon libraries, 3 μL of DNA extract from each sample was transferred to a new strip tube and each combined with 0.1 μL Promega GoTaq DNA polymerase, 3 μL Promega GoTaq buffer, 0.3 μL of each 10 μM primer, 7.88 μL water, 0.12 μL bovine serum albumin (BSA; 20 mg/mL), and 0.3 μL dNTP (10 mM each). Samples were placed in a thermocycler and subjected to the following conditions: initial annealing at 94°C for 3 min; 11 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s (dropping 0.5°C each cycle), extension at 72°C for 1 min; 28 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min; final extension at 72°C for 7 min. Primers used for LSU (28S) barcoding targeted the LR0R (Vilgalys & Hester, 1990) and JH-LSU-369rc sites (You et al., 2015) and were modified similarly to our ITS2 primers for Illumina compatibility. To prepare the LSU amplicon libraries, 3 μL from each DNA-extracted sample were transferred to a new strip tube and combined with 0.1 μL Promega GoTaq DNA polymerase, 3 μL Promega GoTaq buffer, 0.3 μL of each 10 μM primer, 7.88 μL water, 0.12 μL BSA, and 0.3 μL dNTP. The following thermocycler conditions were used: initial annealing at 94°C for 3 min followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, extension at 72°C for 90 s, and final extension at 72°C for 7 min.

Initial PCR amplification was determined by gel electrophoresis. After initial amplification, amplicons were dual indexed by PCR (8 cycles) with Illumina Nextera v2 indices and adapters (Illumina Inc., San Diego, CA, USA). Samples were cleaned (Zymo Select-a-Size Kit), quantified using a Qubit 4.0 Fluorometer and a kit for high sensitivity of double-stranded DNA, equilibrated, and subsequently combined in equimolar concentrations. The libraries, as well as a synthetic mock community control, were sequenced using a 2×300 bp V3 sequencing kit on an Illumina MiSeq at UC Riverside Genomics Core. The inclusion of an equimolar spiked-in mock community of synthetic ITS sequences (Synmock; Palmer et al., 2018) allowed for quantification of index bleed or barcode crossover rates which aided in optimizing the parameterization of our bioinformatics pipeline following recommendations of Palmer et al. (2018).

2.3 | Bioinformatics

Sequence data were processed using the publicly available AMPtk pipeline (version 1.5.3; Palmer et al., 2018; amptk.readthedocs.io) to pre-process reads, cluster into OTUs, filter OTUs, and assign taxonomic identification to OTUs. Pre-processing involved trimming of forward and reverse ITS2 and LSU primer sequences and merging of paired-end reads. For ITS2, reads longer than 300 bp were trimmed to 300, and reads shorter than 300 bp were padded with N's to ensure all reads were of 300-bp length. Reads shorter than 125 bp were discarded. Expected errors <1.0 (Edgar & Flyvbjerg, 2015) were used to quality-filter reads, followed by de-replication of reads and clustering of reads to 97% similarity to construct OTU tables using VSEARCH version 2.18.0 (Rognes et al., 2016). Any padded Ns were then removed from the processed sequences, which were subsequently mapped to the OTUs. For LSU, reads longer than 350 bp were trimmed to 350 and reads shorter than 250 bp were discarded. Expected errors less than 1.0 (Edgar & Flyvbjerg, 2015) were used to quality-filter reads, followed by denoising with DADA2 (Callahan et al., 2016), which performs better for LSU (Skelton et al., 2019) and clustering the resulting ASVs to 99% similarity. Following clustering, our spike-in mock community controls were used to parameterize our bioinformatics process and to detect index bleed, using the AMPtk filter command. A bleed filter was applied to remove observations of OTUs with read counts that fell within the range expected for index bleed alone. The measured percent index bleed was 0.01% and a conservative bleed filter of 0.1% was applied. The hybrid taxonomy algorithm in AMPtk was used to assign taxonomy to ITS2 OTUs. The LSU database compiled by Reynolds et al. (2022) was used to assign taxonomy for LSU within AMPtk. We then assigned OTUs to functional groups using the FungalTraits database version 1.2 (2020-12-14; Põlme et al., 2020). Functional group assignments were made by referencing the genus from our taxonomic

assignments of the LSU data to the primary lifestyle variable in the database. All OTUs not assigned to the generic rank during taxonomy assignment, those with unknown primary lifestyles, and those not listed in the database were omitted from functional diversity analysis. We then tabulated the number of OTUs assigned to each functional group from each sampling site.

2.4 | Statistical analyses

Statistical analyses were conducted in R version 4.0.0 (2020-04-24; R Core Team, 2020). For the purposes of our analyses, only OTUs assigned to the kingdom "Fungi" were retained in both the ITS and LSU datasets. To determine whether the ITS2 marker and the LSU marker gave consistent results, we correlated fungal alpha and beta diversity recovered using each marker in a sample-to-sample comparison, with field replicates treated as separate samples. To compare estimates of alpha diversity, the number of fungal OTUs recovered using ITS2 was plotted against the number of fungal OTUs recovered using LSU, and the strength and statistical significance of the correlation were determined by paired-sample correlation using the *cor.test* function of the *stats* package in R (R Core Team, 2020). To compare beta diversity among samples using the two markers, we plotted the Jaccard distance for all pairwise combinations of samples using the ITS2 marker against the Jaccard distance for all pairwise combinations of samples using the LSU marker. The correlation strength between the two markers and its significance was determined using a Mantel test implemented using the *mantel* function in the *vegan* package (Oksanen et al., 2008).

Multiple linear regression was used to assess relationships between water quality variables and functional diversity. The LSU OTUs recovered in the three field replicate samples from each site were combined for this analysis, meaning that an OTU was considered part of that site's community if it was detected in at least one of the field replicates. Functional diversity of each site was calculated from the tabulated number of OTUs in each functional group as Shannon–Weaver diversity using the *diversity* function in the *vegan* package and then converting Shannon–Weaver diversity to Hill numbers to aid in interpretation. Predictor variables were selected from available water quality data using stepwise backward model selection via the *drop1* function with F-tests to determine the significance of each term. The *manyglm* function in the *mvabund* package (Wang et al., 2012) was then used to assess the effects of selected habitat variables on multivariate functional group composition as well as the individual univariate effects on the OTU richness of each functional group. The default of a negative binomial error distribution was selected after an initial Poisson model showed evidence of heteroscedasticity and overdispersion in residual plots which were resolved in the negative binomial model.

For the remaining analyses, OTUs recovered from the three replicate field samples from each of the 17 sites were combined to obtain a semi-quantitative measure of per-site abundance for each fungal OTU detected. Distinct fungal OTUs in each sample were assigned an "occurrence" value of 1 or 0, with 1 representing OTU presence in that sample (greater than 0 reads) and 0 representing absence, as studies have shown that the number of reads sequenced per OTU is an inadequate measurement of OTU abundance due to biases in PCR amplification (Jusino et al., 2019). We then averaged the OTU occurrence values for the three replicate samples at each site. The resulting proportion represented whether the OTU was detected in zero, one, two, or three of the replicate samples and was used as a proxy for abundance, with the assumption that the detection of an OTU in a greater number of field samples is related to higher abundance of the OTU at each site. This approach, compared with presence/absence data, minimizes the impact of spurious OTUs, as OTUs with a lower proportion were given less weight in the following analyses, but is not as impacted by PCR bias as using raw or relative read numbers.

To assess the effect of habitat type on fungal community composition, we conducted unconstrained ordination by nonparametric multidimensional scaling (NMDS) using the *metaMDS* function in the *vegan* package for R (Oksanen, 2015). We converted the matrix representing OTU abundance to a pairwise Bray–Curtis distance matrix for ordination and for a nonparametric permutational multivariate ANOVA (PERMANOVA) test (Anderson, 2001). The PERMANOVA was performed with the *adonis* function to test for significant differences in fungal community composition between different habitat types (ponds, freshwater streams, and tidal creeks).

We identified which environmental variables were significant predictors of fungal community composition using constrained ordination of the fungal community data and the following environmental variables measured at the same sites by the College Creek Alliance: temperature, conductivity, oxygen saturation, pH, total suspended solids (TSS), total particulate phosphorus, dissolved inorganic phosphorus (DIP), ammonium (NH^4), and Secchi depth (a measure of water clarity) (William & Mary CCA, 2022). We applied distance-based redundancy analysis (dbRDA) using the *capscale* function in the *vegan* package of R (Legendre & Anderson, 1999; Oksanen et al., 2008). We conducted stepwise forward model selection based on maximized adjusted R-squared values (Blanchet et al., 2008) to add environmental variables to the model using the *ordiR2step* function in the *vegan* package. Significance tests of the full model were based on permutations using the *anova.cca* function in the *vegan* package. We repeated this analysis twice, first using only the water quality measurements collected by the College Creek Alliance in April 2021 (hereafter referred to as 'short-term measurements') to coincide with the timeframe during which our eDNA samples were collected, and then again using measurements averaged over 17 years of the College Creek Alliance's quarterly sampling ('long-term averages'). We compared the results of these two analyses and found stronger correspondence between fungal communities and the short-term data than the long-term averages. Given its greater explanatory power, we only used the short-term measurements for subsequent dbRDA analyses on individual taxa.

We predicted that water quality variables would have a much stronger influence on primarily aquatic fungal taxa and a weaker relationship with terrestrial or secondarily aquatic fungi. To test this prediction, we repeated constrained and unconstrained ordination analyses on the following subsets of our data: only OTUs identified to Phylum Chytridiomycota, only Ascomycota, or only Basidiomycota. Chytridiomycota are predominantly aquatic fungi, Basidiomycota are predominantly terrestrial, and Ascomycota are more evenly mixed terrestrial and aquatic fungi (Shearer et al., 2007). Therefore, we predicted the strongest correspondence with habitat type and water quality variables in the Chytridiomycota, weaker correspondence in Ascomycota, and weakest correspondence in Basidiomycota due to the influence of aerial dispersed spores of terrestrial Basidiomycota. To further test our prediction, we also conducted these analyses on OTUs that were identified as genera known to be aquatic hyphomycetes. We retained only OTUs identified to genera listed in the most recent and comprehensive global database of aquatic hyphomycete species (Duarte et al., 2022). Aquatic hyphomycetes contain both fungi from Ascomycota and Basidiomycota and are all specialized aquatic saprotrophs. Therefore, we predicted the strongest correspondence between water quality data and this functional group of aquatic fungi.

3 | RESULTS

3.1 | Detection of fungi compared between ITS and LSU markers

After quality filtering sequences generated using primers targeting the ITS2 region, 6,058,814 reads were retained from an initial 7,087,342 raw reads. Clustering generated 4,309 unique OTUs, of which 2,355 (54.7%) were identified to the kingdom Fungi. Of the 2,355 OTUs identified as Fungi, 2,040 were identified to the phyla level (86.6%), 1004 (42.6%) were identified to 541 genera, and 51 (2.2%) were identified to the functional group aquatic hyphomycetes. Processing of sequences generated using primers targeting the LSU region yielded comparable results. Of 7,768,320 raw reads, 6,349,145 were retained after quality filtering. Clustering generated 2,533 unique OTUs, of which 2,246 (88.7%) were identified to kingdom Fungi. Of those, 2,102 were identified to the phyla level (93.6%), 1,318 (58.7%) were identified to 569 genera, and 46 (2.0%) were identified to the functional group aquatic hyphomycetes.

Taxonomic coverage of the ITS2 and LSU datasets was comparable, but with notable differences (Figure S1). The total numbers of fungal OTUs were similar, but ITS2 recovered more OTUs that could not be assigned to any taxon as well as more OTUs assigned to Viridiplantae. At the phylum level, ITS2 and LSU recovered similar OTU richness in Ascomycota (1176 and 1183, respectively) and Basidiomycota (311 and 340, respectively), though ITS2 recovered slightly more OTUs assigned to Chytridiomycota (465 with ITS2; 350 with LSU). Both markers also recovered OTUs from Rozellomycota (40 with ITS2; 66 with LSU) and Glomeromycota (5 with ITS2; 16 with LSU). Each marker identified OTUs to unique phyla not captured by the other: LSU recovered 113 OTUs from Mucoromycota, 18 from Zoopagomycota, 15 from Blastocladiomycota, and 1 from Microsporidia; ITS recovered 29 OTUs from Monoblepharomycota, 9 from Mortierellomycota, 3 from Olpidiomycota, 1 from Aphelidiomycota, and 1 from Basidiobolomycota (Figure S1). Both the ITS2 and LSU datasets recovered a comparable number of aquatic hyphomycete OTUs. At the genus level, 201 genera were identified by both markers, 340 were unique to the ITS2 dataset, and 368 were unique to the LSU dataset.

Alpha diversity estimates on a sample-to-sample basis showed a significant correlation between fungal OTU richness recovered from ITS2 and OTU richness recovered from LSU (Figure 2; left panel). However, on average, we detected 61 more fungal OTUs per sample in the LSU dataset than in the ITS2 dataset (Paired *t*-test; $t = 4.64$, df = 50, $p < 0.001$). The two markers produced largely congruent estimates of beta diversity among samples; pairwise Jaccard distances in the ITS2 dataset were strongly correlated to pairwise Jaccard distances in the LSU dataset (Figure 2; right panel).

3.2 | Functional diversity

We assigned 1283 of the LSU OTUs to 21 functional groups according to the FungalTraits database. The functional diversity of aquatic fungal communities was significantly correlated with water temperature and total particulate phosphorus concentrations (Figure 3, top panels). Backward model selection retained water temperature (coefficient = −0.194, $t = -2.149$, $p = 0.049$) and total phosphorus (coefficient = 1.632, $t = 3.310$, $p = 0.005$) as significant explanatory variables for functional diversity. The resulting model was significant and explained nearly half the variation in functional diversity (multiple $R^2 = 0.456$, $F_{2,14} = 5.882$, $p = 0.014$). Multivariate generalized linear models showed significant multivariate effects of temperature (deviance = 70.86, $p = 0.007$) and marginally significant effects of total phosphorus (deviance = 41.42, $p = 0.062$) on functional group composition. The univariate tests from the many GLM model showed that the observed decrease in functional diversity with increasing temperature was driven by significant reductions in the OTU richness of eight functional groups, which included algal parasites ($p = 0.007$), animal symbionts ($p = 0.012$), ectomycorrhizae ($p = 0.002$), litter saprotrophs (0.001), mycoparasites ($p = 0.029$), soil saprotrophs ($p = 0.002$), unspecified saprotrophs ($p = 0.012$), and wood saprotrophs ($p = 0.012$; Figure 3; bottom left). The increase in functional diversity with total phosphorus was driven by significant increases in the richness of algal parasites ($p = 0.008$) and animal endosymbionts ($p = 0.032$), and a small decline in arbuscular mycorrhizae ($p = 0.053$; Figure 3; bottom right).

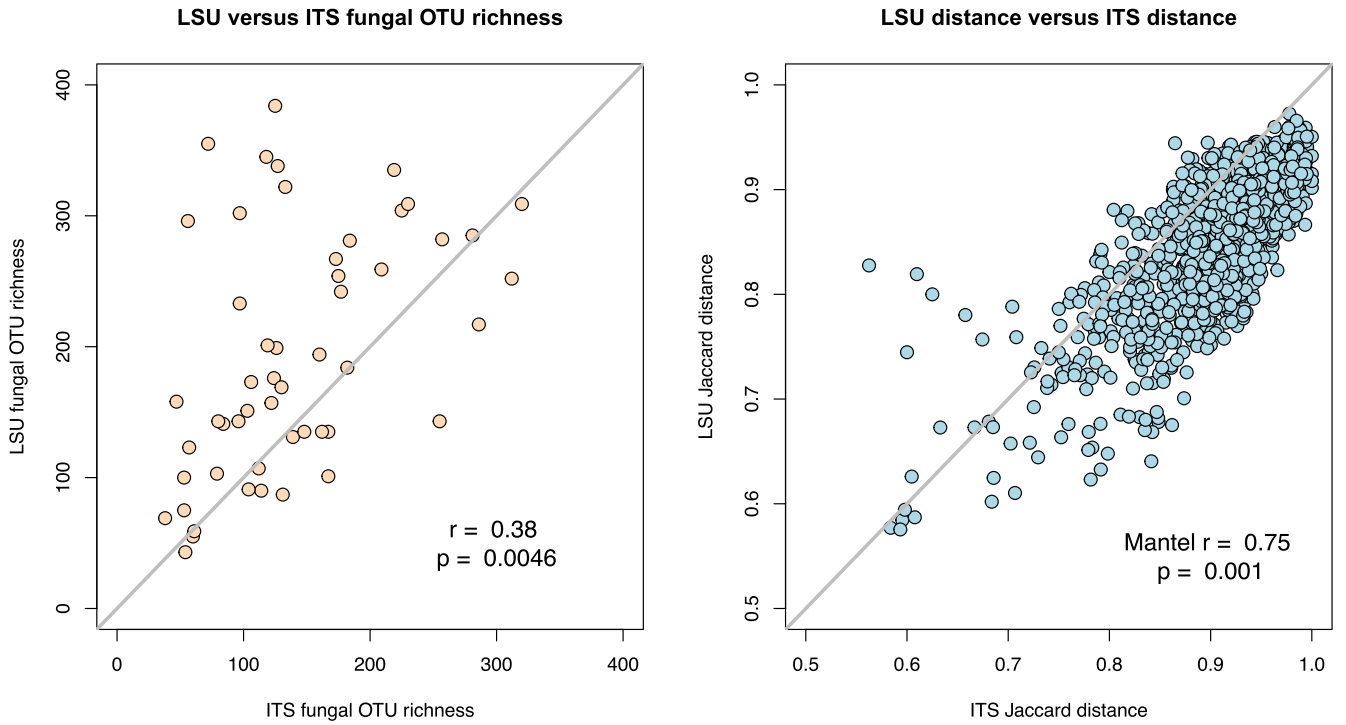

FIGURE 2 Sample-to-sample comparisons of alpha and beta diversity recovered using two different fungal metabarcoding markers, ITS2 and LSU. Left: OTU richness recovered using the ITS2 marker (*x*-axis) plotted against OTU richness recovered from the same samples using the LSU marker (*y*-axis). Right: Jaccard distance for all pairwise comparisons between samples recovered from the ITS2 marker (*x*-axis) and LSU marker (*y*-axis). In both plots, points above the diagonal 1:1 line indicate higher values from the LSU marker, and points below the line indicate higher values for ITS2 marker.

3.3 | Effects of water quality on total fungal community composition

Water quality data explained much of the variation in fungal community composition among pond, stream, and tidal creek habitats. Unconstrained multivariate analysis revealed a significant effect of habitat type on aquatic fungi community composition for both ITS (PERMANOVA; $F=2.09$, $R^2=0.23$, $p=0.001$) and LSU datasets (PERMANOVA; $F=1.89$, $R^2=0.21$, $p=0.001$). Short-term water quality conditions (April 2021) had more explanatory power than long-term conditions (17-year averages) in explaining differences in overall fungal community composition, regardless of marker (Figure 4; Figure S2). With both markers, *capscale* analysis of the short-term environmental data retained the variables TSS ($p=0.002$ with ITS2; $p=0.044$ with LSU), temperature ($p=0.004$ with ITS2; $p=0.002$ with LSU), and DIP ($p=0.014$ with ITS2; $p=0.014$ with LSU). The ITS2 model additionally retained the variable Secchi depth ($p=0.020$). The ITS2 and LSU models resulted in overall model-adjusted R^2 values of 0.23 and 0.21, respectively. This model suggested that stream fungal communities are associated with clearer water (i.e., increased Secchi depth) and cooler water temperatures, whereas pond communities were associated with increased dissolved inorganic phosphorus (DIP), and tidal creek communities were associated with higher total suspended solids and more turbid water (Figure 4, left; Figure S2, left). The variables retained using the ITS2 data and long-term averaged conditions were conductivity ($p=0.002$), temperature ($p=0.004$), and total particulate phosphorus ($p=0.004$) and overall model R^2 was 0.18. In agreement with the short-term model, this model also suggested that stream communities are associated with cooler water temperature, but additionally suggested that pond communities are negatively associated with total phosphorus and conductivity and tidal creek communities have strong positive associations with total phosphorus and conductivity (Figure 4; right). The long-term model using LSU data strongly resembled both the ITS2 and LSU short-term models, retaining the variables TSS ($p=0.002$), temperature ($p=0.01$), and DIP (0.044) but explained less variation in the data (adjusted $R^2=0.19$).

3.4 | Specific effects on each of the major phyla and aquatic hyphomycetes

For concision, we have presented only the result obtained from the ITS2 dataset. The results from LSU were qualitatively similar and are presented in the supplement (Figure S3). Congruent with our prediction, the primarily aquatic Chytridiomycota showed significant variation in community composition. The community composition of Chytridiomycota was significantly different among habitat types (PERMANOVA; $F=2.94$, $R^2=0.30$, $p=0.001$). This variation was explained largely by variation in water quality variables (Figure 5; top left). Constrained ordination model selection retained the variables TSS ($p=0.002$), temperature ($p=0.004$), DIP ($p=0.004$), and Secchi

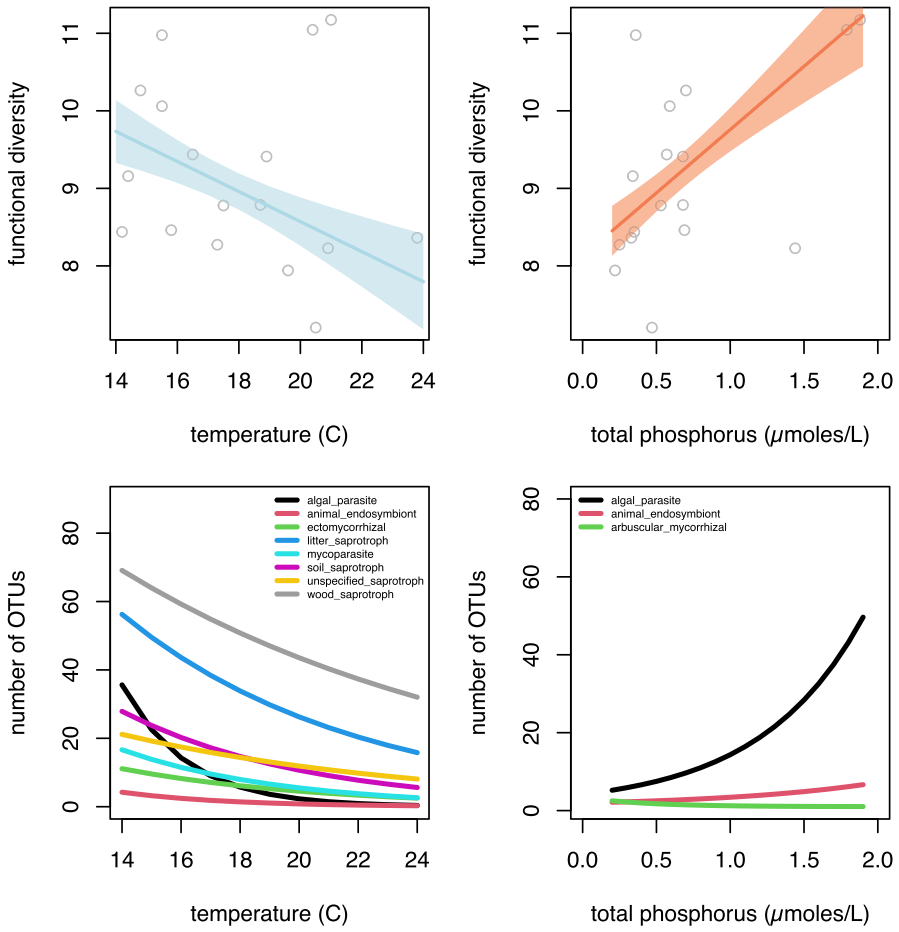

FIGURE 3 Relationships between water quality variables and the functional diversity of aquatic fungi in a coastal watershed. Top panels show Shannon–Weaver diversity of fungal functional groups observed at 17 sites (converted to Hill numbers) versus water temperature (left) and total phosphorus (right). Lines show model fit and shading shows fit ±1 SE. Bottom panels show model fits for the relationships between the OTU richness of each functional group and water temperature (left) and total phosphorus (right). Only those functional groups with statistically significant ($\alpha = 0.05$) relationships are shown.

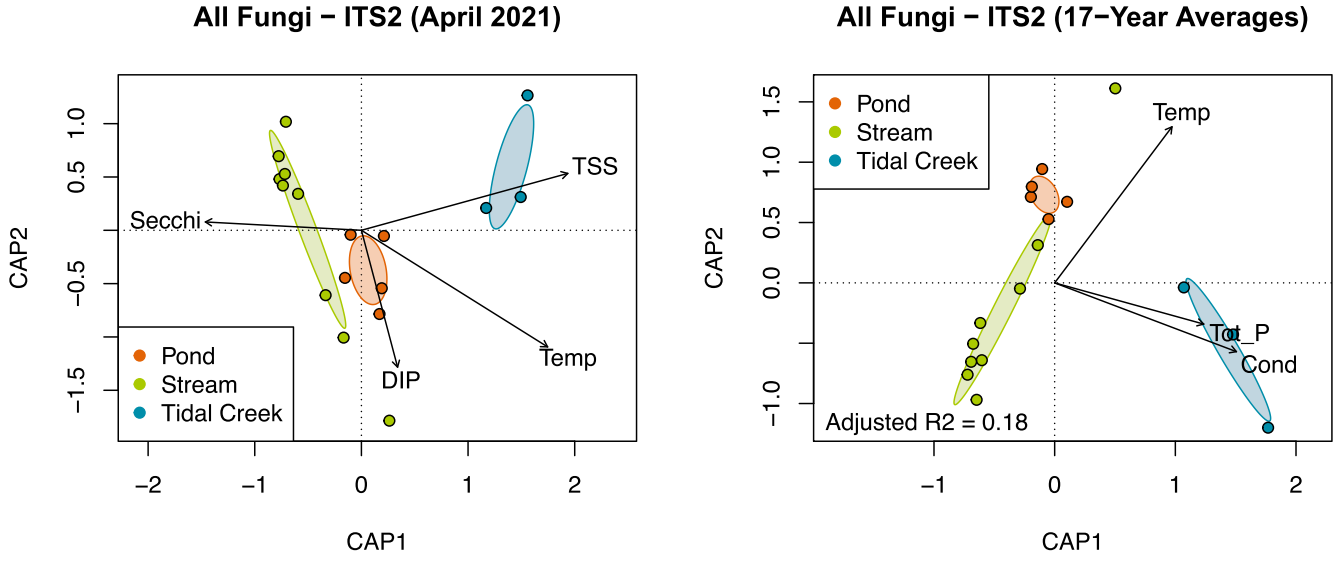

FIGURE 4 Effect of short-term (left) and long-term (right) environmental conditions on fungal community composition inferred from metabarcoding using the ITS2 marker. Both plots show distance-based redundancy analysis (dbRDA) ordination. Points represent site scores of aggregated community data for each of the 17 sampling sites, color coded by habitat type. Ellipses display standard deviation around the centroid for each habitat type. Arrows represent the correlation of environmental variables with community composition, and arrow length represents relationship strength.

depth ($p = 0.030$) and resulted in an overall model with $R^2 = 0.39$, which was much higher than the R^2 for the overall model that included all Fungi (reported above). Again, for concision, we present only the result obtained from the ITS2 dataset, given that the results from LSU were qualitatively similar (Figure S3) with the exceptions noted below.

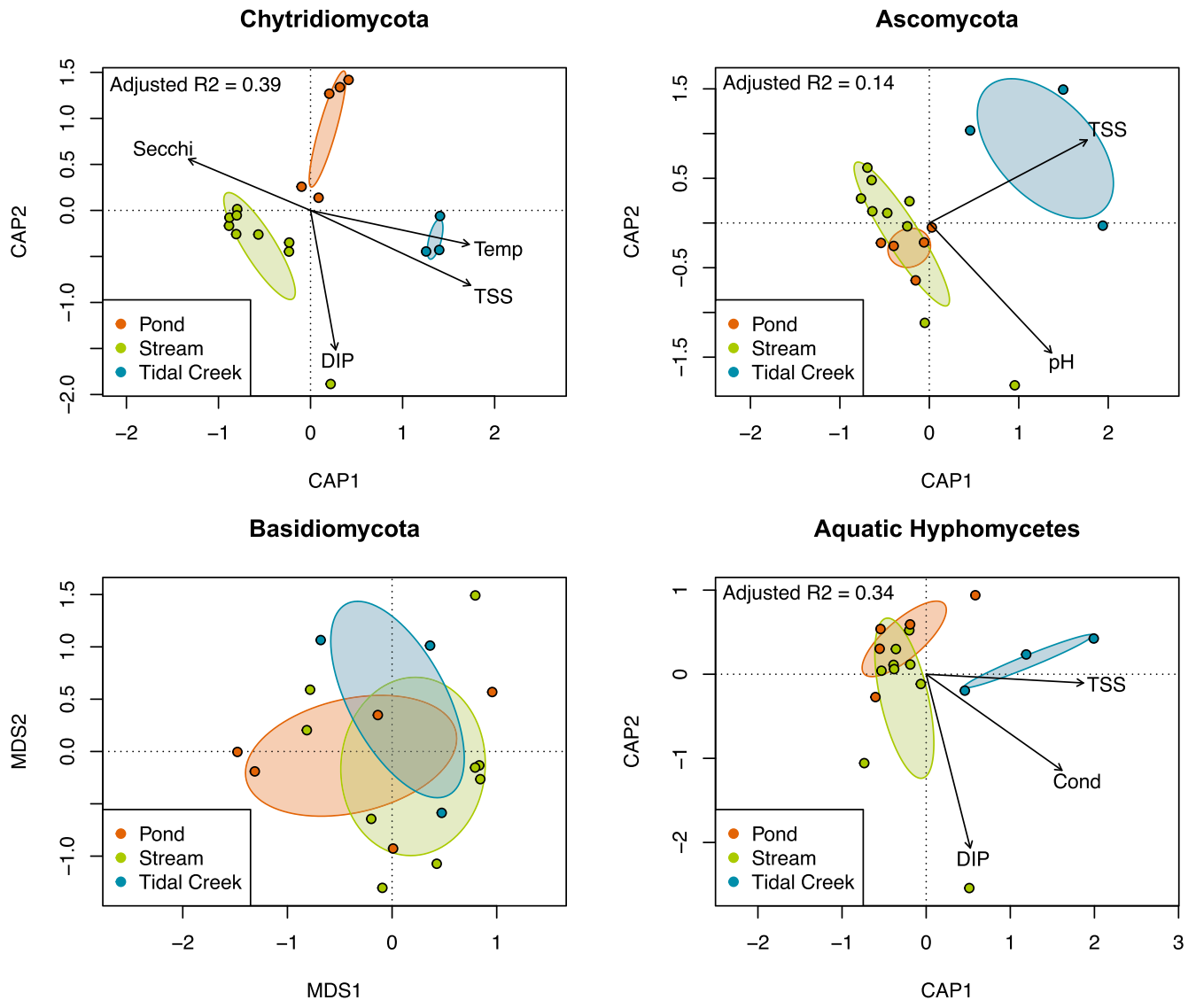

FIGURE 5 Distance-based redundancy analysis (dbRDA) ordination performed with the *capscale* function in R displaying the effect of environmental conditions on Chytridiomycota (top left), Ascomycota (top right), Basidiomycota (bottom left), and Aquatic Hyphomycete (bottom right) community composition, inferred from metabarcoding using the ITS2 marker. Points represent site scores of aggregated community data for 17 sampling sites. Ellipses display standard deviation around the centroid for each habitat type. Arrows represent the correlation between environmental variables with community composition, and arrow length represents relationship strength.

Ascomycota communities also differed significantly among habitat types (PERMANOVA; $F = 1.53$, $R^2 = 0.18$, $p = 0.002$). Ascomycota community composition was significantly related to water quality, but less strongly than Chytridiomycota communities (Figure 5; top right). Model selection retained the variables TSS ($p = 0.006$), and pH ($p = 0.026$), and the overall R^2 of the model was 0.14, considerably lower than the final model for Chytrids (reported above).

In contrast to our models using all fungi, chytrids only, or ascomycetes only, there was no effect of habitat type or water quality variables on Basidiomycota community composition inferred from the ITS2 marker. The PERMANOVA found no significant difference among habitat types (PERMANOVA; $F = 1.18$, $R^2 = 0.14$, $p = 0.096$). Stepwise selection of the constrained ordination using short-term conditions retained no variables, indicating that community composition was not significantly explained by any of the considered environmental variables (Figure 5; bottom left). However, there were some significant relationships between water quality and Basidiomycota composition inferred from the LSU data (Figure S3).

Habitat type was a significant predictor of aquatic hyphomycete community composition (PERMANOVA; $F = 2.06$, $R^2 = 0.23$, $p = 0.008$). Variables retained by stepwise selection of the constrained ordination using short-term conditions were TSS ($p = 0.008$), DIP ($p = 0.024$), and conductivity ($p = 0.018$) and the overall R^2 of the model was 0.34, which was intermediate to the models for chytrids and ascomycetes. Pond and stream communities were more similar to one another than to tidal creek communities, with the former being positively associated with DIP and

negatively associated with TSS and conductivity and the latter being positively associated with TSS and conductivity (Figure 5; bottom right).

4 | DISCUSSION

Our results emphasize the importance of water quality for determining patterns in fungal communities and the great utility of eDNA metabarcoding for assessing the unique drivers of each component of aquatic fungal communities. Aquatic fungi community composition and functional diversity are related to water quality variables, especially temperature, suspended solids, and particulate phosphorus. Each fungal phylum and functional group had unique responses to habitat variables and water quality measures. By comparison, ITS2 and LSU markers revealed somewhat different components of the fungal communities, but strong effects of habitat type and water quality measures were identified in both datasets.

4.1 | Water quality and the composition of aquatic fungal communities

Our results show that aquatic fungal communities respond to relatively small-scale physical and chemical variations within aquatic systems. Variations in habitat type, temperature, nutrient concentrations, and other physicochemical factors within our study area were associated with distinct taxonomic assemblages. Temperature and total particulate phosphorus were the strongest predictors of functional assemblages. Due to the polyphyletic nature of freshwater fungi, taxa responses to habitat type and water quality varied greatly, with Chytridiomycota and aquatic hyphomycete communities being most strongly associated with habitat type, while Ascomycota had a significant but lesser association and Basidiomycota only demonstrated an association with habitat type using the LSU dataset.

Potential explanations for the responsiveness of aquatic fungal community composition to habitat type and water quality are that fungi active in the system are either metabolically influenced by water chemistry or are contributing to water chemistry variability through ecological roles. Although prior studies have shown that terrestrial vegetation is a strong predictor of terrestrial fungal DNA assemblages, we found poor representation of terrestrial fungal communities in our stream samples. Likewise, Matsuoka et al. (2019) found no relationship between catchment vegetation and freshwater fungal community composition, suggesting minor proportions of terrestrial fungi in their water samples. Furthermore, our study found that fungi in groups that are typically considered to be exclusively aquatic, such as Chytridiomycota and aquatic hyphomycetes, were highly responsive to water chemistry, while Basidiomycota, a group that is mainly terrestrial, showed weak responses to water chemistry and habitat type. Thus, though fungi of terrestrial origin were present in the aquatic samples, the stronger responses of aquatic fungal taxa to habitat type and water quality suggest that the identified relationships between these environmental variables and fungal community composition in water are driven by aquatic taxa and are not an artifact of the surrounding terrestrial fungal ecology. These findings imply that changes to freshwater ecosystems may influence the structure of aquatic fungal communities.

High habitat specificity between temperate ponds, streams, and oligohaline tidal creeks as found in our study is congruent with a recent study demonstrating that Arctic aquatic fungal communities differ between stream, pond, melting ice water, and estuary sites (Zhang et al., 2016). Aquatic fungal community composition has also been shown to be biome-specific, with freshwater community composition differing significantly from all other biomes inhabited by aquatic fungi (Panzer et al., 2015). Strong specificity to habitat type suggests that physical alterations to water bodies, such as damming a stream which shifts it from a lotic to a lentic system, may result in shifts in the fungal community structure, with unknown impacts on energy and nutrient cycling in the system.

We found temperature to be a strong correlate with aquatic fungal composition and functional diversity, supporting previous findings of temperature regulation of aquatic fungal community composition (Yuan et al., 2020; Zhang et al., 2016). In both our long-term and short-term datasets, temperature was a major factor separating the community composition of cooler streams from the typically warmer ponds and tidal creeks (Figure 4), particularly among the Chytridiomycota (Figure 5; top left). Temperature was the strongest correlate of functional diversity, with major declines in the OTU richness of many important functional groups, including various saprotrophs and algal parasites, at warmer temperatures. Temperature changes have been shown to drive seasonal shifts in aquatic saprotrophic species (Suberkropp, 1984) and algal parasites whose prevalence peaks in spring and declines with increasing temperatures (Gsell et al., 2022). Seasonal trends could conflict with the spatial patterns observed in our study, as aquatic saprotrophs have been observed to reach peak richness during spring and summer, and lows in winter (Iqbal, 1997). Because we sampled exclusively in late spring, when aquatic saprotrophs are expected to be at their peak, our assessment likely reflects local variation in maximum seasonal diversity. However, new patterns may emerge if fungal sampling were conducted during other seasons. Matsuoka et al. (2021) reported seasonal changes in freshwater fungal communities, finding that community assemblages were season-specific and exhibited annual periodic patterns. Our findings reflect this seasonal effect, with variance in fungal community composition in April 2021 being explained more strongly by short-term environmental conditions (measurements from April 2021 only) than by long-term environmental conditions (measurements averaged over 17 years of quarterly sampling). Future work should incorporate year-round fungal sampling with experiments to further test the relationships revealed in the current study between fungal composition, functional diversity, temperature, and

seasonality. Although surveys are correlational and controlled experiments are needed to verify causal relationships, our study does suggest that warming temperatures—because of either climate change, impoundments, or removal of riparian vegetation—could cause major shifts in aquatic fungal communities and potential declines in functional diversity.

Chemical changes such as nutrient pollution might alter fungal composition via cascading effects on trophic webs. For instance, particulate phosphorus was a significant predictor of total fungal composition and functional diversity. It is widely known that phosphorus enrichment is a major cause of nutrient pollution and eutrophication in aquatic systems, often leading to harmful blooms of algae and cyanobacteria (Correll, 1998). Indeed, the increased functional diversity that we observed with increasing total particulate phosphorus was largely driven by fungi that were classified as algal parasites. This suggests that the changes we observed in fungal communities with increasing total phosphorus are a response to increased primary production of algae, and could support recent work that suggests that fungi may play an important role in governing harmful algal blooms (Gleason et al., 2015). In contrast, other recent findings have shown a negative correlation between phosphorus and the prevalence of algal parasites (Gleason et al., 2015; McKindles et al., 2023). These studies measured soluble reactive phosphorus, which is quickly assimilated by phytoplankton leading to unpredictable fluctuations due to variation in the composition of primary producers and concomitant variation in nutrient demand. Our study showed increases in fungal communities associated with total particulate phosphorus derived from total suspended solids, including algal cells. In this sense, particulate phosphorus concentration may be a better measure of the trophic state of the system, whereas soluble reactive phosphorus concentrations may reflect short-term dynamics. The sensitivity of fungal community composition to nutrient concentrations shown in our study suggests that freshwater fungi may be an ideal group to be used as indicators and moderators of ecosystem disturbance and environmental change (Barros & Seena, 2022; Vatova et al., 2022).

Variation in overall fungal community composition was also explained strongly by total suspended solids in the water (TSS), which was generally higher in tidal creek and pond sites than stream sites. TSS is a measure of all solid particles greater than 1 micron suspended in the water, and TSS often consists of sediments, bacteria, and particulate organic matter, including algae. In streams, TSS is mostly inorganic sediments eroded and transported by stream flow; in ponds, TSS is mostly organic matter in the form of plankton; in tidal creeks, the solids are a mix of both inorganic and organic matter. Previous studies have shown the responsiveness of fungal communities to organic matter in water, with Pietryczuk et al. (2018) finding that diversity increased with increasing organic matter, while Solé et al. (2008) identified several species that do not tolerate high organic matter concentrations. Our results are congruent with these findings, but it remains unclear whether inorganic sediments correlate with aquatic fungal community composition.

4.2 | Efficacy of eDNA to characterize aquatic fungal diversity

Our study shows that high-throughput amplicon sequencing of environmental DNA is an effective tool for identifying many fungal taxa in freshwater through efficient and cost-effective sampling methods. In both our ITS and LSU datasets, we captured over 2000 fungal OTUs among our 17 sites and were able to assign at least phylum-level taxonomy to 87% of those fungal OTUs using ITS and 94% using LSU. While large gaps still exist in taxonomic databases, as demonstrated by the proportions of fungal OTUs that were able to be assigned to genus level with each marker (43% with ITS2; 59% with LSU), this method allowed us to survey fungal diversity within a drainage network while overcoming limitations in traditional sampling methods such as the time-, labor-, and expertise-intensive nature of culturing.

We found strong correlations between local water quality and fungal community composition. This finding strongly indicates that DNA-based sampling of aquatic fungal communities effectively characterizes fine-scale geographic variation in aquatic fungal community composition driven by local environmental conditions, and that DNA-based aquatic fungal community inferences are not overwhelmed by spurious sources of fungal DNA such as windblown spores and other terrestrial inputs. Our findings contrast previous studies which used molecular methods to capture fungal diversity in freshwater but lacked evidence to show that fungal DNA present in the water originated from fungi that were active in the system, rather than from inactive fungal matter that entered the water from land through wind, surface runoff, or other means (LeBrun et al., 2018; Matsuoka et al., 2019). However, the pervasive influence of water quality on aquatic fungal communities is not specific to our study system, as previous studies conducted in a range of biomes also documented freshwater fungal community composition to be strongly influenced by environmental variability (Heino et al., 2014; Ortiz-Vera et al., 2018; Panzer et al., 2015; Pietryczuk et al., 2018; Zhang et al., 2016).

4.3 | Comparison of ITS and LSU markers

Researchers are increasingly aware of the importance of marker selection and the utility of multi-marker approaches for metabarcoding studies of fungal communities (Skelton et al., 2019; Tedersoo et al., 2022). However, direct, sample-for-sample comparisons are relatively scarce. Here, we provide a direct comparison of aquatic fungal communities inferred from two popular metabarcoding markers, ITS2 and LSU. Comparison of community dissimilarity analyses conducted using ITS2 and LSU yielded similar outcomes. If a future eDNA study of aquatic fungal communities was solely interested in broad patterns of beta diversity, then either marker alone may produce analogous results. However, we did see considerable differences in alpha diversities between the two markers, with certain taxonomic groups being more or less emphasized by each marker

(see Figure S1). Similar to a previous study that sampled a diversity of aquatic and terrestrial fungal habitats, we recovered more OTUs from several early diverging fungal lineages using the LSU marker, likely because of decreased primer bias and length variability of the LSU marker in early diverging groups such as Zoopagomycota (Reynolds et al., 2022). Interestingly, we recovered greater Chytridiomycota OTU richness from the ITS2 marker, suggesting that this may be a preferable marker for studies focused on this very important phylum of aquatic fungi. Previous work has shown that multi-marker approaches often recover diversity that would be missed in by a single-marker approach, and that diversity may comprise taxa that are disproportionately important to the research goals of the study (Reynolds et al., 2022; Skelton et al., 2019). Therefore, future studies targeting patterns in alpha diversity or specific taxonomic groups should strongly consider using multi-marker approaches.

5 | CONCLUSIONS

The taxonomic composition and functional diversity of freshwater fungal communities respond to variation in water quality variables and are therefore likely to be vulnerable to habitat alterations such as nutrient pollution and increasing temperatures. Our work shows that metabarcoding of environmental DNA can provide comprehensive surveys of these cryptic organisms and provides sufficient community-level resolution to characterize fine-scale geographic variation in fungal community composition and functional diversity. Globally, anthropogenic processes are creating chemical and physical changes to freshwater systems, with known consequences for biodiversity and ecosystem functions. Our findings indicate that freshwater fungi are highly responsive to environmental conditions, though their responses differ greatly among taxonomic and functional groups. Understanding how freshwater fungal communities are affected by water quality variability is crucial for identifying and predicting how aquatic fungal diversity and roles in habitats may shift in response to changing freshwater conditions.

ACKNOWLEDGMENTS

We heartily thank Madeline Reinsel and other members of the Keck Lab for providing the long-term water quality data. A draft of the manuscript was improved by the insightful comments of Matthias Leu and Rowan Lockwood. Funding was provided by the Department of Biology at William & Mary's Mary E. Ferguson Memorial Research Grant and the Mycological Society of America's Undergraduate Research Award.

CONFLICT OF INTEREST STATEMENT

We have no conflicts of interest.

DATA AVAILABILITY STATEMENT

Raw sequences and sample metadata are available on NCBI SRI under project number PRJNA976210. Water quality data are provided in the online supplement and are available from the William & Mary College Creek Alliance website – https://www.wm.edu/as/kecklab/watershedmonitoring/waterqualitydata/.

ORCID

Michelle A. Jusino https://orcid.org/0000-0002-3284-4254
James Skelton https://orcid.org/0000-0002-3017-5843

REFERENCES

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecology*, *26*(1), 32–46. https://doi.org/10.1111/j.1442-9993.2001.01070.pp.x

Apitz, S. E. (2012). Conceptualizing the role of sediment in sustaining ecosystem services: Sediment-ecosystem regional assessment (SEcoRA). *Science of the Total Environment*, *415*, 9–30. https://doi.org/10.1016/j.scitotenv.2011.05.060

Bärlocher, F. (1992). *The ecology of aquatic hyphomycetes*. Springer.

Barros, J., & Seena, S. (2022). Fungi in freshwaters: Prioritising aquatic hyphomycetes in conservation goals. *Watermark*, *14*(4), 605. https://doi.org/10.3390/w14040605

Blanchet, F. G., Legendre, P., & Borcard, D. (2008). Forward selection of explanatory variables. *Ecology*, *89*(9), 2623–2632. https://doi.org/10.1890/07-0986.1

Brazee, N. J., & Lindner, D. L. (2013). Unravelling the *Phellinus pini* s.l. complex in North America: A multilocus phylogeny and differentiation analysis of *Porodaedalea*. *Forest Pathology*, *43*(2), 132–143. https://doi.org/10.1111/efp.12008

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Ceballos-Escalera, A., Richards, J., Arias, M. B., Inward, D. J. G., & Vogler, A. P. (2022). Metabarcoding of insect-associated fungal communities: A comparison of internal transcribed spacer (ITS) and large-subunit (LSU) rRNA markers. *MycoKeys*, *88*, 1–33. https://doi.org/10.3897/mycokeys.88.77106

Comeau, A. M., Vincent, W. F., Bernier, L., & Lovejoy, C. (2016). Novel chytrid lineages dominate fungal sequences in diverse marine and freshwater habitats. *Scientific Reports*, *6*(1), 30120. https://doi.org/10.1038/srep30120

Correll, D. L. (1998). The role of phosphorus in the eutrophication of receiving waters: A review. *Journal of Environmental Quality*, *27*(2), 261–266. https://doi.org/10.2134/jeq1998.00472425002700020004x

Duarte, R., Fernandes, I., Gulis, V., Cássio, F., & Pascoal, C. (2022). New ITS rDNA barcodes clarify phylogenetic relationships and biogeography of aquatic hyphomycetes. *Research Square*, *20*, 377. https://doi.org/10.21203/rs.3.rs-1428377/v1

Edgar, R. C., & Flyvbjerg, H. (2015). Error filtering, pair assembly and error correction for next-generation sequencing reads. *Bioinformatics*, *31*(21), 3476–3482. https://doi.org/10.1093/bioinformatics/btv401

Elosegi, A., & Sabater, S. (2013). Effects of hydromorphological impacts on river ecosystem functioning: A review and suggestions for assessing ecological impacts. *Hydrobiologia*, *712*(1), 129–143. https://doi.org/10.1007/s10750-012-1226-6

Ferreira, V., Gulis, V., Pascoal, C., & Graca, M. (2014). Stream pollution and fungi. In *Freshwater fungi* (pp. 389–412). De Gruyter.

Findlay, S. E. G., & Arsuffi, T. L. (1989). Microbial growth and detritus transformations during decomposition of leaf litter in a stream. *Freshwater Biology*, *21*(2), 261–269. https://doi.org/10.1111/j.1365-2427.1989.tb01364.x

Finlay, B. J., Maberly, S. C., & Cooper, J. I. (1997). Microbial diversity and ecosystem function. *Oikos*, *80*(2), 209. https://doi.org/10.2307/3546587

Frenken, T., Alacid, E., Berger, S. A., Bourne, E. C., Gerphagnon, M., Grossart, H.-P., Gsell, A. S., Ibelings, B. W., Kagami, M., Küpper, F. C., Letcher, P. M., Loyau, A., Miki, T., Nejstgaard, J. C., Rasconi, S., Reñé, A., Rohrlack, T., Rojas-Jimenez, K., Schmeller, D. S., … Agha, R. (2017). Integrating chytrid fungal parasites into plankton ecology: Research gaps and needs: Research needs in plankton chytridiomycosis. *Environmental Microbiology*, *19*(10), 3802–3822. https://doi.org/10.1111/1462-2920.13827

Frenken, T., Wolinska, J., Tao, Y., Rohrlack, T., & Agha, R. (2020). Infection of filamentous phytoplankton by fungal parasites enhances herbivory in pelagic food webs. *Limnology and Oceanography*, *65*(11), 2618–2626. https://doi.org/10.1002/lno.11474

Gerphagnon, M., Agha, R., Martin-Creuzburg, D., Bec, A., Perriere, F., Rad-Menéndez, C., Gachon, C. M. M., & Wolinska, J. (2019). Comparison of sterol and fatty acid profiles of chytrids and their hosts reveals trophic upgrading of nutritionally inadequate phytoplankton by fungal parasites. *Environmental Microbiology*, *21*(3), 949–958. https://doi.org/10.1111/1462-2920.14489

Gleason, F. H., Jephcott, T. G., Küpper, F. C., Gerphagnon, M., Sime-Ngando, T., Karpov, S. A., Guillou, L., & van Ogtrop, F. F. (2015). Potential roles for recently discovered chytrid parasites in the dynamics of harmful algal blooms. *Fungal Biology Reviews*, *29*(1), 20–33. https://doi.org/10.1016/j.fbr.2015.03.002

Grami, B., Rasconi, S., Niquil, N., Jobard, M., Saint-Béat, B., & Sime-Ngando, T. (2011). Functional effects of parasites on food web properties during the spring diatom bloom in Lake Pavin: A linear inverse modeling analysis. *PLoS One*, *6*(8), e23273. https://doi.org/10.1371/journal.pone.0023273

Grossart, H.-P., Van den Wyngaert, S., Kagami, M., Wurzbacher, C., Cunliffe, M., & Rojas-Jimenez, K. (2019). Fungi in aquatic ecosystems. *Nature Reviews Microbiology*, *17*(6), 339–354. https://doi.org/10.1038/s41579-019-0175-8

Gsell, A. S., Wolinska, J., Preuß, K., Teurlincx, S., Özkundakci, D., Hilt, S., van Donk, E., Ibelings, B. W., & Adrian, R. (2022). Long-term trends and seasonal variation in host density, temperature, and nutrients differentially affect chytrid fungi parasitising lake phytoplankton. *Freshwater Biology*, *67*(9), 1532–1542. https://doi.org/10.1111/fwb.13958

Gulis, V., Kuehn, K., & Suberkropp, K. (2006). The role of fungi in carbon and nitrogen cycles in freshwater ecosystems. In G. M. Gadd (Ed.), *Fungi in biogeochemical cycles* (1st ed., pp. 404–435). Cambridge University Press. https://doi.org/10.1017/CBO9780511550522.018

Heino, J., Tolkkinen, M., Pirttilä, A. M., Aisala, H., & Mykrä, H. (2014). Microbial diversity and community-environment relationships in boreal streams. *Journal of Biogeography*, *41*(12), 2234–2244. https://doi.org/10.1111/jbi.12369

Ihrmark, K., Bödeker, I. T. M., Cruz-Martinez, K., Friberg, H., Kubartova, A., Schenck, J., Strid, Y., Stenlid, J., Brandström-Durling, M., Clemmensen, K. E., & Lindahl, B. D. (2012). New primers to amplify the fungal ITS2 region—Evaluation by 454-sequencing of artificial and natural communities. *FEMS Microbiology Ecology*, *82*(3), 666–677. https://doi.org/10.1111/j.1574-6941.2012.01437.x

Ingold, C. (1942). Aquatic hyphomycetes of decaying alder leaves. *Transactions of the British Mycological Society*, *25*(4), 339.

Iqbal, S. H. (1997). Species diversity of freshwater hyphomycetes in some streams of Pakistan. II. Seasonal differences of fungal communities on leaves. *Annales Botanici Fennici*, *34*(3), 165–178.

Jobard, M., Rasconi, S., Solinhac, L., Cauchie, H.-M., & Sime-Ngando, T. (2012). Molecular and morphological diversity of fungi and the associated functions in three European nearby lakes: True fungi (eumycota) in pelagic freshwaters. *Environmental Microbiology*, *14*(9), 2480–2494. https://doi.org/10.1111/j.1462-2920.2012.02771.x

Jones, E. B. G., Hyde, K. D., & Pang, K.-L. (Eds.). (2014). *Freshwater Fungi: And Fungal-like Organisms*. Walter De Gruyter GmbH & Co KG.

Jusino, M. A., Banik, M. T., Palmer, J. M., Wray, A. K., Xiao, L., Pelton, E., Barber, J. R., Kawahara, A. Y., Gratton, C., Peery, M. Z., & Lindner, D. L. (2019). An improved method for utilizing high-throughput amplicon sequencing to determine the diets of insectivorous animals. *Molecular Ecology Resources*, *19*(1), 176–190. https://doi.org/10.1111/1755-0998.12951

Jusino, M. A., Lindner, D. L., Cianchetti, J. K., Grisé, A. T., Brazee, N. J., & Walters, J. R. (2014). A minimally invasive method for sampling Nest and Roost cavities for fungi: A novel approach to identify the fungi associated with cavity-nesting birds. *Acta Ornithologica*, *49*(2), 233–242. https://doi.org/10.3161/173484714X687127

Kagami, M., Miki, T., & Takimoto, G. (2014). Mycoloop: Chytrids in aquatic food webs. *Frontiers in Microbiology*, *5*, 166. https://doi.org/10.3389/fmicb.2014.00166

Kagami, M., Motoki, Y., Masclaux, H., & Bec, A. (2017). Carbon and nutrients of indigestible pollen are transferred to zooplankton by chytrid fungi. *Freshwater Biology*, *62*(5), 954–964. https://doi.org/10.1111/fwb.12916

LeBrun, E. S., Taylor, D. L., King, R. S., Back, J. A., & Kang, S. (2018). Rivers may constitute an overlooked avenue of dispersal for terrestrial fungi. *Fungal Ecology*, *32*, 72–79. https://doi.org/10.1016/j.funeco.2017.12.003

Lefèvre, E., Letcher, P., & Powell, M. (2012). Temporal variation of the small eukaryotic community in two freshwater lakes: Emphasis on zoosporic fungi. *Aquatic Microbial Ecology*, *67*(2), 91–105. https://doi.org/10.3354/ame01592

Legendre, P., & Anderson, M. J. (1999). Distance-based redundancy analysis: Testing multispecies responses in multifactorial ecological experiments. *Ecological Monographs*, *69*(1), 1–24. https://doi.org/10.1890/0012-9615(1999)069[0001:DBRATM]2.0.CO;2

Lindner, D. L., & Banik, M. T. (2009). Effects of cloning and root-tip size on observations of fungal ITS sequences from *Picea glauca* roots. *Mycologia*, *101*(1), 157–165. https://doi.org/10.3852/08-034

Liu, J., Yu, Y., Cai, Z., Bartlam, M., & Wang, Y. (2015). Comparison of ITS and 18S rDNA for estimating fungal diversity using PCR–DGGE. *World Journal of Microbiology and Biotechnology*, *31*(9), 1387–1395. https://doi.org/10.1007/s11274-015-1890-6

Matsuoka, S., Sugiyama, Y., Sato, H., Katano, I., Harada, K., & Doi, H. (2019). Spatial structure of fungal DNA assemblages revealed with eDNA metabarcoding in a forest river network in western Japan. *Metabarcoding and Metagenomics*, *3*, e36335. https://doi.org/10.3897/mbmg.3.36335

Matsuoka, S., Sugiyama, Y., Shimono, Y., Ushio, M., & Doi, H. (2021). Evaluation of seasonal dynamics of fungal DNA assemblages in a flow-regulated stream in a restored forest using eDNA metabarcoding. *Environmental Microbiology*, *23*(8), 4797–4806. https://doi.org/10.1111/1462-2920.15669

McKindles, K. M., McKay, R. M. L., Bullerjahn, G. S., & Frenken, T. (2023). Interactions between chytrids cause variable infection strategies on harmful algal bloom forming species. *Harmful Algae*, *122*, 102381. https://doi.org/10.1016/j.hal.2023.102381

Monchy, S., Sanciu, G., Jobard, M., Rasconi, S., Gerphagnon, M., Chabé, M., Cian, A., Meloni, D., Niquil, N., Christaki, U., Viscogliosi, E., & Sime-Ngando, T. (2011). Exploring and quantifying fungal diversity in freshwater lake ecosystems using rDNA cloning/sequencing and SSU tag pyrosequencing. *Environmental Microbiology*, *13*(6), 1433–1453. https://doi.org/10.1111/j.1462-2920.2011.02444.x

Nava, V., & Leoni, B. (2021). A critical review of interactions between microplastics, microalgae and aquatic ecosystem function. *Water Research*, *188*, 116476. https://doi.org/10.1016/j.watres.2020.116476

Oidtmann, B., Heitz, E., Rogers, D., & Hoffmann, R. W. (2002). Transmission of crayfish plague. *Diseases of Aquatic Organisms*, *52*(2), 159–167.

Oksanen, J. (2015). Vegan: An introduction to ordination (Version 8) [Computer software].

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Simpson, G., Solymos, P., Stevens, M. H., & Wagner, H. (2008). The vegan package: Community ecology package (1.15–1) [Computer software].

Ortiz-Vera, M. P., Olchanheski, L. R., da Silva, E. G., de Lima, F. R., Martinez, L. R., Sato, M. I. Z., Jaffé, R., Alves, R., Ichiwaki, S., Padilla, G., & Araújo, W. L. (2018). Influence of water quality on diversity and composition of fungal communities in a tropical river. *Scientific Reports*, *8*(1), 14799. https://doi.org/10.1038/s41598-018-33162-y

Paerl, H. W., Dyble, J., Moisander, P. H., Noble, R. T., Piehler, M. F., Pinckney, J. L., Steppe, T. F., Twomey, L., & Valdes, L. M. (2003). Microbial indicators of aquatic ecosystem change: Current applications to eutrophication studies. *FEMS Microbiology Ecology*, *46*(3), 233–246. https://doi.org/10.1016/S0168-6496(03)00200-9

Palmer, J. M., Jusino, M. A., Banik, M. T., & Lindner, D. L. (2018). Non-biological synthetic spike-in controls and the AMPtk software pipeline improve mycobiome data. *PeerJ*, *6*, e4925. https://doi.org/10.7717/peerj.4925

Panzer, K., Yilmaz, P., Weiß, M., Reich, L., Richter, M., Wiese, J., Schmaljohann, R., Labes, A., Imhoff, J. F., Glöckner, F. O., & Reich, M. (2015). Identification of habitat-specific biomes of aquatic fungal communities using a comprehensive nearly full-length 18S rRNA dataset enriched with contextual data. *PLoS One*, *10*(7), e0134377. https://doi.org/10.1371/journal.pone.0134377

Pietryczuk, A., Cudowski, A., Hauschild, T., Świsłocka, M., Więcko, A., & Karpowicz, M. (2018). Abundance and species diversity of fungi in Rivers with various contaminations. *Current Microbiology*, *75*(5), 630–638. https://doi.org/10.1007/s00284-017-1427-3

Põlme, S., Abarenkov, K., Henrik Nilsson, R., Lindahl, B. D., Clemmensen, K. E., Kauserud, H., Nguyen, N., Kjøller, R., Bates, S. T., Baldrian, P., Frøslev, T. G., Adojaan, K., Vizzini, A., Suija, A., Pfister, D., Baral, H.-O., Järv, H., Madrid, H., Nordén, J., … Tedersoo, L. (2020). FungalTraits: A user-friendly traits database of fungi and fungus-like stramenopiles. *Fungal Diversity*, *105*(1), 1–16. https://doi.org/10.1007/s13225-020-00466-2

R Core Team. (2020). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing.

Rasconi, S., Grami, B., Niquil, N., Jobard, M., & Sime-Ngando, T. (2014). Parasitic chytrids sustain zooplankton growth during inedible algal bloom. *Frontiers in Microbiology*, *5*, 229. https://doi.org/10.3389/fmicb.2014.00229

Reynolds, N. K., Jusino, M. A., Stajich, J. E., & Smith, M. E. (2022). Understudied, underrepresented, and unknown: Methodological biases that limit detection of early diverging fungi from environmental samples. *Molecular Ecology Resources*, *22*(3), 1065–1085. https://doi.org/10.1111/1755-0998.13540

Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: A versatile open source tool for metagenomics. *PeerJ*, *4*, e2584. https://doi.org/10.7717/peerj.2584

Rosi-Marshall, E. J., & Royer, T. V. (2012). Pharmaceutical compounds and ecosystem function: An emerging research challenge for aquatic ecologists. *Ecosystems*, *15*(6), 867–880. https://doi.org/10.1007/s10021-012-9553-z

Rothermel, B. B., Walls, S. C., Mitchell, J. C., Dodd, C. K., Jr., Irwin, L. K., Green, D. E., Vazquez, V. M., Petranka, J. W., & Stevenson, D. J. (2008). Widespread occurrence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in the southeastern USA. *Diseases of Aquatic Organisms*, *82*(1), 3–18.

Schoch, C. L., Seifert, K. A., Sabine, H., Vincent, R., Spouge, J. L., André, L. C., Wen, C., Elena, B., Kerstin, V., Crous Pedro, W., Miller Andrew, N., Wingfield Michael, J., Catherine, A. M., Kwang-Deuk, A., Feng-Yan, B., Barreto Robert, W., Dominik, B., & David, S. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. *Proceedings of the National Academy of Sciences*, *109*(16), 6241–6246. https://doi.org/10.1073/pnas.1117018109

Shearer, C. A., Descals, E., Kohlmeyer, B., Kohlmeyer, J., Marvanová, L., Padgett, D., Porter, D., Raja, H. A., Schmit, J. P., Thorton, H. A., & Voglymayr, H. (2007). Fungal biodiversity in aquatic habitats. *Biodiversity and Conservation*, *16*(1), 49–67. https://doi.org/10.1007/s10531-006-9120-z

Skelton, J., Jusino, M. A., Carlson, P. S., Smith, K., Banik, M. T., Lindner, D. L., Palmer, J. M., & Hulcr, J. (2019). Relationships among wood-boring beetles, fungi, and the decomposition of forest biomass. *Molecular Ecology*, *28*(22), 4971–4986. https://doi.org/10.1111/mec.15263

Solé, M., Fetzer, I., Wennrich, R., Sridhar, K. R., Harms, H., & Krauss, G. (2008). Aquatic hyphomycete communities as potential bioindicators for assessing anthropogenic stress. *Science of the Total Environment*, *389*(2–3), 557–565. https://doi.org/10.1016/j.scitotenv.2007.09.010

Suberkropp, K. (1984). Effect of temperature on seasonal occurrence of aquatic hyphomycetes. *Transactions of the British Mycological Society*, *82*(1), 53–62. https://doi.org/10.1016/S0007-1536(84)80211-9

Tedersoo, L., Bahram, M., Zinger, L., Nilsson, H., Kennedy, P., Yang, T., Anslan, S., & Mikryukov, V. (2021). *Best practices in metabarcoding of fungi: From experimental design to results*. https://doi.org/10.22541/au.163430390.04226544/v1

Tedersoo, L., Bahram, M., Zinger, L., Nilsson, R. H., Kennedy, P. G., Yang, T., Anslan, S., & Mikryukov, V. (2022). Best practices in metabarcoding of fungi: From experimental design to results. *Molecular Ecology*, *31*(10), 2769–2795. https://doi.org/10.1111/mec.16460

Thomas, A. C., Howard, J., Nguyen, P. L., Seimon, T. A., & Goldberg, C. S. (2018). eDNA sampler: A fully integrated environmental DNA sampling system. *Methods in Ecology and Evolution*, *9*(6), 1379–1385. https://doi.org/10.1111/2041-210X.12994

Toju, H., Tanabe, A. S., Yamamoto, S., & Sato, H. (2012). High-coverage ITS primers for the DNA-based identification of ascomycetes and basidiomycetes in environmental samples. *PLoS One*, *7*(7), e40863. https://doi.org/10.1371/journal.pone.0040863

You, L., Simmons, D. R., Bateman, C. C., Short, D. P., Kasson, M. T., Rabaglia, R. J., & Hulcr, J. (2015). New fungus-insect symbiosis: culturing, molecular, and histological methods determine saprophytic Polyporales mutualists of Ambrosiodmus ambrosia beetles. *PLoS One*, *10*(9), e0137689.

Vatova, M., Rubin, C., Grossart, H., Gonçalves, S. C., Schmidt, S. I., & Jarić, I. (2022). Aquatic fungi: Largely neglected targets for conservation. *Frontiers in Ecology and the Environment*, *20*(4), 207–209. https://doi.org/10.1002/fee.2495

Vilgalys, R., & Hester, M. (1990). Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several cryptococcus species. *Journal of Bacteriology*, *172*(8), 4238–4246. https://doi.org/10.1128/jb.172.8.4238-4246.1990

Wang, Y., Naumann, U., Wright, S. T., & Warton, D. I. (2012). Mvabund – an R package for model-based analysis of multivariate abundance data: *The* mvabund R package. *Methods in Ecology and Evolution*, *3*(3), 471–474. https://doi.org/10.1111/j.2041-210X.2012.00190.x

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR protocols: A guide to methods and applications* (pp. 315–322). Academic Press. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

William & Mary CCA. (2022). *College Creek Alliance water quality data*. William & Mary.

Woodward, G., Gessner, M. O., Giller, P. S., Gulis, V., Hladyz, S., Lecerf, A., Malmqvist, B., McKie, B. G., Tiegs, S. D., Cariss, H., Dobson, M., Elosegi, A., Ferreira, V., Graça, M. A. S., Fleituch, T., Lacoursière, J. O., Nistorescu, M., Pozo, J., Risnoveanu, G., … Chauvet, E. (2012). Continental-scale effects of nutrient pollution on stream

ecosystem functioning. *Science*, *336*(6087), 1438–1440. https://doi.org/10.1126/science.1219534

Wurzbacher, C., Warthmann, N., Bourne, E., Attermeyer, K., Allgaier, M., Powell, J. R., Detering, H., Mbedi, S., Grossart, H.-P., & Monaghan, M. (2016). High habitat-specificity in fungal communities in oligo-mesotrophic, temperate Lake Stechlin (north-East Germany). *MycoKeys*, *16*, 17–44. https://doi.org/10.3897/mycokeys.16.9646

Yuan, T., Zhang, H., Feng, Q., Wu, X., Zhang, Y., McCarthy, A. J., & Sekar, R. (2020). Changes in fungal community structure in freshwater canals across a gradient of urbanization. *Watermark*, *12*(7), 1917. https://doi.org/10.3390/w12071917

Zhang, T., Wang, N.-F., Zhang, Y.-Q., Liu, H.-Y., & Yu, L.-Y. (2016). Diversity and distribution of aquatic fungal communities in the Ny-Ålesund region, Svalbard (high Arctic): Aquatic fungi in the Arctic. *Microbial Ecology*, *71*(3), 543–554. https://doi.org/10.1007/s00248-015-0689-1

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: French, L. C., Jusino, M. A., Chambers, R. M., & Skelton, J. (2024). Community structure and functional diversity of aquatic fungi are correlated with water quality: Insights from multi-marker analysis of environmental DNA in a coastal watershed. *Environmental DNA*, *6*, e576. https://doi.org/10.1002/edn3.576