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Analysis of eDNA to Assess Effects of Water Quality on Freshwater Fungal Diversity in a Virginia Coastal Watershed

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Analysis of eDNA to Assess Effects of Water Quality on Freshwater Fungal Diversity in a Virginia Coastal Watershed

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ABSTRACT

Freshwater fungi comprise a phylogenetically and functionally diverse group which contributes to wide-ranging ecosystem processes in aquatic systems. Saprotrophic fungi convert detritus into nutrient-rich food sources for fish and invertebrates, whereas pathogenic and parasitic fungi can cause disease and population declines of other aquatic organisms. With their diverse and important roles, changes in freshwater fungal community structure may have far-reaching impacts on ecosystems. To understand how natural and anthropogenic stressors to freshwater systems impact fungal-mediated ecosystem processes, a greater understanding of the taxonomic and functional composition of freshwater fungal communities is needed. We assessed relationships among freshwater habitat types, water quality variables, and fungal community composition using multi-marker DNA metabarcoding and water quality monitoring data from a Chesapeake Bay watershed in Virginia, USA. Standardized water samples were collected using eDNA backpack samplers at 17 stream, pond, and tidal creek sites exhibiting a range of pH, dissolved oxygen, salinity, temperature, and organic and inorganic nitrogen and phosphorus profiles. We targeted two regions of the ribosomal RNA gene, ITS2 and LSU, and sequence data were processed using the AMPtk pipeline. The resulting fungal community data from each site were correlated with habitat type and water quality data using distance-based redundancy analysis and generalized linear models. Habitat type and water quality factors were found to be significant drivers of freshwater fungal community composition. Fungal community composition differed significantly among streams, ponds, and tidal creeks, with stream and pond communities being more similar to one another than to tidal creek communities. Average fungal richness measured in number of Operational Taxonomic Units (OTUs) per site was highest in ponds and lowest in tidal creeks. Effects of habitat type and water quality varied between fungal phyla, with

habitat type and water quality explaining a significant proportion of variation in Chytridiomycota and Ascomycota community composition, but not variability in Basidiomycota community composition. Water quality factors also had significant power in explaining community composition of aquatic hyphomycetes, a functional group that contributes largely to leaf litter decomposition in streams. The responsiveness of freshwater fungal communities to their environment can help us predict how important fungal communities may be altered as freshwater ecosystems face natural and anthropogenically-driven changes to water quality.

INTRODUCTION

Since the rise of industrialization, freshwater systems have faced novel environmental stressors, ranging from nutrient and heavy metal pollution to altered levels of pH, dissolved oxygen, and temperature. These factors have direct effects on ecosystems, such as toxicity to organisms, and far-reaching indirect effects, including shifts in species composition and ecosystem function (Dudgeon et al., 2006; Reid et al., 2019). While it is well understood that environmental conditions place controls on species distributions and influence species interactions, changing environmental conditions have widely ranging effects on different taxa. Responses to water composition changes can differ drastically, even between closely related species. In contrast to macroflora and fauna, microbial communities are difficult to detect visually and therefore are often overlooked in ecological studies, which can disguise significant responses of microbial groups to altered freshwater systems (Debroas et al., 2017). Novel detection methods, such as sequencing of environmental DNA, provides new opportunities for higher resolution investigation into individual taxonomic groups' responses to environmental change (Bohmann et al., 2014; Deiner et al., 2017; Ruppert et al., 2019).

Freshwater 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). Freshwater fungi are functionally important, with some groups converting detritus to food for fish and invertebrates and other groups releasing bioactive, and potentially toxic, compounds into the environment (Jones et al., 2014). Aquatic hyphomycetes (Ingold, 1942) is a paraphyletic group of exclusively aquatic fungi that are functional detrivores specializing on leaf litter in streams. A member of the zoosporic Chytridiomycota phylum, *Batrachochytrium dendrobatidis,* can cause a fatal infectious disease in amphibians which has devastated

populations of vulnerable species worldwide (Oidtmann et al., 2002; Rothermel et al., 2008). Chytrids are also hypothesized to play an important role in transferring energy and nutrients from phytoplankton to higher trophic levels by parasitizing inedible algae and releasing zoospores which can be eaten by zooplankton, a cycle referred to as the "mycoloop" (Grossart et al., 2019). Whereas aquatic hyphomycetes and Chytridiomycota are often the focus of studies on freshwater fungi due to their documented roles in ecosystems, fungi from numerous phyla are known to be either fully or partially reliant on aquatic systems.

Due to taxonomic complexity of freshwater fungi, previously reported impacts of changing environmental parameters on community composition of freshwater fungi are variable. Some studies found that higher diversity and fungal biomass in aquatic systems were negatively related to levels of heavy metals, sulfate, and nitrate in the system (Solé et al., 2008; DiLeo et al., 2010). In contrast, Gorniak et al. (2013) found that fungal biomass increased with increased ammonium, total nitrogen, and dissolved organic phosphorus, while Pietryczuk et al. (2018) found that fungal diversity was lowest in rivers with lower levels of anthropogenic pollutants. Interesting relationships between potentially pathogenic fungi and anthropogenic pollutant levels have also been suggested, but these relationships require further study (Pietryczuk et al. 2018). The differing niches and ecological roles occupied by various divergent lineages of freshwater fungi indicate that groups within this broad taxon will respond differently to environmental changes. Freshwater fungal diversity may be an important indicator of freshwater system health, but understanding taxa-specific responses to water quality requires further study using novel tools such as DNA metabarcoding.

Detection of organisms using DNA isolated from the environment is a new and emerging field that has been applied as a tool for monitoring and assessing species diversity, particularly in

the context of rapidly changing ecosystems (Pawlowski et al., 2020; Taberlet et al., 2018). Current research aims to answer crucial questions related to environmental DNA (eDNA), including effects of abiotic and biotic factors on the distribution, degradation, and persistence of DNA in aquatic systems (Stewart, 2019). A handful of studies have applied eDNA and metabarcoding methods to aquatic fungi species diversity and have successfully captured species presence in aquatic systems using primers targeting gene regions such as the nuclear ribosomal internal transcribed spacer region (Schoch et al., 2012; Toju et al., 2012). Matsuoka et al. (2019) surveyed aquatic fungi in terrestrial river networks in Japan using eDNA metabarcoding and identified spatial patterns in DNA assemblages across the entire river network, finding greater similarities in fungal assemblages within river branches than between river branches. Few studies, however, have investigated variations in fungal communities due to water quality or freshwater habitat type using eDNA. Zhang et al. (2016) detected distinctions between fungal communities in Arctic ponds, streams, estuaries, and ice melt water, and found significant effects of salinity, conductivity, and temperature on community composition. Applications of eDNA techniques to additional freshwater systems will likely reveal complex trends between physical and chemical characteristics of freshwater ecosystems and their respective fungal communities.

We know of no previous species surveys of freshwater fungi that have been conducted in the southeastern portion of Virginia, USA. Freshwater fungi are present in Virginia and can regulate populations of invertebrates, such as midges (Martin, 1981), but knowledge of whether fungal communities in this region are habitat-specific or are responsive to water quality is lacking. To fill this gap in understanding, our study aims to assess the diversity and distribution of freshwater fungi in the College Creek Watershed, a coastal drainage network of the James River near Williamsburg, Virginia, by applying high-throughput sequencing techniques to

environmental DNA (eDNA) and correlating the resulting fungal communities with local water quality. We predict that freshwater fungal communities are responsive to differing habitat types and environmental variables, with the exclusively aquatic groups, Chytridiomycota and aquatic hyphomycetes, exhibiting the strongest responses. We investigated relationships between fungal communities and environmental variables in this watershed to infer whether habitat type and water quality affect the occurrence and diversity of freshwater fungi in southeastern Virginia.

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 in Williamsburg, Virginia, and included 9 streams, 5 ponds, and 3 tidal creeks (Table 1; Figure 1). Our 17 sites coincide with locations for which water quality data has been collected by the College Creek Alliance for 17 years (William & Mary CCA, 2022). Sites consisted of a mix of exposed ponds and streams in residential neighborhoods, recreational ponds and tidal creeks subject to fishing and boat traffic, and densely wooded streams.

Table 1. Coordinate information for each of the 17 sites sampled within the College Creek watershed in Williamsburg, VA.

Site ID	Site Name	Site Type	Latitude	Longitude
1	CW Ponds	Pond	37.2597152	-76.6996529
2	Kingsmill Pond	Pond	37.2363937	-76.6723110
3	Kingspoint Pond	Pond	37.2439774	-76.7107777
4	Lake Matoaka	Pond	37.2636487	-76.7223596
5	Tutter's Neck	Pond	37.2518458	-76.6858300
6	Bloody Ravine	Stream	37.2592586	-76.6729417
7	College Campus	Stream	37.2685504	-76.7196001
8	Colonial Williamsburg	Stream	37.2690886	-76.6902152
9	Compton Dr.	Stream	37.2764142	-76.7243965
10	Holly Hills	Stream	37.2509872	-76.7246507
11	Kingsmill Creek	Stream	37.2396322	-76.6635858
12	Mimosa Dr.	Stream	37.2638012	-76.7105830
13	New Hope Rd.	Stream	37.2844167	-76.7256017
14	Papermill Creek	Stream	37.2577542	-76.7022158
15	College Landing	Tidal creek	37.2513461	-76.7101797
16	James River	Tidal creek	37.2262611	-76.6948829
17	Kingspoint Dock	Tidal creek	37.2450675	-76.7127811

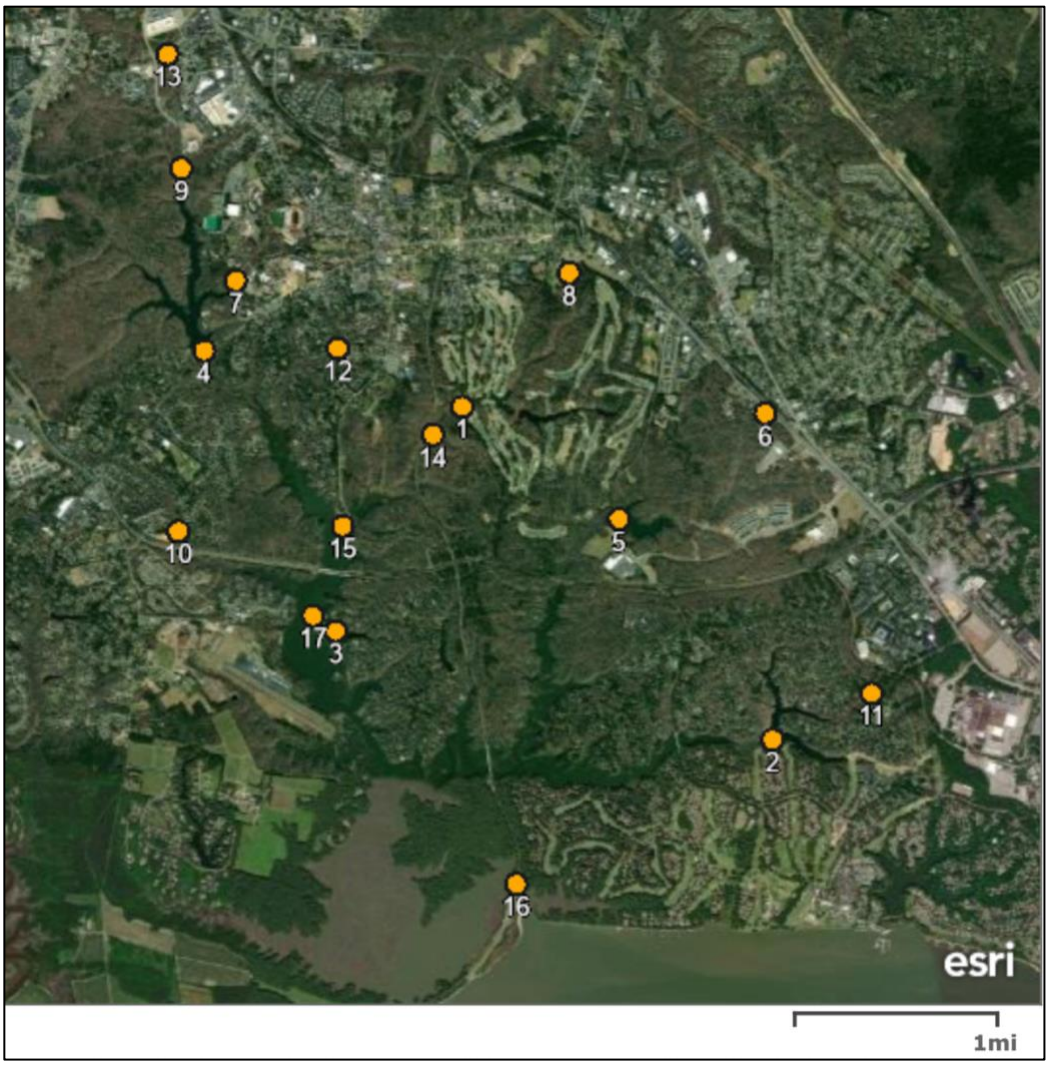

Figure 1. Map of the 17 study sites sampled within the College Creek watershed in Williamsburg, VA.

Using the Smith-Root eDNA Sampler Backpack (Thomas et al., 2018), three 2-liter samples were collected at each site between March and May of 2021. The three samples were collected roughly three meters apart at each site. Water from 3 inches below the surface was vacuum-filtered at a maximum pressure of 12 psi through a 5 micrometer Polyethersulfone (PES) membrane filter using Smith-Root Single Use eDNA Filter Packs. Each filter was then

placed in a 1.5 mL tube with 1.0 mL filter-sterilized cell lysis solution (CLS; Lindner & Banik, 2009) and frozen at -20 °C prior to DNA extraction.

2.2 Molecular Analyses

DNA extraction followed the glassmilk extraction protocol of Brazee & Lindner (2013). Samples were thawed in a water bath at 65 °C for 15 minutes, agitated with vortexing, and returned to the water bath for 45 minutes. Samples were then centrifuged at 10,000 rcf (relative centrifugal force) for 1 minute before 100 µl of supernatant was removed from each sample and transferred to 0.2 ml strip tubes. DNA extraction protocol proceeded following Brazee & Lindner (2013) with the inclusion of a negative control sample. The negative control sample, which lacked DNA, was included to identify any contamination during the extraction process.

Initial PCR amplification was performed on all 51 samples and the negative extraction control 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 ITS amplicon libraries, 3 µl from each DNA-extracted sample were 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 fITS7_ill primer, 0.3 µl ITS4_ill primer, 7.88 µl water, 0.12 µl bovine serum albumin (BSA), and 0.3 µl dNTP. Samples were placed in a thermocycler and subjected to the following conditions: initial annealing at 94 °C for 3 minutes; 11 cycles of denaturing at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds (dropping 0.5 °C each cycle), extension at 72 °C for 1 minute; 28 cycles of denaturing at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for one minute; final extension at 72 °C for 7 minutes. Primers used for LSU (28S) barcoding targeted the LR0R (Vilgalys & Hester, 1990) and JH-LSU-369rc sites (Li et al., 2015) and were modified

similarly to our ITS 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 LROR_ill primer, 0.3 µl JH-LSU-369rc_ill primer, 7.88 µl water, 0.12 µl bovine serum albumin (BSA), and 0.3 µl dNTP. The following thermocycler conditions were used: initial annealing at 94 °C for 3 minutes followed by 40 cycles of denaturing at 94 °C for 30 seconds, annealing at 55°C for 45 seconds, extension at 72 °C for 90 seconds, and final extension at 72 °C for 7 minutes.

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 (SynMock; Palmer et al., 2018) was 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 allowed for detection of index bleed or barcode crossover which aids in parameterization of our bioinformatics pipeline.

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 ITS and LSU primer sequences, merging of paired end reads. For ITS, reads longer than 300 bp were trimmed to 300, and reads shorter than 300 bp were padded with Ns to

ensure all reads were of 300 bp length. Reads shorter than 125 bp were discarded. Expected errors less than 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. 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 clustering with DADA2 (Callahan et al., 2016), which performs better for LSU (Skelton et al., 2019). 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. The hybrid taxonomy algorithm in AMPtk was used to assign taxonomy to the OTUs.

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 kingdom "Fungi" were retained in our dataset for both ITS and LSU datasets. The number of OTUs in each sample were then converted to 1 or 0, with 1 representing OTU presence in that sample 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 converted OTUs to a presence/absence matrix. To obtain a semi-quantitative measure of fungal OTU abundance from the occurrence data, we then averaged the OTU occurrence data for the three replicates taken at each site to convert occurrence from each sample to a proportion of occurrence among replicates (0, 0.33, 0.66, or 1) for each site (17 sites total). This proportion represented whether the OTU was detected in zero, one, two, or three of the samples collected at each given site and

was used as a proxy for abundance, with the assumption that detection of an OTU in a greater number of samples is related to higher abundance of the OTU at each site.

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 et al. 2015). We converted the matrix representing OTU occurrence 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). To assess the differences in community composition as explained by environmental variables, we conducted a constrained ordination using the environmental variables: temperature, conductivity, oxygen saturation, pH, total suspended solids (TSS), total phosphorus, dissolved inorganic phosphorus (DIP), NH4, and Secchi depth. The ordination method applied was a distance-based redundancy analysis (dbRDA) which is a type of constrained ordination using non-Euclidean distance measures, and was performed with the *capscale* function in R (Legendre & Anderson, 1999). We conducted stepwise forward model selection based on maximized adjusted R-squared (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 conducted two initial analyses to determine whether fungal community composition was more strongly related to short-term or long-term environmental conditions. First, we used predictor variables measurements taken in April 2021, coinciding with the collection of our fungal samples. Then we then conducted the same analysis using the averages of environmental

data collected as part of a long-term water quality monitoring program (William & Mary CCA, 2022) at 4-month intervals for 17 years at each site. For all subsequent analyses we used only the April measurements because we found the strongest correspondence between fungal communities and the April data (Figure 3).

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 community composition 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. Therefore, we predicted the strongest correspondence with water quality variables in the Chytridiomycota, weaker correspondence in Ascomycota, and the weakest correspondence in Basidiomycota. To further test our prediction, we also conducted community analysis on OTU 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 Ascomycota and Basidiomycota fungi and are all specialized aquatic saprotrophs. Therefore, we predicted the strongest correspondence between water quality data and this functional group of aquatic fungi.

To investigate whether environmental variables predicted fungal OTU richness, we used generalized linear modeling (GLM). A negative binomial (NB) error distribution was chosen over a Poisson error distribution by initially fitting models with both distributions and comparing residual deviance using the *glm* and *glm.nb* functions in the *mass* package in R. We created five GLMs, with the response variable being number of OTUs at each site identified to either Fungi

(all fungal OTUs), Chytridiomycota, Ascomycota, Basidiomycota, or Aquatic Hyphomycete. Each model was initially constructed with the predictor variables: temperature, conductivity, oxygen saturation, pH, total suspended solids (TSS), total phosphorus, dissolved inorganic phosphorus (DIP), NH4, and Secchi depth. Backwards stepwise selection was then conducted on each of the models using the *step* function in R which uses AIC as criterion for retaining or eliminating variables. To assess the explanatory power of the models, we calculated a "pseudo-R^2" based on measures of null and residual deviance.

RESULTS

After processing the ITS dataset, 6,058,814 reads were retained from an initial 7,087,342 raw reads. We retained 4,309 unique OTUs, of which 2,355 were identified to the kingdom Fungi. Of those, 1,176 OTUs were identified to the phylum Ascomycota, 465 identified to phylum Chytridiomycota, 311 identified to phylum Basidiomycota, and 315 could not be identified to a phylum. Of the remaining fungal OTUs that were assigned phyla taxonomy, 40 were identified to phylum Rozellomycota, 29 to Monoblepharomycota, 9 to Mortierellomycota, 5 to Glomeromycota, 3 to Olpidiomycota, 1 to Basidiobolomycota, and 1 to Aphelidiomycota. Of the 2355 OTUs identified to the Fungi kingdom, 1,004 could be identified to the genus level, and 51 could be identified to the functional group aquatic hyphomycetes, or synonymously Ingolian hyphomycetes.

3.1 Site Diversity

Fungal richness varied considerably among the three habitat types sampled. The mean number of OTUs detected and identified to Fungi were 305.6 at pond sites (n = 5; SE = 80.7), 292.3 at stream sites (n = 9; SE = 46.8), and 226.3 at tidal creek sites (n = 3; SE = 67.9), with 586 being the maximum number of OTUs detected at a site and 102 being the minimum (Table 2). Overall fungal diversity as measured in number of OTUs per site was most strongly associated with Secchi depth, conductivity, DIP, pH, and total phosphorus concentration (NB GLM; pseudo-R^2 = 0.56; Table 3). The mean number of OTUs identified to Chytridiomycota was 89.3 in tidal creeks (n = 3; SE = 36.7), 58.9 in streams (n = 9; SE = 6.8), and 51.2 in ponds (n = 5; SE = 12.2). Conductivity, pH, TSS, DIP, temperature, and oxygen saturation were the strongest predictors of Chytridiomycota OTU richness (NB GLM; pseudo-R^2 = 0.86; Table 3). Streams had a mean of 165.1 OTUs identified to Ascomycota (n = 9; SE = 32.5), while ponds

had 163.6 (n = 5; SE = 40.4), and tidal creeks had 83.7 (n = 3; SE = 24.5) (Table 2). Roughly 64% of the variance in Ascomycota OTU richness could be explained by conductivity, pH, total phosphorus, DIP, and Secchi depth (NB GLM; pseudo-R^2 0.64; Table 3). Ponds had a mean of 49.2 OTUs identified to Basidiomycota (n = 5; SE = 17.0), while streams had 31.0 (n = 9; SE = 6.7), and tidal creeks had 14.3 (n = 3; SE = 2.3) (Table 2). An approximated 46% of the variance in Basidiomycota OTU richness could be explained by the total phosphorus and Secchi depth (NB GLM; pseudo-R^2 = 0.46; Table 3). The mean OTUs identified to aquatic hyphomycetes was 14.3 in streams (n = 9; SE = 2.2), 14 in ponds (n = 5; SE = 4.0), and 6 in tidal creeks (n = 3; SE = 2.9) (Table 2). Conductivity, pH, total phosphorus, DIP, and Secchi depth explained roughly 70% of the variance in richness of aquatic hyphomycete OTUs (NB GLM; pseudo-R^2 = 0.70; Table 3).

Table 2. Number of OTUs detected at sites by phyla, or functional group in the case of aquatic hyphomycetes.

Site ID	Site Name	Site type	Total Fungi	Chytridiomycota	Ascomycota	Basidiomycota	Aquatic Hyphomycete
1	CW Ponds	Pond	256	42	142	34	6
2	Kingsmill Pond	Pond	102	33	47	11	9
3	Kingspoint Pond	Pond	231	32	147	32	8
4	Lake Matoaka	Pond	353	51	185	59	21
5	Tutter's Neck	Pond	586	98	297	110	26
6	Bloody Ravine	Stream	172	67	86	15	10
7	College Campus	Stream	395	46	240	57	19
8	Colonial Williamsburg	Stream	225	19	138	40	7
9	Compton Dr.	Stream	423	89	231	45	24
10	Holly Hills	Stream	273	70	139	19	16
11	Kingsmill Creek	Stream	249	71	121	25	9
12	Mimosa Dr.	Stream	221	61	112	17	12
13	New Hope Rd.	Stream	113	43	52	2	8
14	Papermill Creek	Stream	560	64	367	59	24
15	College Landing	Tidal creek	329	124	129	18	11
16	James River	Tidal creek	98	16	45	15	1
17	Kingspoint Dock	Tidal creek	252	128	77	10	6

Table 3. Summaries of five GLMs using negative binomial distributions which model the relationship between OTU richness and environmental variables.

Overall aquatic fungal OTU richness

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	19.098	4.816	3.966	0.000***
Conductivity	-0.001	0.000	-3.053	0.002**
pH	-1.578	0.670	-2.356	0.018*
Total phosphorous	-0.680	0.272	-2.502	0.012*
DIP	0.467	0.142	3.281	0.001**
Secchi	-0.013	0.004	-3.765	0.000***

Dispersion parameter for Negative Binomial (9.96) family taken to be 1.

Null deviance: 40.813 on 16 degrees of freedom. Residual deviance: 17.298 on 11 degrees of freedom.

Chytridiomycota OTU richness

	Estimate	Std. error	z value	Pr(>|z|)
(Intercept)	21.734	3.932	5.527	0.000***
Temperature	-0.139	0.048	-2.919	0.004**
Conductivity	-0.002	0.000	-6.131	0.000***
Oxygen saturation	0.015	0.008	1.980	0.048*
pH	-2.185	0.589	-3.710	0.000***
TSS	0.056	0.011	5.007	0.000***
DIP	0.671	0.146	4.601	0.000***
Secchi	-0.006	0.002	-2.808	0.005**

Dispersion parameter for Negative Binomial (41.4268) family taken to be 1.

Null deviance: 114.500 on 16 degrees of freedom. Residual deviance: 15.878 on 9 degrees of freedom.

Ascomycota OTU richness

	Estimate	Std. error	z value	Pr(>|z|)
(Intercept)	19.494	5.220	3.734	0.000***
Conductivity	-0.001	0.000	-2.653	0.008**
pH	-1.656	0.725	-2.285	0.022*
Total phosphorous	-1.145	0.292	-3.925	0.000***
DIP	0.492	0.156	3.157	0.002**
Secchi	-0.016	0.004	-4.281	0.000***

Dispersion parameter for Negative Binomial (8.919) family taken to be 1.

Null deviance: 47.951 on 16 degrees of freedom. Residual deviance: 17.367 on 11 degrees of freedom.

Basidiomycota OTU richness

	Estimate	Std. error	z value	Pr(>|z|)
(Intercept)	6.101	0.759	8.034	0.000***
Total phosphorous	-1.779	0.473	-3.762	0.000***
Secchi	-0.018	0.006	-3.214	0.001**

Dispersion parameter for Negative Binomial (3.6715) family taken to be 1.

Null deviance: 34.213 on 16 degrees of freedom. Residual deviance: 18.462 on 14 degrees of freedom.

Aquatic Hyphomycete OTU richness

	Estimate	Std. error	z value	Pr(>|z|)
(Intercept)	32.230	7.904	4.078	0.000***
Conductivity	-0.003	0.001	-3.012	0.003**
pH	-3.747	1.053	-3.559	0.000***
Total phosphorous	-0.982	0.309	-3.184	0.001**
DIP	0.857	0.294	2.915	0.004**
Secchi	-0.012	0.004	-3.301	0.001***

Dispersion parameter for Negative Binomial(26.8133) family taken to be 1.

Null deviance: 49.941 on 16 degrees of freedom. Residual deviance: 15.160 on 11 degrees of freedom.

3.2 Effects of Habitat Type on Fungal Community Composition

Overall fungal community composition differed significantly among streams, ponds, and tidal creeks. Unconstrained ordination by NMDS ($k = 2$; stress = 0.14) revealed a significant effect of site type in aquatic fungi community composition (PERMANOVA; $F = 2.09$, $R^2 = 0.23$, $p = 0.001$; Figure 2). The largest source of variation was the difference between tidal creeks and ponds/streams, which were separated along the first NMDS axis. The second largest source of variation was between ponds and streams, which were separated along the second NMDS axis.

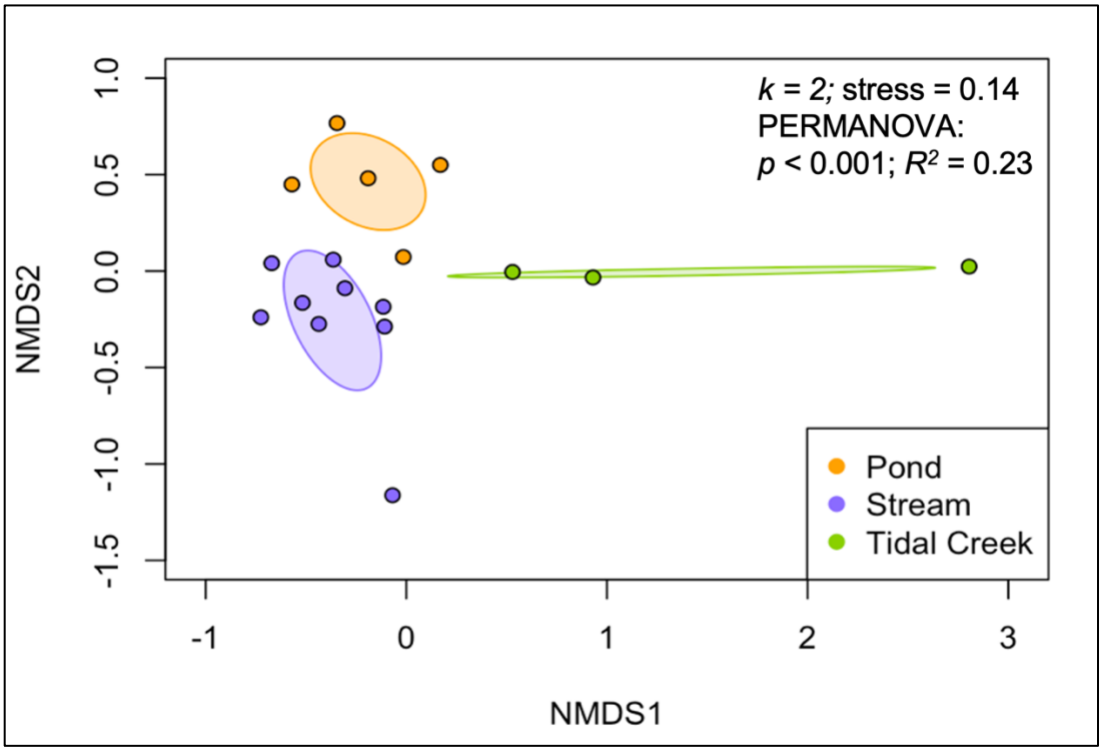

Figure 2. Nonmetric multidimensional scaling (NMDS) ordination displaying effect of habitat type on fungal community composition, inferred from metabarcoding of the ITS2 rDNA marker from filtered water samples. Each point represents aggregated community data for 3 replicate samples per sampling site taken from 17 sampling sites with the

College Creek Watershed, Williamsburg VA during spring of 2022. Ellipses display standard deviation around the centroid for each habitat type.

3.3 Effects of Environmental Conditions on Fungal Community Composition

Short-term environmental conditions (April 2021) had more explanatory power than long-term conditions (17-year averages) in explaining differences in overall fungal community composition. Stepwise selection of our constrained ordination using short-term conditions retained the variables TSS ($p = 0.002$), temperature ($p = 0.004$), DIP ($p = 0.014$), and Secchi depth ($p = 0.020$) and resulted in an overall model adjusted R^2 of 0.23. Our model suggested that stream fungal communities are associated with clearer water (Secchi depth) and cooler water temperatures, whereas pond communities are associated with increased dissolve inorganic phosphorus (DIP), and tidal creek communities are associated with higher total suspended solids and more turbid water (Figure 3A). The variables retained in stepwise selection of our constrained ordination using long-term conditions were conductivity ($p = 0.002$), temperature ($p = 0.004$), and total phosphorus ($p = 0.004$) and overall model R^2 was 0.18. This model suggested that stream communities are negatively associated with temperature, pond communities are negatively associated with total phosphorus and conductivity, and tidal creek communities have strong positive associations with total phosphorus and conductivity (Figure 3B).

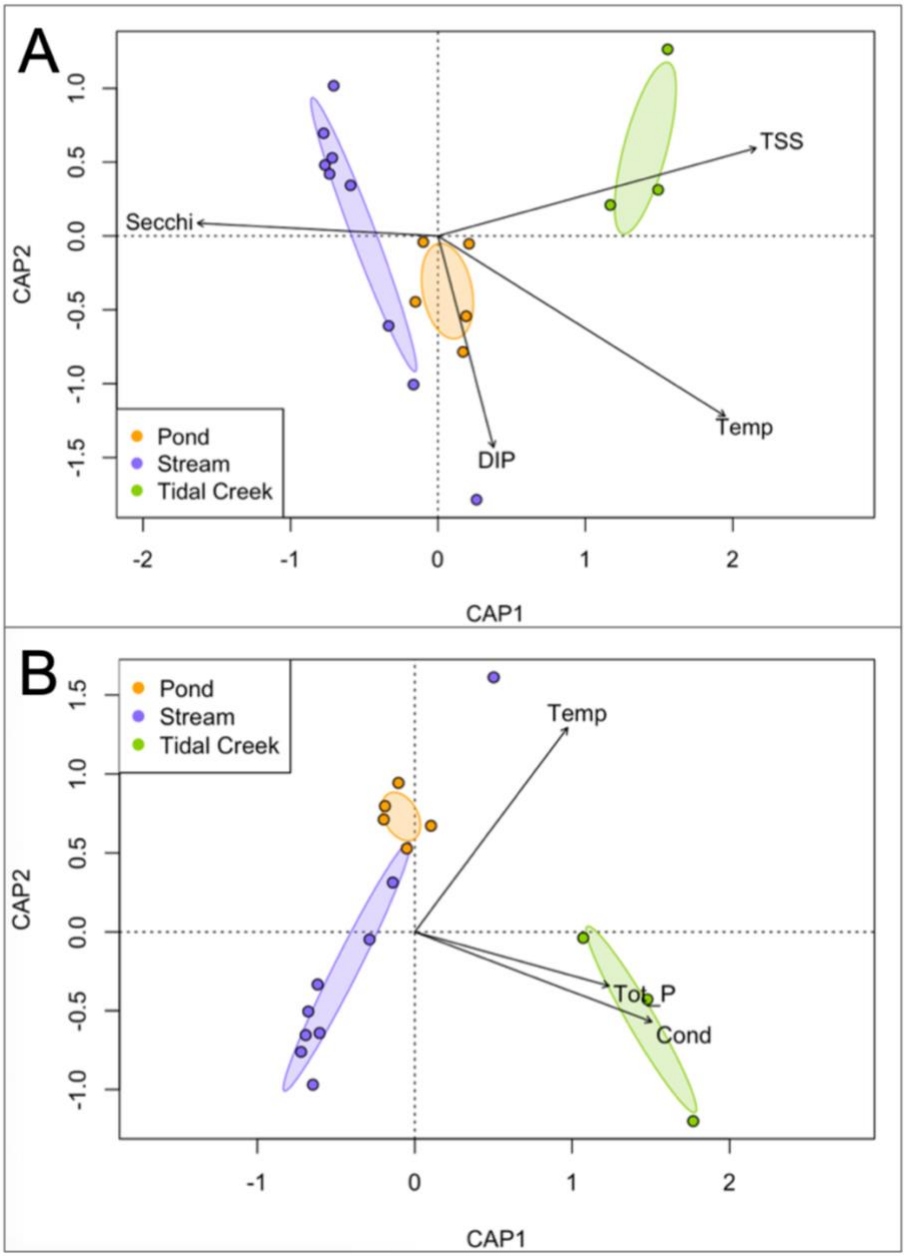

Figure 3. Distance-based redundancy analysis (dbRDA) ordination performed with the *capscale* function in R displaying effect of short-term (A) and long-term (B) environmental conditions on fungal community composition. Points represent site scores of aggregated community data for 17 sampling sites. Ellipses display standard deviation around the centroid for each habitat type. Arrows represent correlation of environmental variables with community composition, and arrow length represents relationship strength.

3.4 Environmental Conditions on Chytridiomycota Community Composition

Congruent with our prediction, Chytridiomycota showed significant variation among habitat types which was largely explained by variation in water quality. The results of PERMANOVA showed that community composition of Chytridiomycota was significantly different among habitat types (PERMANOVA; $F = 2.94$, $R^2 = 0.30$, $p = 0.001$). Stepwise selection of our constrained ordination using short-term conditions retained the variables TSS ($p = 0.002$), temperature ($p = 0.004$), DIP ($p = 0.004$), and Secchi depth ($p = 0.030$) and resulted in an overall model R^2 of 0.39, which was much higher than the R^2 for the overall model that included all Fungi (reported above). This model suggests that stream communities of Chytridiomycota are positively associated with Secchi depth and negatively associated with temperature and TSS, while pond communities are negatively associated with DIP, and tidal creek communities are positively associated with TSS and temperature and negatively associated with Secchi depth (Figure 4A).

3.5 Environmental Conditions on Ascomycota Community Composition

Ascomycota community composition was significantly related to habitat type and water quality, but less strongly than Chytridiomycota communities. Results of PERMAONVA showed that Ascomycota communities were significantly different among habitat types (PERMANOVA; $F = 1.53$, $R^2 = 0.18$, $p = 0.002$). Variables retained by stepwise selection of the constrained ordination using short-term conditions were 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). Stream and pond community composition were similar to one another but dissimilar from tidal creek communities, which varied highly around the centroid (Figure 4B). Tidal creek

communities of Ascomycota were positively associated with TSS while pond and stream communities were negatively associated with TSS.

3.6 Environmental Conditions on Basidiomycota Community Composition

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. 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 environmental variables that were considered (Figure 4C).

3.7 Environmental Conditions on Aquatic Hyphomycete Community Composition

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. Pond and stream communities were more similar to one another than 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 4D).

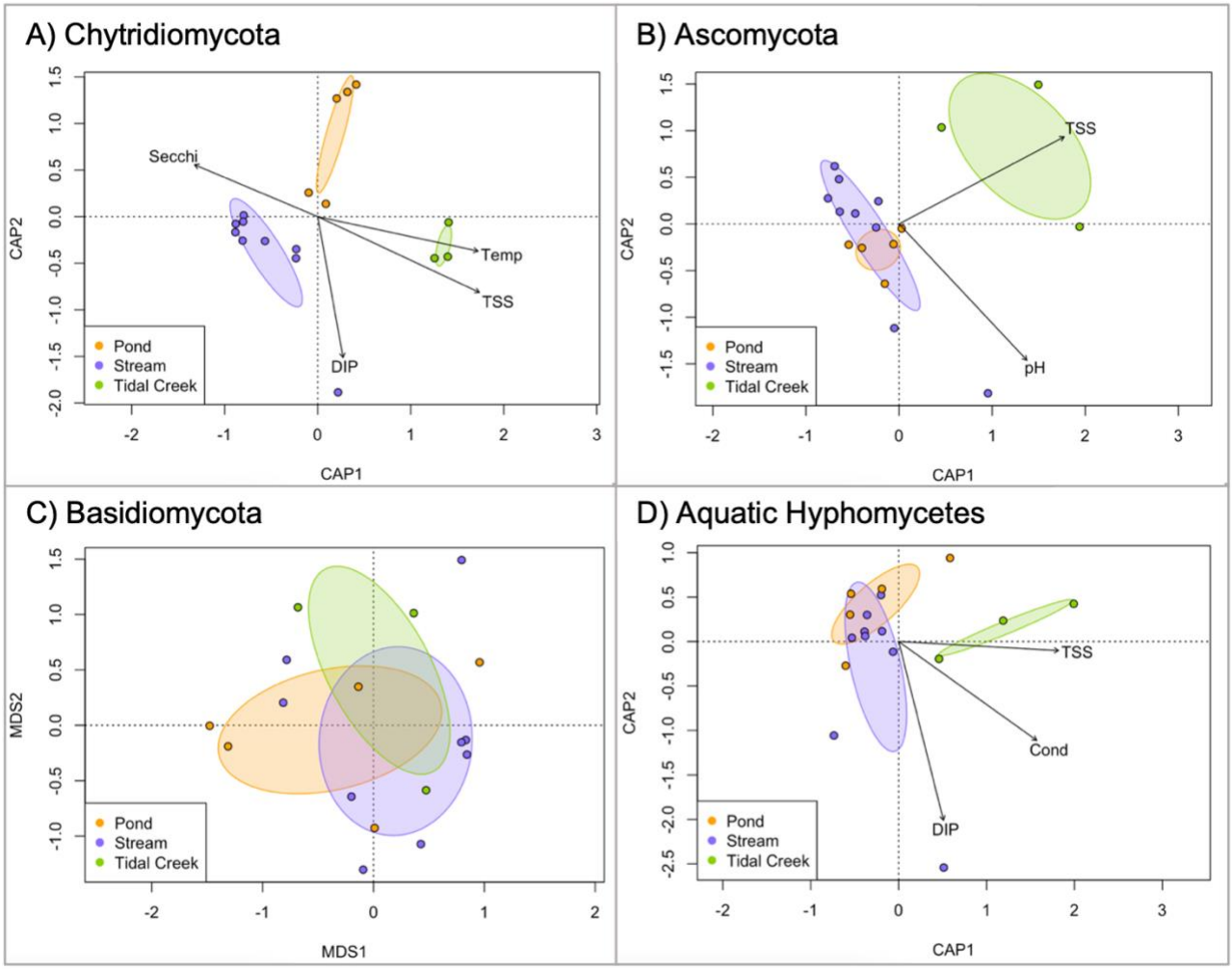

Figure 4. Distance-based redundancy analysis (dbRDA) ordination performed with the *capscale* function in R displaying effect of environmental conditions on A) Chytridiomycota, B) Ascomycota, C) Basidiomycota, and D) Aquatic Hyphomycete community composition. Points represent site scores of aggregated community data for 17 sampling sites. Ellipses display standard deviation around the centroid for each habitat type. Arrows represent correlation environmental variables with community composition, and arrow length represents relationship strength.

DISCUSSION

4.1 Overall Fungal Community Composition and Richness

Our results clearly show that the three habitat types studied here have distinct fungal communities and that water quality variables are significant drivers of the differences in aquatic fungal community composition observed in those habitat types. The sensitivity of fungal community composition to environmental factors and habitat type observed in our study suggests that freshwater fungal communities are likely to be responsive to human-driven changes to freshwater systems, such as nutrient pollution and the conversion of flowing streams to ponds or lakes through dam construction. This sensitivity of fungal community composition to water conditions makes freshwater fungi an ideal group to be used as indicators of ecosystem disturbance and environmental change. 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 suggest that aquatic fungal community composition is strongly influenced by environmental variability (Heino et al., 2014; Panzer et al., 2015; Pietryczuk et al., 2018; Zhang et al., 2016).

Variance in fungal community composition was explained more strongly by short-term environmental conditions than by long-term conditions, suggesting that community composition responds rapidly to fluctuations in water quality. This result suggests that freshwater fungal communities are likely to respond quickly to environmental perturbations but may also be resilient and rapidly recover if environmental condition improve. Previously reported seasonal changes in fungal communities confirm their responsiveness to short-term variation in environmental conditions. Seasonal variability in freshwater fungal community composition has been documented in the literature, with Matsuoka et al. (2020) finding that community

assemblages were season-specific and exhibited annual periodic patterns (Matsuoka et al., 2020; Yuan et al., 2020). The seasonal variability of freshwater fungi may be driven by certain fungal taxa such as aquatic hyphomycetes, which are highly associated with leaf litter decomposition cycles (Matsuoka et al. 2020). Future work that includes additional eDNA sampling of our sites throughout the year is needed to confirm the seasonality of particular groups and whether it contributes to the higher explanatory power of the short-term water quality dataset over the long-term dataset.

Overall fungal community composition differed significantly between streams, ponds, and tidal creeks, with stream and pond communities being more similar to one another than to tidal creek communities. While aquatic fungal community composition has been shown to be biome specific, with freshwater community composition differing significantly from all other biomes inhabited by aquatic fungi (Panzer et al., 2015), high habitat specificity between small ponds, streams, and estuaries has not been widely documented in the literature. A recent study, however, demonstrated Arctic aquatic fungal communities differing between stream, pond, melting ice water, and estuary sites, and identified strong relationships between environmental variables in the habitats and the fungal community, congruent with our findings (Zhang et al., 2016). In our study, variation in overall fungal community composition due to environmental factors was explained most 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. Previous studies have shown 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. Temperature was also a significant factor contributing to variance in fungal community composition, with stream communities being associated with cooler temperatures. This finding is congruent with that of Zhang et al. (2016) who found relationships between temperature and community composition, though the temperature ranges were much cooler in Zhang et al.'s study (0.4 - 11.0 °C) than in our sites. Dissolved inorganic phosphorus was also a significant predictor of aquatic fungal community composition, with pond communities being associated with increased DIP.

Furthermore, average fungal OTU richness was comparable for ponds and streams (305.6 and 292.3 OTUs, respectively) but was lower in tidal creeks (226.5 OTUs). Overall fungal OTU richness was predicted by Secchi depth, DIP, pH, conductivity, and total phosphorus. Zhang et al. (2016) also found relationships between OTU numbers and conductivity, with OTU richness increasing with conductivity and temperature, but did not find a significant relationship between OTU richness and pH.

Due to the paraphyletic nature of freshwater fungi, phyla responses to environmental variables vary in both the degree of the effect on community composition and nature of association with OTU richness. Congruent with our predictions, we saw the strongest response to water quality variables in the Chytridiomycota which are comprised of nearly exclusively obligately aquatic species, a weaker response in the Ascomycota which comprise many aquatic and non-aquatic species, and no significant response in the Basidiomycota, which are primarily terrestrial taxa. Also congruent with our predictions was a strong relationship between water quality and the polyphyletic functional group of aquatic hyphomycetes.

4.2 Chytridiomycota and Aquatic Hyphomycetes

Chytridiomycota and aquatic hyphomycetes, both exclusively aquatic groups, showed the strongest relationships between community composition and environmental variability. Chytrids are an important group known to be parasites of freshwater algae and a range of animals and are hypothesized to play an important role in cycling organic matter from phytoplankton to zooplankton, yet there is a lack of knowledge regarding environmental factors driving Chytrid community structure (Comeau et al., 2016; Gleason et al., 2008). Variability in Chytrid community composition was most strongly related to TSS, temperature, DIP, and Secchi depth. Chytrid OTU richness per site was highest in two of the three tidal creek sites and was significantly associated with nearly all of the environmental variables in our dataset, with richness predicted most greatly by pH, conductivity, TSS, and DIP. Overall, the highest OTU richness of Chytrids was found in two of the three tidal creek sites which tended to be lower in overall fungal richness.

On the other hand, aquatic hyphomycete OTU richness was lowest in two of the three tidal creek sites, but was also predicted most strongly by pH, conductivity, and DIP. In addition, aquatic hyphomycete richness was also predicted by total phosphorus and Secchi depth. Reflecting their dependence on detritus, aquatic hyphomycete OTU richness was highest in pond and stream habitats, likely due to detritus-based nature of these systems. Similarly, the increased Chytrid richness in tidal creeks reflects their dependence on phytoplankton, which are much more prevalent in tidal creeks than streams. The distinct ecosystem roles of chytrids and aquatic hyphomycetes leads to the two groups inhabiting distinct niches of freshwater habitats, with chytrids typically dominating in the water column and aquatic hyphomycetes dominating on

benthic substrate (Yuan et al., 2020), and our findings show that they also dominate in differing habitat types.

4.3 Ascomycota

In contrast to the Chytrids, Ascomycota community composition, a large phylum divided between aquatic and terrestrial fungi, was influenced by environmental variability to a lesser degree than the Chytrids and aquatic hyphomycetes. It should be noted that aquatic hyphomycetes are a functional paraphyletic group and most of its members fall into phylum Ascomycota, though the aquatic hyphomycetes represent a small minority of the highly diverse Ascomycota taxa observed in our study. In terms of OTU richness, Ascomycota were the dominant phyla, which is consistent with Lepère et al. (2019) but differs from a handful of studies in which Chytridiomycota were the dominant taxonomic group in freshwater (Monchy et al., 2011; Panzer et al., 2015). Ascomycota OTU richness was most strongly predicted by DIP, total phosphorus, Secchi depth, conductivity, and pH.

4.4 Basidiomycota

While aquatic Basidiomycota have been described and a small percentage of aquatic hyphomycetes members are from this phylum, Basidiomycota are considered to occur far less often in water and are a predominantly terrestrial species (Shearer et al., 2007). Our results support this understanding since neither variation of Basidiomycota community composition nor Basidiomycota OTU richness was significantly influenced by aquatic habitat type or water quality. Given that Basidiomycete communities were not significantly correlated with the factors related to the aquatic environment while taxonomic groups that are known to be aquatic are strongly associated with those factors, we believe that a majority of fungi identified as Basidiomycetes in our dataset originated in terrestrial ecosystems. While not dependent on water

for any or all of their life cycle, terrestrial fungi that enter water as spores through wind, flooding, or as attached to leaf litter have been hypothesized to contribute to aquatic food webs and other ecosystem processes, though further research is needed to understand their roles in aquatic systems (Voronin, 2014).

4.5 Implications

Our results clearly show that aquatic fungal communities respond to physical and chemical variation in aquatic systems, which has implications for our understanding of how human-driven change to freshwater ecosystems may influence fungal communities. For instance, fungal community composition and richness was distinct between pond and stream sites. This suggests that if a stream is dammed and shifts from a lotic to lentic system, the fungal community structure will likely shift as well, with unknown impacts on energy and nutrient cycling in the system. Furthermore, chemical changes such as nutrient pollution might have cascading effects on trophic webs by altering fungal community structure. For instance, DIP was a significant predictor of both Chytrid and aquatic hyphomycete community composition. If DIP increases in a system and alters community composition of these two taxonomic groups, nutrient cycles deriving from both planktonic and detritus-based sources will likely be affected. Continued research on roles of various groups of aquatic fungi, as well as further study of how water quality drives fungal community composition, can uncover how aquatic fungi interact with their surroundings and other organisms in freshwater ecosystems.

CONCLUSIONS

Freshwater fungal communities, which contribute largely to the cycling of energy and nutrients in aquatic ecosystems, are sensitive to the conditions of the environments they inhabit. Globally, anthropogenic processes are creating chemical and physical changes to freshwater systems, with known consequences for biodiversity. Though freshwater fungi are vital to ecosystem health, their microscopic nature and complex taxonomy make them difficult to study. As demonstrated by our work, metabarcoding of DNA from the environment can be used to conduct comprehensive surveys of these organisms that otherwise may not be detected in the system due to cryptic morphology or limitations to culturing. By correlating fungal community composition and richness with water quality data, we found that freshwater fungi are highly responsive to variable environmental conditions, with responses differing greatly by taxonomic group. Understanding how freshwater fungal communities are specific to particular habitats and 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.

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APPENDIX

Table A1. Water quality data from 17 sites in a small coastal tributary in Southeastern, VA, USA. Sites were sampled in April 2021 (William & Mary CCA, 2022).

Site Name	Site Type	Temperature (C)	Conductivity	Oxygen Saturation	pH	TSS	Total P	DIP	NH4	Secchi
Tutter's Neck	Pond	17.5	409	111	7.27	5.4	0.53	0.25	0.47	57
Lake Matoaka	Pond	17.3	352	107	7.22	2.3	0.25	0.05	1.4	120
Kingspoint Pond	Pond	19.6	275	120	7.28	1.2	0.22	0.05	0	120
CW Ponds	Pond	18.9	642	99.6	7.25	7	0.68	0.25	1.23	45
Kingsmill Pond	Pond	20.5	391	122	7.28	3.2	0.47	0.1	3.98	120
Papermill Creek	Stream	18.7	702	98.9	7.16	5.6	0.68	0.71	1.81	39
College Campus	Stream	15.8	634	74.9	7.05	4.6	0.69	0.2	1.58	46
Compton Dr.	Stream	14.2	439	89.8	7.1	2.5	0.35	0.05	2.75	120
Holly Hills	Stream	15.5	480	91.7	7.09	1.8	0.59	0.56	1.87	120
Colonial Williamsburg	Stream	23.8	1566	88.8	7.67	10.8	0.33	4.95	0.94	120
Kingsmill Creek	Stream	16.5	167	92.1	7.41	3.8	0.57	0.45	5.21	120
Mimosa Dr.	Stream	15.5	739	99.7	6.98	3.5	0.36	0.2	0.23	120
Bloody Ravine	Stream	14.8	486	97.5	7.16	4.4	0.7	0.2	1.11	83
New Hope Rd.	Stream	14.4	427	91.4	7.15	3.2	0.34	0.25	2.4	120
College Landing	Tidal creek	21	673	82	7.17	21.2	1.88	0.86	0.47	20
Kingspoint Dock	Tidal creek	20.4	696	88.3	7.23	31.5	1.79	0.45	0.7	20
James River	Tidal creek	20.9	1231	97.7	7.46	20.33	1.44	0.35	2.63	30

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