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Zooplankton Community Composition And Abundance Is Structured By Aquatic Fungi In A Large Estuary

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ABSTRACT

Fungi are a little known but important part of pelagic aquatic ecosystems. A recent hypothesis – the "mycoloop hypothesis" – proposes that fungal parasites of algae improve energy transfer to the zooplankton and higher trophic levels by converting the biomass of inedible algae to highly nutritious fungal biomass and by separating phytoplankton aggregates into smaller and more ingestible fragments. This interaction can form an important resource for zooplankton during blooms of inedible or graze-resistant algae. Previous work in freshwater lakes and marine environments suggests that the mycoloop is most influential in highly productive systems such as estuaries, though no previous study has focused on this pathway in estuaries. To address this research gap we conducted a survey of the aquatic fungi within the third largest estuary and one of the most productive bodies of water in the world, Chesapeake Bay, USA. We compared the efficacy of two widely used fungal metabarcoding markers, the ITS and LSU regions, and examined correlations between the algal parasites and zooplankton abundance and community composition. Sampling was conducted over 3 months (June, July, August) in 2023 at 24 sites throughout the estuary, totaling 70 sampling events. We collected samples of zooplankton using traditional methods and characterized fungal communities in the water and the gut contents of zooplankton by DNA metabarcoding of the fungal LSU (28S) region. We found that zooplankton and aquatic fungal communities both had spatial and temporal variation in diversity and community structure that correlated with environmental variables. Congruent with our predictions based on the mycoloop hypothesis, the density of zooplankton was positively correlated with the diversity of algal parasites. A trophic connection between algal parasites and zooplankton was confirmed by zooplankton gut DNA contents. This study improves our understanding of the functional roles of fungi in estuarine systems and supports the importance of the aquatic fungi in the lower food web of a major estuary.

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I would like to dedicate this thesis to my parents, Melissa and Clay Kirkendall, as well as Andrea Negele, who provided endless support and love throughout my time at William and Mary.

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Chapter 1

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1.1 Introduction

1.1.1 Origins of fungi in water

Fungi are an old and diverse lineage that began in aquatic environments. A 2017 estimate proposes that there may be as many as 2.2-3.8 million species of fungi present on earth [53], though a 2020 survey estimated that less than 150,000 of these have been described [17]. Fungi first diverged from other life roughly 1.5 billion years ago [107], before land had been colonized. During the subsequent several hundred million years, fungal diversity radiated to fill various niches in the marine environment. Estimates of aquatic fungal diversity likely vastly underestimate the true diversity in aquatic ecosystems. One study of fungal biodiversity in aquatic ecosystems suggested that there will "...always be fewer fungal taxa in freshwater and marine habitats compared to terrestrial habitats..." [98] due to the exclusion of Dikarya (a fungal group containing Basidiomycota and Ascomycota, the dominant groups on land) as well as the physiological stresses of an aquatic lifestyle. However, Wiens and Donoghue (2004) argue that the geographical distribution of a given clade (in this case, the kingdom Fungi) is governed by the ancestral ecological niche of

that clade, among other factors [112]. Current hypotheses suggest Fungi evolved in marine environments [10, 77], indicating that the diversity of aquatic, particularly marine, fungi is likely underestimated. This is likely due to the difficulty in collection of aquatic fungi compared to terrestrial fungi, as well as highly conserved morphology among aquatic fungal lineages, making fungal species identification without the use of molecular tools challenging. As molecular tools improve and reference databases expand to include a greater diversity of early-diverging and zoosporic lineages, the recorded diversity of aquatic fungi is likely to increase greatly.

1.1.2 Diversity of fungi in rivers and streams

Research on lotic fungi generally has focused on the role of decomposers, particularly aquatic hyphomycetes. For example, one study sampled streams along a eutrophication gradient within a river basin in Portugal and found that 78% of their total sequencing reads belonged to taxa closely related to aquatic hyphomycete species [29]. Aquatic hyphomycete diversity varies spatially along a latitudinal gradient, though not traditionally (i.e. increasing towards the equator) as temperate zones tend to be more species rich [47]. However, other researchers have found different patterns. Yang et al. (2021) sampled along the Elbe river from freshwater to through brackish to marine sites, and found that >80% of relative sequence abundance in their freshwater sites was from zoosporic groups such as Chytridiomycota and Rozellomycota [115].

1.1.3 Fungi in lakes and ponds

Much about the fungal community of lentic systems (lakes, ponds, wetlands, etc.) is still unknown. Chytridiomycota and other zoosporic groups such as Rozellomycota appear to be more diverse in these systems than in marine or lotic systems [57, 64]. Dikarya are still very species rich, though most of their diversity comes from Ascomycota [21, 64]. The general understanding is that zoosporic lineages of fungi (Chytridiomycota and Rozellomycota in particular) tend to be more abundant in freshwater lentic fungal communities than

previously known [48, 98]. For instance, Khomich et al. (2017) looked at aquatic fungal communities in 77 lakes in Scandinavia along a longitudinal gradient. They found that while Dikarya composed the majority (70%) of recovered OTUs, most of the sequences (63%) belonged to Chytridiomycota suggesting that Chytridiomycota may be numerically dominant over more diverse groups in freshwater lakes [64].

1.1.4 Marine and estuary fungi

The primary environmental characteristics structuring marine fungal biogeography are temperature and salinity [96, 98]. Yang et al. (2021) sampled along the Elbe river down to the river plume and the nearby marine waters and found that fungal community composition varied widely along the salinity gradient. The relative abundance of Chytridiomycota decreased from 85%-0% along the fresh-marine transect while Dikarya showed the opposite pattern, increasing from 7.5% to 95% of OTUs detected [115]. Marine fungi have been a lively topic of recent research. Current understandings of marine fungal communities have Ascomycota dominating coastal and open water environments, while polar systems and hydrothermal vents tend to have a greater representation of early diverging fungi like Chytridiomycota and Rozellomycota [41, 48, 52, 85].

1.1.5 Importance of aquatic fungi

The ability of fungi to produce secondary metabolites that are useful for human medicine is widely known, and this talent is not lost in the aquatic fungi. The first secondary metabolite isolated from an aquatic fungus was an antibiotic (Cephalosporin) [2]. To date, over 1000 compounds have been isolated from aquatic fungi, helping to manage conditions such as cancer, heart disease, and diabetes, as well as to create drugs for immune suppression and antivirals [35, 46, 109, 111]. Additionally, aquatic fungi have been shown to have some application in bioremediation of recalcitrant compounds. Some have been shown to be useful in the treatment of water and sediment contaminated with heavy metals [97, 118], while others have been effective in the cleanup and decomposition of plastics and

oils [24, 25, 65, 100]. Our understanding of the true diversity of aquatic fungi is still limited, there are huge potential benefits to exploring new aquatic fungi.

1.1.6 Study goals

This study addresses a critical knowledge gap in our current understanding of the biogeography of aquatic fungi: the community present in a productive estuary. Additionally, we compare the efficacy of two widely used fungal metabarcoding primer sets, the large subunit (LSU) and internal transcribes spacer (ITS), in surveying the community of aquatic fungi, with an eye to early diverging lineages. Here, we sample the community present in the Chesapeake Bay, the third largest estuary and one of the most productive water bodies in the world [34]. We hypothesized that we would see a greater diversity of Chytridiomycota and other basal groups of fungi in the lower salinity waters of the Chesapeake because of their apparent preference for fresh water that has been demonstrated through previous work [115, 64, 98, 48]. Additionally, we expect that environmental factors such as water temperature, salinity, and dissolved oxygen will be critical in structuring community composition and fungal richness.

1.2 Methods

1.2.1 Study Area

Chesapeake Bay is roughly 320 kilometers long, only 4.5 km wide at its narrowest point and 48 km at its widest. It contains 18,804 km of shoreline and has a 166,534 km2 watershed, encompassing over 18 million people [7]. Chesapeake Bay encompasses a wide range of salinity and nutrients gradients that fluctuate with rainfall, freshwater inputs from rivers, nutrient runoff, and other factors. The Bay also features a high level of productivity having been described as "hypertrophic" (>500 g C m-2 y-1) [50], which is a result of its wide variety of habitats, the interface between freshwater and saltwater and the resulting high diversity of species, as well as the massive effect of anthropogenic eutrophication.

The Chesapeake is affected by multiple anthropogenic stressors. Chesapeake Bay receives nutrient runoff from agriculture, stormwater, wastewater, and other sources that cause eutrophication and frequent algal blooms, resulting in periodic dead zones [103, 113, 114]. These problems are widespread – more than 75% of Virginia's estuaries and tidal rivers have been declared impaired as of the most recent Department of Environmental Quality Integrated Report [80] – and can be extreme. The Virginia Institute of Marine Science (VIMS) releases an annual report on dead zones in Chesapeake Bay and found that in 2021 the dead zone covered an average of 1.5 cubic miles, and lasted for 141 days, 46 days longer than in 2020 [104].A better understanding of the role of aquatic fungi and algal parasitism on bloom dynamics and trophic transfer could provide a better understanding of the complex host of factors that contribute to form algal blooms and dead zones.

1.2.2 Field Survey Design

Sampling was conducted monthly during summer 2023 (June, July, and August). For each month sampling was conducted at 8 stations in the Chesapeake Bay (Fig. 1.1). We chose our sampling stations by using historical water quality data provided by the Chesapeake Bay Program Data Hub [87] to separate all 49 monitoring stations into with 9 groups by k-means-clustering, using the "kmeans" command in the "stats package, of the scaled and centered environmental variables. One station was selected from each cluster, apart from one cluster that was primarily located in the Susquehanna and north Chesapeake. Each of the 8 stations was sampled at three locations at least 100m from each other, forming 24 total sampling sites. Chlorophyll concentration and other water quality variables (total phosphorus, dissolved inorganic phosphorus, dissolved inorganic nitrogen, nitrate, ammonium) were gathered via the Chesapeake Bay Program Data Hub. [87].

Samples of eDNA were collected using a Smith-Root eDNA Backpack Sampler (Smith-Root, Mount Vista, WA, USA; Thomas et al., 2018). Two liters of water from 10 cm below the surface were vacuum filtered with a maximum pressure of 82.7 kPa through a 45 mm polyethersulfone (PES) membrane filter with 5 µm pores (Sterlitech, Auburn, WA,

USA). The filters were then placed in 5 ml microcentrifuge tubes with 2.4 mL DNA-sterile CTAB cell lysis solution and then stored at -80 °C until DNA extraction, within 30 d. We collected 3 samples at each station on each sampling data resulting in 72 total samples.

1.2.3 DNA extraction and sequencing

Environmental DNA (eDNA) was extracted from the filters using a modified glassmilk procedure [70]. This procedure is effective for extracting and sequencing chytrid and other fungi from PES filtered water samples [36]. The frozen filters in CTAB cell lysis solution were thawed, incubated in a water bath for 15 min at 65 °C, agitated by vortexing, and returned to the water bath for an additional 45 min. Samples were then centrifuged at 10,000 rcf (relative centrifugal force) for 1 min before 100 µL of supernatant was removed and transferred to 1.5 mL microcentrifuge tubes. A negative control including un-used CTAB lysis solution was included to identify any contamination present during each batch of extractions.

Initial PCR amplification of the fungal ITS2 marker was performed on all samples as well as the negative control samples. The primers used for ITS2 barcoding targeted the fITS7 [56] and ITS4 [110] priming sites and were modified for Illumina high-throughput sequencing by the addition of the Illumina adapters [83]. Amplicon libraries were prepared by transferring 3 µL from each DNA-extracted sample to a new strip tube and combining 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 then placed in a thermal cycler under 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.

Initial amplification of the LSU gene region (28S) used primers targeting the LR0R [102] and the JH-LSU-369rc [117] priming sites. These primers were modified for Illumina high-

throughput sequencing by the addition of the Illumina adaptors. PCR reactions included 3 µL template eDNA, 0.1 µl Promega GoTaq DNA polymerase, 3 µl Promega GoTaq buffer, 0.3 µl of each primer at 10 µM, 7.88 µl water, 0.12 µl bovine serum albumin (BSA), and 0.3 µl dNTP (0.2 mM). Samples were then placed in a thermal cycler under the following conditions: initial annealing at 94 °C for 3 min; 40 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 45 s, extension at 72 °C for 90 s, and a final extension at 72 °C for 7 min. Amplification of the targeted regions were verified using gel electrophoresis. The ITS and LSU amplicons were then dual indexed by 8 cycles of PCR with Illumina Nextera v2 indices and adapters (Illumina Inc., San Diego, CA, USA) following the manufacturer's protocol. Indexed amplicon libraries were quantified using a Qubit 4.0 Fluorometer using a dsDNA HS kit, equilibrated and cleaned using the Zymo select-a-size DNA clean and concentrator mag-bead kit (Zymo Research, Catalog# D4085), and combined in equal volumes (4 µl per library). The combined libraries were sequenced using a 2 x 300 V3 sequencing kit on an Illumina MiSeq at UC Riverside Genomics Core, Riverside CA, USA. For our LSU sequencing run we included a mock community composed of early diverging groups of fungi to help parameterize our bioinformatics [93].

1.2.4 Bioinformatics

Raw sequence data for both the ITS and LSU data sets was processed using the open access AMPtk pipeline [83] to pre-process reads, cluster into OTUs, filter OTUs, and assign taxonomy to OTUs. Pre-processing included trimming of the forward and reverse primers, then merging paired end reads. Expected errors less than 1% [30] were used to quality-filter reads, followed by de-replication and clustering of reads to 98% similarity for the LSU data set and 97% for the ITS data set. Clustering to OTUs with LSU was done with the DADA2 pipeline, while the ITS data we used VSEARCH to build OTU tables. We used the hybrid taxonomy algorithm in AMPtk to assign taxonomy to the LSU OTUs using a database developed for use with LSU primers and curated to include a wide diversity of early diverging taxa [93]. We determined the composition of algal-parasitic fungi in each

sample by searching the fungal genera against the FungalTraits database [88]. FungalTraits is the most up-to-date and comprehensive database to link fungal genera with functional life history information. We considered all genera with a primary lifestyle in the database of "algal parasite" to represent the algal parasite community. OTUs that were not identified to the generic rank, or OTUs that were not found in the FungalTraits database, were not included in subsequent analysis.

1.2.5 Fungal community analysis

We determined OTU richness by using the 'specnumber()' function in the Vegan package in R [81]. We tested for differences in OTU richness by month as well as by station using ANOVA tests. Significant differences were parsed using a Tukey post-hoc test.

Community analysis of the aquatic fungal community was conducted separately for each data set and done on presence/absence data using the Jaccard distance metric. To visualize how communities were related to each other we utilized an unconstrained ordination with non-metric multidimensional scaling (NMDS) with the 'metaMDS' function in Vegan [81]. The "dimcheckMDS" function from the package "goeveg" was used to produce a stressplot to determine the optimal value of k, and trymax was set to 1000. To test for differences in community composition among months and sites, a nonparametric permutational multivariate ANOVA (PERMANOVA) [5] was performed using the "adonis2" function [81]. To test whether community composition was more variable by site or by month a beta dispersion test was performed using the "betadisper" function [6].

To determine how environmental variables correlated with fungal community composition we used a canonical redundancy analysis (RDA) using the 'capscale' function in the Vegan package [81]. The most important environmental variables were selected based on p value during forward model selection using the 'forward.sel' function from the "packfor" package [27, 11]. Significance of selected environmental variables was confirmed using the 'anova.cca' function with 999 permutations. To determine the proper error structure for modeling how environmental variables impact aquatic fungal OTU richness we began by

creating two candidate models, both with the full suite of environmental variables, one with the Poisson distribution and one with the negative binomial distribution, to test for overdispersion. The AIC values of the two candidate modls were compared, and the poisson distribution was chosen. We chose a generalized linear mixed effects model (GLMM) using the 'glmmTMB' command from the R package "glmmTMB" [13] with a random effect of station to account for variation due to the location sampled. We conducted stepwise backward model selection using the drop1 function and a Chi-square test, as recommended by Zuur et al. (2009).

The final model was validated by plotting the residuals versus fitted values for each predictor variable including those discarded during model selection to check for evidence of non-independence, testing for overdispersion of residuals using the "testDispersion" function for the R package "DHARMa" version 0.4.6 [51], checking for multicollinearity among predictors using the "check collinearity" function for the R package "performance" [71]. The "r2 nakagawa" function from the "performance" package was used to estimate the variance explained by the fixed and random effects components of the model.

1.3 Results

1.3.1 Bioinformatics results

LSU sequencing returned 12,118,702 raw reads of which 9,467,602 reads passed quality filtering (78.1%). The DADA2 pipeline identified a total of 3,411 ASVs, of which 633 were identified as chimeras, leaving 2,778 valid ASVs. ASVs were clustered at 98% to generate biological OTUs, creating 780 OTUs. 91% of reads were successfully mapped to OTUs. Our samples were pooled with samples from other projects for sequencing and when our samples were subset we were left with 464 OTUs and 7,538,230 fungal reads from our Chesapeake Bay sampling.

ITS sequencing returned a total of 8,691,445 raw reads, of which 6,094,843 passed quality filtering (70.1%). Clustering and de novo chimera detection identified a total

of 3,246 OTUs, of which 7,452,126 reads (86%) were successfully mapped to OTUs. Our samples were pooled with samples from other projects for sequencing and when our samples were subset we were left with 30 OTUs and 93,820 fungal reads from our Chesapeake Bay sampling.

1.3.2 Comparison of two primer sets

With the LSU data set we identified a total of 464 OTUs from 5 different phyla (Figure 1.2, Table 1.2). 148 OTUs (31.8%) belong to Ascomycota, 81 (17.4%) belong to Basidiomycota, 95 (20.4%) belong to Chytridiomycota, 107 (23%) from Mucoromycota, 1 from Zoopagomycota, and 32 OTUs were only classified to the Kingdom level. Using the ITS data set we identified a total of 30 fungal OTUs from 3 different phyla (Figure 1.2, Table 1.1). 12 OTUs (40%) belong to Ascomycota, 7 (23%) belong to Basidiomycota, 8 (27%) belong to Chytridiomycota, and 3 OTUs were only classified to the Kingdom level (Fungi).

There was a large disparity between primer sets in the proportion of fungal reads to total reads. The ITS data returned a total of 1,676,386 reads of which 93,820 (5.6%) belonged to the kingdom Fungi, whereas the LSU region returned a total of 10,849,972 reads of which 7,540,182 (69.5%) were fungal (Figure 1.3).

We found no correlation on a sample-to-sample basis of fungal OTU richness recovered from the ITS and LSU data sets, and on average we detected 26 more fungal OTUs per sample in the LSU data set than we did with the ITS (Paired t-test; t=11.14; df=69; p=2.2e-16). We did find a correlation in a measure of beta diversity; there was a weak correlation between pairwise Jaccard distances in the LSU and ITS data sets (Figure 1.4). The ITS data set appeared to be a poor representation of the aquatic fungal community because of the issues with non-specific amplification and poor agreement between the two data sets, therefore we only used LSU data for all subsequent analyses.

1.3.3 Aquatic fungal diversity

In the LSU data set we detected OTUs belonging to 9 classes of Ascomycota: Dothideomcyetes (54 OTUs), Sordariomycetes (35 OTUs), Eurotiomycetes (13 OTUs), Leotiomycetes (12 OTUs), Saccaromycetes (10 OTUs), Lecanoromycetes (6 OTUs), Orbilomycetes (2 OTUs), one OTU each of Arthoniomycetes and Taphrinomycetes, and 14 Ascomycota OTUs were unable to be identified past phylum level. We detected OTUs belonging to 7 classes of Basidiomycota: Agaricomycetes (53 OTUs), Exobasidiomycetes (8 OTUs), Tremellomycetes (8 OTUs), Microbotryomycetes (4 OTUs), Cystobasidiomycetes (2 OTUs), one OTU from Pucciniomycetes and Wallemiomycetes, and 4 Basidiomycota OTUs were unable to be identified past phylum. We detected OTUs belonging to 3 classes of Chytridiomycota: Chytridiomycetes (46 OTUs), Monoblepharidomycetes (30 OTUs), Neocallimastigomycetes (13 OTUs), and 6 unable to be identified past phylum. We detected 6 classes within Mucoromycota, Mortierellomycetes (51 OTUs), Mucoromycetes (23 OTUs), Endogonomycetes (19 OTUs), Glomeromycetes (6 OTUs), Umbelopsidomycetes (4 OTUs), Archaeosporomycetes (2 OTUs), and 2 with the class unknown. We collected only one OTU from Zoopagomycota belonging to the class Zoopagomycetes.

Our lifestyle assignment for the LSU data set using the FungalTraits database identified 18 lifestyles within the Chesapeake Bay (Table 1.3, Figure 1.5). The most common lifestyle was soil saprotroph, 82 of the OTUs (17.6%) belonged to that group. The remaining lifestyles were: litter saprotroph (45 OTUs, 9.6%), wood saprotroph (44 OTUs, 9.4%), plant pathogen (27 OTUs, 5.8%), algal parasite (24 OTUs, 5.2%), animal endosymbiont (12 OTUs, 2.6%), unspecified saprotroph (12 OTUs, 2.6%), ectomycorrhizal (9 OTUs, 1.9%), animal parasite (7 OTUs, 1.5%), arbuscular mycorrhizal (6 OTUs, 1.3%), pollen saprotroph (6 OTUs, 1.3%), lichenized (4 OTUs, 0.8%), foliar endophyte (2 OTUs, 0.4%), lichen parasite (2 OTUs, 0.4%), and one OTU each (0.2%) from the lifestyles mycoparasite, nectar saprotroph, protistan parasite, and root endophyte. 178 (38%) of the OTUs were unable to be assigned a lifestyle due to lack of taxonomic assignment.

LSU fungal OTU richness was not significantly different by station (ANOVA, $F_{7,62}$ = 1.3, p = 0.26) however there was a significant difference by month (ANOVA, $F_{2,67}$ = 24.5, p = 1.05e-8). June had a significantly greater richness of OTUs than July and August

The LSU fungal community NMDS showed variation temporally as well as spatially (k = 2, stress = 0.17). Community composition varied both by month ($F_{2,67}$ = 7.72, R2 = 0.19, p = 0.001) (Figure 1.6) and by station ($F_{7,62}$ = 2.12, R2 = 0.19, p = 0.001). Community variability recovered using the LSU primers did not vary significantly by month ($F_{2,67}$ = 2.82, p = 0.06), though it did by station ($F_{7,62}$ = 2.56, p = 0.02).

Ordinations by specific phyla revealed differences in the spatial temporal distribution of the major taxa groups. The ordination for Basidiomycota had insufficient data and the ordination did not converge.

PERMANOVA testing the ordination for Ascomycota (k = 2, stress = 0.11) showed significant differences in community composition by month ($F_{2,59}$ = 1.92, R2 = 0.06, p = 0.008) as well as by station ($F_{7,54}$ = 1.38, R2 = 0.15, p = 0.009). Ascomycota beta dispersion did vary significantly both by month ($F_{2,59}$ = 5.13, p = 0.009) as well as station ($F_{7,54}$ = 3.53, p = 0.003).

The Chytridiomycota ordination (k = 2, stress = 0.21) PERMANOVA testing showed significant differences in community composition by month ($F_{2,67}$ = 9.03, R2 = 0.21, p = 0.001) as well as station ($F_{7,62}$ = 2.14, R2 = 0.19, p = 0.001). However there were not significant differences in beta dispersion for either month ($F_{2,67}$ = 1.01, p = 0.37) or station ($F_{7,62}$ = 1.1, p = 0.37).

For the NMDS ordination for Mucoromycota (k = 2, stress = 0.11) PERMANOVA testing revealed significant differences in community composition for both month ($F_{2,67}$ = 8.31, R2 = 0.20, p = 0.001) and station ($F_{7,62}$ = 2.73, R2 = 0.24, p = 0.001). There was a significant difference in beta dispersion by station ($F_{7,62}$ = 3.86, p = 0.001), however there was not by month ($F_{2,67}$ = 0.22, p = 0.81).

Our RDA model selection identified 7 environmental variables significant in structuring Chesapeake Bay fungal communities and explained 31% of the variance in fungal commu-

nity composition (Figure 1.7, Table 1.4).

The best model to explain fungal OTU richness included DO (z = 7.06, p = 1.7e-12), Chlorophyll concentration (z = -3.94, p = 8.2e-5), and salinity (z = -2.41, p = 0.016) as fixed effects, with a random effect of station. DO had a positive relationship with fungal OTU richness, while chlorophyll and salinity were negatively associated. The fixed effects explained 44% of the variance in LSU fungal OTU richness.

We constructed simpler models without the random effect for the major phyla found during our sampling since the random effect models would not converge. Our model explain LSU Basidiomycota richness identified water temperature (z = -4.19, p = 2.8e-5), bottom depth (z = -2.02, p = 0.04), and nitrate (z = -1.89, p = 0.059) as significant effects, all negatively associated with OTU richness. The model explained 36% of the variance in Basidiomycota OTU richness. For the Ascomycota OTU richness model we identified 5 significant predictors: ammonium (z = -4.8, p = 1.4e-6), chlorophyll concentration (z = -3.89, p = 0.0001), TP (z = 3.3, p = 0.0009), nitrate (z = -2.62, p = 0.008), and salinity (z = -2.61, p = 0.008). Of the significant variables, only TP had a positive relationship with OTU richness. The Ascomycota model explained 30% of the variance. The best model to explain Chytridiomycota OTU richness included DO (z = 4.26, p = 2.1e-5) and TP (z = -2.78, p = 0.005). Dissolved oxygen had a positive relationship with OTU richness while TP was negative, and the model explained 32% of the variance in Chytridiomycota OTU richness. Our model to explain LSU Mucoromycota OTU richness identified 4 water quality variables as significant: water temperature (z = -4.55, p = 5.4e-6), salinity (z = -3.004, p = 0.002), ammonium (z = -2.22, p = 0.026), and DO (z = 2.2, p = 0.027). Only DO was positively correlated with OTU richness, and the model explained 56% of the variance in LSU Mucoromycota OTU richness.

1.4 Discussion

1.4.1 Taxonomic comparison between primer sets

Primer choice is a crucial decision in metabarcoding studies of environmental fungal communities. The two primer sets used in our study returned different pictures of the fungal community of Chesapeake Bay. From the same samples, the ITS2 primers returned 30 OTUs from 3 phyla, while the LSU primer set returned 464 OTUs from 5 phyla. The ITS dataset missed the diversity of Mucoromycota that was detected by the LSU primers and did not provide the level of taxonomic resolution for many of the Chytridiomycota lineages that was observed with LSU. The ITS region has historically performed poorly for detecting zoosporic fungal lineages [93], particularly in aquatic environments where taxonomic representation in databases can be sparse. The database used for the LSU data set has been curated with a focus towards basal groups that may not yet be present in the ITS database which may account for some of the difference. Some patterns were conserved between the two data sets. Ascomycota was the richest fungal phyla in with both primer sets, adding to the body of knowledge that supports the rich community of Ascomycota present in coastal environments [48, 85, 98]. OTUs belonging to Chytridiomycota represented similar proportions of the community with both data sets, 26% in the ITS data set and 20% in the LSU.

Primer choice has a major influence on trait-based inferences of functional diversity in aquatic fungi. We recovered a much greater diversity of functional groups from the FungalTraits database using the LSU primer set than we did using the ITS primer set. One aspect of that difference is simply the difference in OTUs recovered using each set of primers, however with both data sets roughly 40% of OTUs were not able to be assigned a primary lifestyle (12/30 in ITS; 178/464 in LSU). Soil saprotrophs were the richest lifestyle in the LSU data set, though almost 90% of the soil saprotrophs belonged to Mucoromycota which was missed with the ITS primers. There were no soil saprotrophs observed in the ITS data set. This oversight illustrates the importance of primer choice in ecological studies of

marine fungi. A study that sets out to describe the functional roles in an aquatic system may be missing a large proportion of functional diversity with ITS primers. Despite the disparity in the diversity of saprotrophic groups collected, saprotrophs were the dominant trophic mode and made up roughly 40% of the lifestyle richness with both primer sets (37% ITS; 41% LSU). A study looking at trophic modes in the South China Sea using the FUNGuild database found that saprotrophs were the most abundant trophic mode and made up 26% of the total abundance [69], markedly less than what was observed in our study though perhaps the freshwater inputs and intermixing of fresh and marine waters in the Chesapeake provide more raw material for decomposition and a more diverse community of saprotrophs. Given the improved taxonomic diversity and detection of OTUs from zoosporic lineages, we will be focusing on the LSU data set for the remainder of the discussion.

1.4.2 Community dynamics

Current understandings of mycoplankton dynamics indicate that the community composition changes temporally, showing some degree of seasonality [9, 91]. For zoosporic fungi from the phylum Chytridiomycota these seasonal changes often track the phenology of their phytoplankton host taxa, or the timing of pollen release from the trees [61, 91]. For other groups of marine fungi, such as Ascomycota or Basidiomycota, these seasonal changes are believed to be due to changes in environmental or nutrient conditions, or the amount of rainfall or other freshwater input to the system [28, 108]. However, there is no consensus on how these communities vary. A study done using ITS primers in the South China Sea found that mycoplankton richness was lowest in summer and fall, while Winter and Spring showed significantly greater OTU richness [69]. Another study in the Yellow River and Yellow Sea using ITS primers found that OTU richness during one winter and spring was significantly lower than every other sampled date, while the next winter/spring OTU richness at those sites was not statistically different from the rest of the year [108]. Wang et al (2021) found that seasonality was responsible for about 18% of the community

variation in their study. In our study we found evidence of seasonality in fungal community composition over the duration of sampling (Figure 1.6). Adonis testing showed that month was responsible for 19% of the variation in community composition in the LSU data set, in line with what has been observed in previous research.

The literature varies on the expected representation of Chytridiomycota in coastal communities. One study that sampled along the Elbe River from a shallow to a marine environment using the 18S region found that the relative abundance of Chytridiomycota in their samples steadily decreased from 85% to 0% as the salinity increased to a maximum of 31 ppt [115], whereas the salinity in our study ranged from 18-30 ppt and did not appear to influence chytrid distribution. A study of mycoplankton diversity in Hawaiian coastal waters in 2010 using the SSU region recovered 0 OTUs outside of dikarya, though this could potentially be due to limitations in sequencing methods or reference databases available at that time [41]. However, another study in coastal North Carolina, nearby to our study site Chesapeake Bay, using LSU primers found that Chytridiomycota composed roughly 25% of OTUs detected in their study [85].

Several studies have also taken a more specific look at the contribution of environmental and physical factors in structuring mycoplankton communities [64, 69, 96, 108] . Yang et al (2021), in their study looking at mycoplankton diversity of the Elbe river, found that environmental variables explained roughly 50% of the variation in community composition. They found that the two most impactful factors were water temperature and salinity, though these two effects were separated on different axes [115]. Li et al (2023) used Mantel tests to determine the contribution of a suite of environmental and water quality parameters to fungal community composition, and found that every variable they considered had a significant relationship with community composition, and together explained up to 90% of the variance, depending on season [69].

Reinforcing the findings of the studies mentioned above, our study also showed that water temperature was the most impactful variable in our RDA model (determined by F statistic) structuring mycoplankton community composition in the Chesapeake, followed

by salinity. Constrained ordination showed that water temperature was strongly associated with the CAP1 axis, while salinity is separated primarily along the CAP2 axis, indicating that effects of these two variables on community structure are independent. The biplot shows June and July clustering together, largely driven by changes in water temperature and total phosphorus, while August is distinct, and variability is primarily driven by levels of dissolved oxygen 1.7. We found a much greater diversity of Chytridiomycota and Mucoromycota in June and July using the LSU primers than was observed with the ITS primers, and in July these fungi are responding strongly to water temperature and TP in the same way that communities in June do. Environmental factors had a significant role in determining fungal OTU richness. We predicted that we would see significant effects of water temperature and salinity, based both on previous research looking at environmental drivers of mycoplankton distribution as well as our understanding of the critical role of salinity in an estuarine environment such as Chesapeake Bay. Our model identified a different set of environmental factors as significant: chlorophyll concentration, dissolved oxygen, and salinity. Salinity has frequently been shown to be a significant predictor of aquatic fungal diversity. Yang et al. (2021) found that salinity was a driving factor in structing fungal communities along a transect of fresh to marine water in the Elbe River. A survey of the mycoplankton along a transect in the Delaware Bay found that changes in fungal diversity tracked primarily with salinity [14]. A study in Lake Erie found that increasing conductivity, which is associated with higher levels of salinity, may even regulate the infectivity of parasitic fungi [74]. We found that increasing salinity was associated with decreasing richness of fungal OTUs. This tracks with previous research that has observed declines in the richness of early diverging fungi, such as Chytridiomycota and Mucoromycota, along transects from fresh to marine water [14, 108, 115].

Curiously, we found a negative effect of chlorophyll concentration and a positive effect of dissolved oxygen in our LSU model results. Dissolved oxygen is a common factor determining mycoplankton diversity and has been found to be positively associated with the abundance of aquatic fungi [23, 69], indeed many lineages of Chytridiomycota in particular

appear to be obligate aerobes [45]. However, the assumption is that increasing chlorophyll concentration, and the corresponding increase in dissolved oxygen due to oxygenic photosynthesis, would result in a greater richness of fungal OTUs. A possible explanation for this discrepancy is that top down pressure by aquatic fungi that parasitize phytoplankton is actually suppressing photosynthetic activity.

1.5 Figures and Tables

Ascomycota		Basidiomycota	
Dothideomycetes	7	Agaricomycetes	3
Sordariomycetes	2	Cystobasidiomycetes	1
Eurotiomycetes	1	Microbotryomycetes	1
Saccaromycetes	1	Pucciniomycetes	1
Unknown Ascomycota	1	Unknown Basidiomycota	1
Chytridiomycota			
Chytridiomycetes	3		
Rhizophydiomycetes	1		
Unknown Chytridiomycota	4		

Table 1.1: Phyla and classes detected using the ITS primer set. Total of 30 OTUs.

Ascomycota		Basidiomycota	
Dothideomycetes	54	Agaricomycetes	53
Sordariomycetes	35	Exobasidiomycetes	8
Eurotiomycetes	13	Tremellomycetes	8
Leotiomycetes	12	Microbotryomycetes	4
Saccaromycetes	10	Cystobasidiomycetes	2
Lecanoromycetes	6	Pucciniomycetes	1
Orbilomycetes	2	Wallemiomycetes	1
Arthoniomycetes	1	Unknown Basidiomycota	4
Taphrinomycetes	1		
Unknown Ascomycota	14		
Chytridiomycota		Mucoromycota	
Chytridiomycetes	46	Mortierellomycetes	51
Monoblepharidomycetes	30	Mucoromycetes	23
Neocallimastigomycetes	13	Endogonomycetes	19
Unknown Chytridiomycota	6	Glomeromycetes	6
		Umbelopsidomycetes	4
Zoopagomycota		Archaeosporomycetes	2
Zoopagomycetes	1	Unknown Mucoromycota	2

Table 1.2: Phyla and classes detected using the LSU primer set. Total of 464 OTUs.

Figure 1.1: 8 stations sampled in Chesapeake Bay during Summer 2023. Each station was sampled 3 times at least 100m apart, once in June, July, and August. Two types of samples were taken during each visit, fungal eDNA samples as well as zooplankton samples.

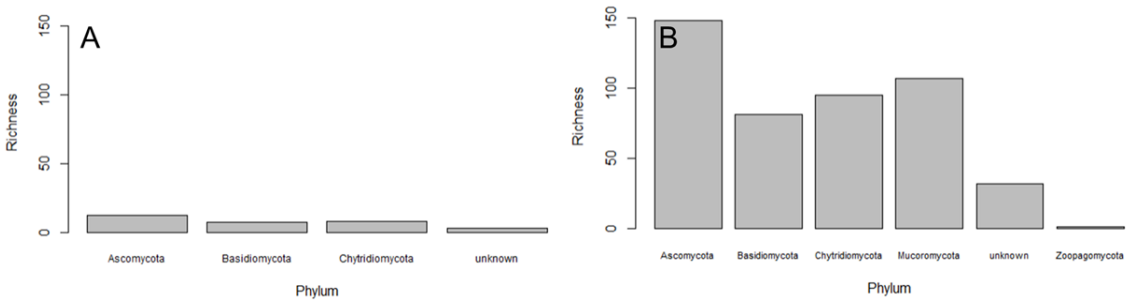

Figure 1.2: Bar graphs displaying the detected OTU richness of each primer set, broken down by phylum. Panel A shows the ITS data set (30 OTUs), and panel B shows the LSU data set (464 OTUs)

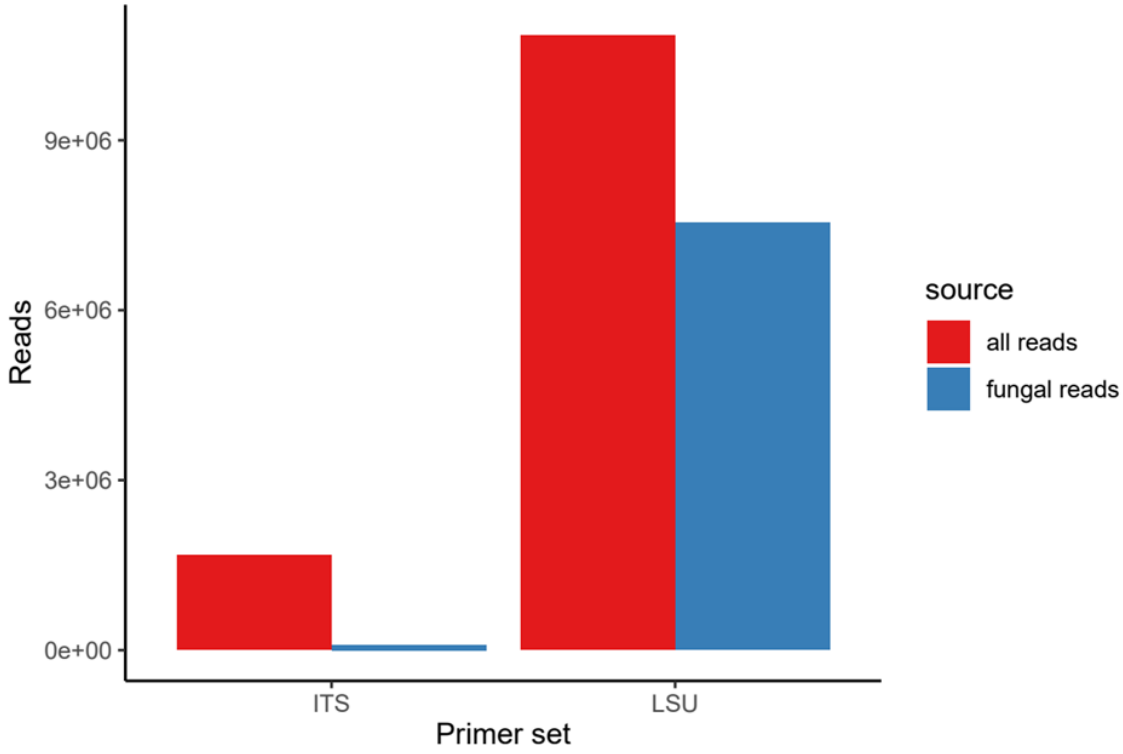

Figure 1.3: Bar graphs displaying the breakdown of all returned reads and reads belonging to the Kingdom Fungi for both the ITS and LSU primer sets. All returned reads are displayed in red, and fungal reads are displayed in blue. 5.6% of ITS reads were fungal and 69.5% of LSU reads were fungal.

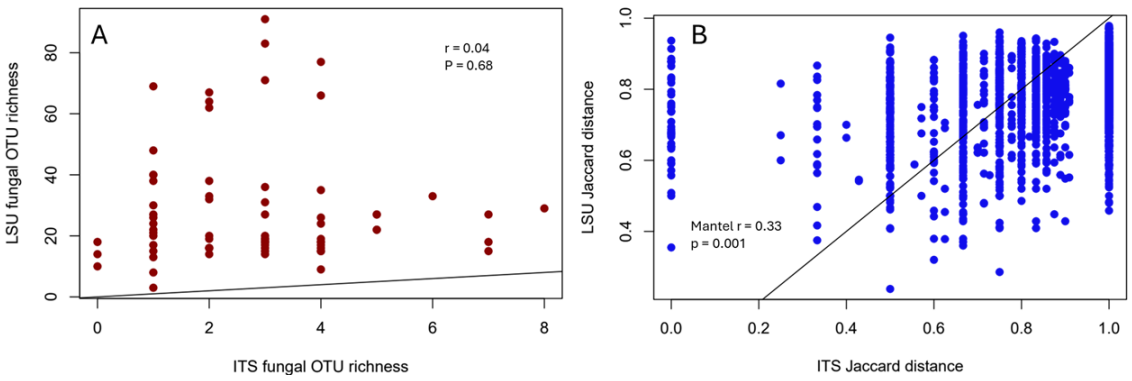

Figure 1.4: Sample to sample comparisons of OTU richness and Jaccard distance (alpha and beta diversity) recovered using two different primers, ITS and LSU. A) OTU richness for the ITS primer (x-axis) and the LSU primer (y-axis) from the same samples. B) Jaccard distance for all pariwise comparisons with the ITS primer (x-axis) and LSU primer (y-axis). For both plots points above the 1:1 line indicate greater values from the LSU primer and points below indicate higher values for the ITS primer.

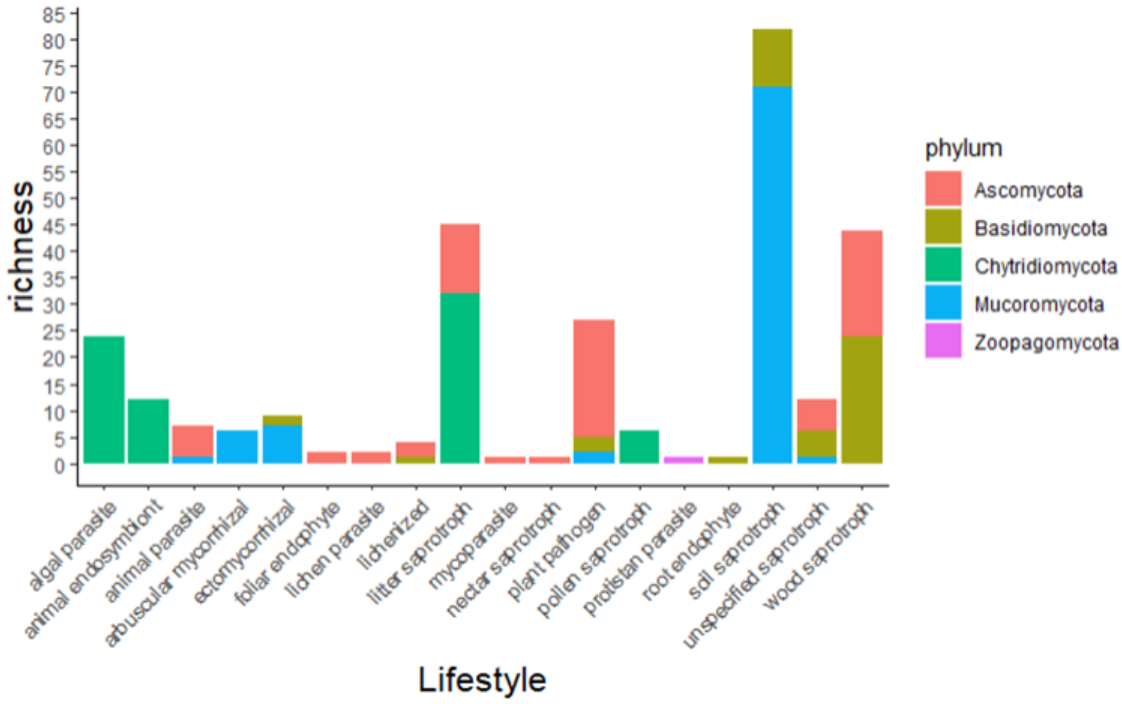

Figure 1.5: Stacked bar graphs displaying the primary lifestyle assignments of each primer set, with coloration by phylum. 178 OTUs (38%) were unable to be assigned a primary lifestyle.

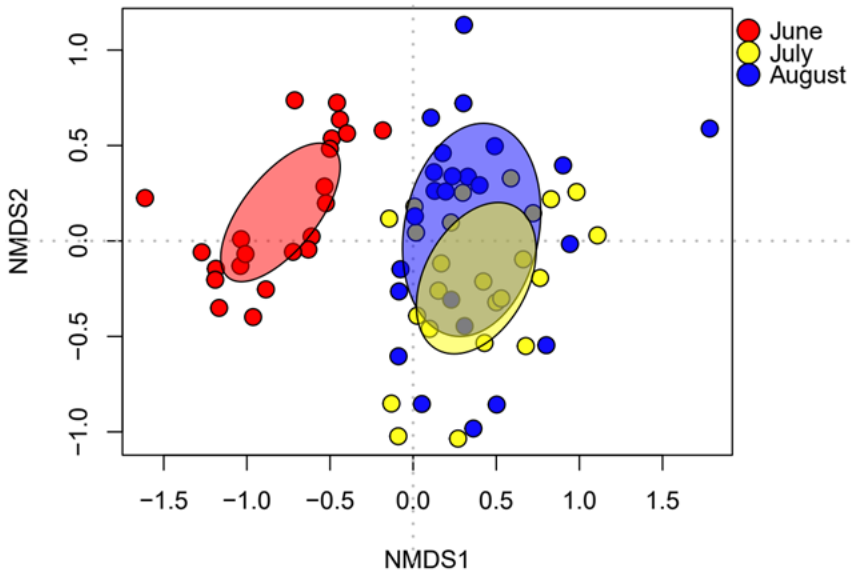

Figure 1.6: Non-metric multidimensional scaling (NMDS) ordination showing the effect of month on the fungal community composition using the LSU primer set. Each point represents the total community at a specific site (presence/absence). The ellipses indicate the standard deviation around the centroid for each grouping variable and are colored by month.

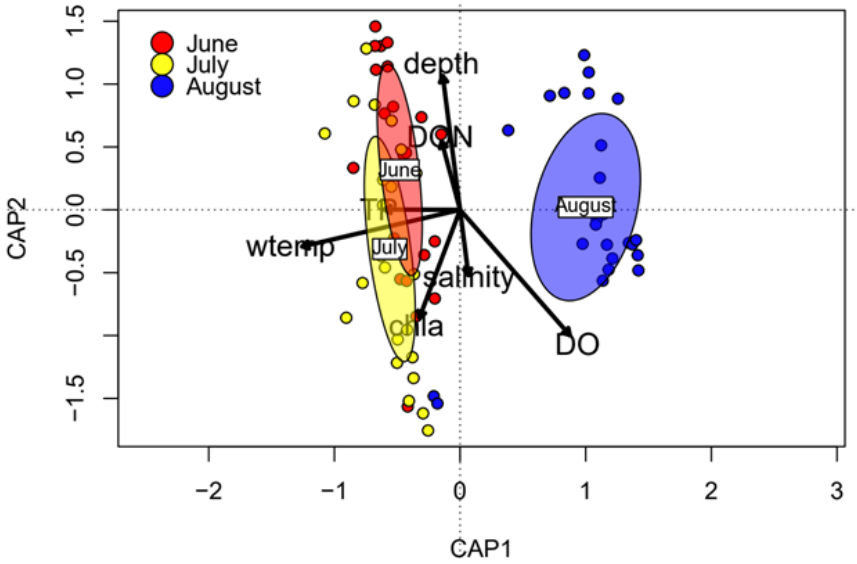

Figure 1.7: Plot of the redundancy analysis (RDA) of the fungal communities recovered using LSU primers of 24 sampling locations sampled over 3 months showing the effects of the environmental variables. Arrows indicate variables that explain a significant portion of the fungal communities. Points represent the fungal community at a specific site, and are colored according to the month collected, red for June, yellow for July, and blue for August.

ITS		LSU	
Lifestyle	Richness	Lifestyle	Richness
algal parasite	3	algal parasite	24
animal parasite	1	animal endosymbiont	12
epiphyte	1	animal parasite	7
litter saprotroph	7	arbuscular mycorrhizal	6
nectar saprotroph	1	ectomycorrhizal	9
plant pathogen	2	foliar endophyte	2
unknown	12	lichen parasite	2
unspecificed saprotroph	1	lichenized	4
wood saprotroph	2	litter saprotroph	45
		mycoparasite	1
		nectar saprotroph	1
		plant pathogen	27
		pollen saprotroph	6
		protistan parasite	1
		root endophyte	1
		soil saprotroph	82
		unknown	178
		unspecificed saprotroph	12
		wood saprotroph	44

Table 1.3: Primary lifestyle assignments as determined by the FungalTraits database for both the ITS and LSU data sets.

LSU RDA				
	Df	SumOfSqs	F	p
Water temperature	1	2.59	10.81	0.001
Dissolved Oxygen	1	1.46	6.08	0.001
Salinity	1	0.78	3.25	0.001
Total Phosphorus	1	0.77	3.19	0.001
Chlorophyll concentration	1	0.42	1.76	0.013
Dissolved Organic Nitrogen	1	0.39	1.62	0.031
Depth	1	0.38	1.58	0.034

Table 1.4: Results of the permutation tests of the RDA analysis for relationships between fungal communities and environmental factors at 70 sites in Chesapeake Bay. Results are based on 999 permutations.

Chapter 2

Evidence for the Mycoloop in a large productive estuary

2.1 Introduction

Phytoplankton and zooplankton compose the majority of the biomass in the world's oceans and estuaries [34]. Plankton are organisms that are unable to swim against currents. They can range in size from very small, like marine viruses, $< 0.2\mu m$ (femtoplankton) to larger organisms, such as jellyfish, that are $>$ 20 cm (megaplankton). Phytoplankton are the photoautotrophic primary producers in plankton communities and include prokaryotes (cyanobacteria, also called blue-green algae) and eukaryotes (such as diatoms, dinoflagellates, and coccolithophores, among others). Phytoplankton contribute approximately half (49%) of global primary production and of oxygen production [33]. Phytoplankton drive carbon dioxide fixation in the world's oceans, which is one of the planet's most significant carbon sinks, absorbing approximately 20-35% of atmospheric CO2 annually [49, 63]. This flux is four times greater than the amount of CO2 captured by the Amazon rainforests each year [16].

Zooplankton are planktonic heterotrophic animals. Zooplankton include crustaceans, mollusks, chordates, and cnidarians, among others. Zooplankton are an important linkage

between the organic carbon and nutrients generated by phytoplankton and higher trophic levels such as fish. While some phytoplankton are high quality food for zooplankton, others are inedible or low quality food [59]. Some phytoplankton resist grazing by forming large multi-cellular aggregates, some phytoplankton lack essential fatty acids and therefore have low nutritive value, and some phytoplankton produce toxins [40]. Zooplankton typically avoid eating low-quality phytoplankton. However, phytoplankton blooms are sometimes dominated by algae that are resistant to grazing or avoided by zooplankton, which can have serious ramifications for higher trophic levels [38]. High resistance in the phytoplankton to zooplankton grazing leads to decoupling between primary and secondary production and inefficient carbon transfer to zooplankton [3]. This decoupling of production can lead to the loss of biomass at higher trophic levels, and could impact fish productivity and diversity [94].

Algal blooms are common occurrences. Every US coastal and Great Lakes state experiences occasional algal blooms [78]. An algal bloom is a significant population increase [101] that occurs when the growth and/or immigration rate is much greater than the mortality rate for a given population of algae. Algal blooms can be formed by mechanical processes such as wave concentration, a phenomenon where waves or currents push phytoplankton from offshore where they are more diffuse into the coast where densities can rise sharply [54]. Blooms can also result from ecological processes including bottom-up controls such as inputs of a limiting nutrient, and top-down controls such as a reduction in grazing by zooplankton [92]. When algal blooms cause significant damage to the environment or economy they are classified as harmful algal blooms (HABs) [78].

Algal blooms sometimes cause dead zones [78]. Dead zones occur when oxygen concentrations in the water become too low to support aerobic life. Death and decomposition of blooms of algal cells can reduce oxygen concentrations sufficient to cause a dead zone. Additionally, some harmful algal blooms are formed by species that produce compounds called cyanotoxins which have negative impacts on the local fauna and on human health [20]. Human health effects range from sore throat and nausea, to kidney damage, liver

damage, and death [20]. Cyanotoxins can remain in the water after the bloom has passed, so the absence of a bloom does not mean that the water is safe [20]. One study found that 3 different microcystins declined in concentration only about 30-37% after 21 days of constant illumination [73].

HABs are projected to increase as the global climate warms. At elevated temperatures cyanobacteria outcompete eukaryotic primary producers like diatoms and dinoflagellates [72, 82]. Consequently, warming climates are expected to shift phytoplankton communities towards dominance by cyanobacteria [72]. Rising temperatures will also intensify vertical stratification, which gives cyanobacteria an additional competitive advantage over other phytoplankton due to their unique ability to form gas filled vesicles that give them some measure of buoyancy control [82].

The increasing dominance of cyanobacteria and the rising likelihood of harmful algal blooms can have serious ramifications for human industry, recreation, and even for the quality of human drinking water [31]. Cyanobacterial blooms can diminish zooplankton productivity because they have low nutritional quality, some cyanobacteria taxa produce toxins, and they competitively displace more nutritious phytoplankton [40]. Because cyanobacteria resist grazing by zooplankton, HABs of cyanobacteria decouple primary and secondary production, leading to fewer zooplankton and reductions in biomass at higher trophic levels [3].

The "mycoloop hypothesis" proposes that fungal parasites, primarily in the phylum Chytridiomycota, transfer nutrients from phytoplankton to higher trophic levels by converting phytoplankton biomass to fungal zoospores which are consumed by, and highly nutritious to zooplankton ([60], Fig. 2.1). Additionally, chytrids are hypothesized to enhance trophic transfer by fragmenting phytoplankton resulting in up to a 50% reduction in filament length of filamentous species. The formation of phytoplankton aggregates and filaments is a quality that increases resistance to grazing by zooplankton [38].

Chytrids play a role in structuring planktonic food webs through the consumption of their zoospores by zooplankton [60, 58]. Zoospores are a high-quality food for zooplankton

because they synthesize sterols that are rich in polyunsaturated fatty acids, and they have high cholesterol concentrations [40, 59]. Grazing on chytrid zoospores can therefore alleviate dietary stress on the zooplankton caused by inedible phytoplankton blooms by supplementing their diets. Chytrid infection channels an estimated 20% of total primary production in terms of carbon from the phytoplankton to the zooplankton and provide up to 57% of zooplankton dietary requirements [90]. Thus, the mycoloop pathway may act to stabilize pelagic food webs during algal blooms. A dead zone that kills the zooplankton will necessarily reduce biomass at all higher trophic levels by halting carbon transfer.

Previous work testing the mycoloop hypothesis has been largely focused on foundational laboratory studies as proof of concept [39, 40, 37] and studies of mesotrophic inland lake habitats [91]. This work has generally supported the importance of this pathway for fungal mediated trophic transfer from the phytoplankton to the zooplankton, however models based on empirical observations from mesotrophic systems predict that the mycoloop pathway is even more important in highly productive eutrophic systems [60]. The Chesapeake Bay is the largest estuary in the United States [34] and one of the most productive aquatic habitats on Earth, yet no previous work has examined the potential importance of aquatic fungi to secondary production in Chesapeake Bay or other major estuaries. Given that previous research has indicated that the mycoloop may be stronger/more important in more productive environments [59, 91] the mycoloop is likely an important part of such a large and productive system as Chesapeake Bay. Understanding the potential importance of aquatic fungi to the base of productive estuarine food webs could greatly improve the ability of managers to understand complex estuary ecosystems and guide more effective management decision-making. Current tools and models that are available do not consider this significant flux of energy and nutrients and may therefore produce flawed predictions. Studies that investigate how changes in water quality affect the zooplankton community may be best served by also considering the fungal community as well [106, 8].

This study sought to provide a new understanding of how potentially fungal-mediated energetic pathways impacts higher trophic levels in a large and productive estuarine food

web. Specifically, we aimed to 1) Determine the drivers of fungal parasite diversity throughout the summer season across the Chesapeake Bay using environmental DNA (eDNA). 2) Confirm a trophic linkage between aquatic fungi and zooplankton by examining zooplankton guts using metabarcoding to confirm ingestion of algal parasites. And 3) test a key prediction of the mycoloop hypothesis by correlating fungal parasite diversity with zooplankton abundance. Based on the mycoloop hypothesis, we predicted that the richness of fungal parasites in the phytoplankton will correspond to higher abundances of zooplankton as there will be more availability of high-quality food in the form of fungal zoospores. Our results provide supportive observational evidence of the mycoloop hypothesis in Chesapeake Bay and provide new insight into the mechanisms that support secondary production in highly productive estuary ecosystems.

2.2 Specific Methods

2.2.1 Zooplankton sample collection

Zooplankton were sampled using a 300 mm diameter, 153 µm mesh zooplankton net (WILDCO, Yulee, FL, USA; SKU: 3-426-A32). Zooplankton were sampled in a vertical tow from 2 m above bottom to the water surface and preserved in 95% ETOH. We collected 2 zooplankton tows each visit, one for abundance analysis and one for gut extraction and metabarcoding. We collected 3 samples at each station on each sampling date resulting in 72 samples of each sample type.

2.2.2 Zooplankton Sample Processing

Zooplankton samples were sieved through 153 µm mesh to remove the ethanol preservative and then diluted with de-ionized water in a beaker to a known volume. The sample was mixed in a figure-8 pattern with a stirring rod and a 1 ml aliquot was taken using a Hensen-Stempel pipette and placed on a zooplankton counting wheel. Using a dissecting scope, all zooplankton in the 1 ml sub-sample were identified and enumerated, with the total body

length of the first 20 individuals of each taxon measured by ocular micrometer under a stereoscopic microscope at 2X magnification. If needed, multiple aliquots of 1-mL subsamples will be processed until a combined count of at least 100 individuals was reached, not including immature copepodites or nauplii.

Zooplankton density was determined using the depth the net sampled to, the diameter of net, and subsamples processed during identification. The biomass of certain taxa (mg/m3) (calanoid copepods, cyclopoid copepods, cladocerans) for a given sample was estimated using length-weight regressions for dry weight summarized by EPA [32]. Regressions were not available for the other taxa.

2.2.3 Zooplankton diet analysis

Zooplankton samples for gut analysis were split into 3 taxonomic groups; calanoid copepods, cladocerans, and decapods. Calanoid copepods were chosen for their abundance and importance in aquatic food webs, cladocerans for their abundance and common use as a model organism in mycoloop studies, and decapods for their commerical importance. Zooplankton samples for gut analysis were collected using identical methods to the samples for abundance counts. From each sample 30 of each group, when available, were processed for gut content analysis. Samples for gut content analysis were washed in sterile 95% ethanol by repeatedly dunking each individual zooplankton in several wash beakers, then pulverized with a small sterile pestle (Research products international; item# 199222) in a 1.5mL microcentrifuge tubes. DNA extraction, PCR amplification, dual indexing and sequencing were performed using the methods described in section 1.2.3.

2.2.4 Statistical Analysis

2.2.4.1 Drivers of algal parasite diversity in Chesapeake Bay

All statistical analyses were conducted in R version 4.3.2 [89]. Environmental data was collected from the Chesapeake Bay Data Hub ([87], downloaded 4/9/24). We downloaded

data corresponding to each of our sampling stations from June, July and August 2023. All variables were standardizied to have a mean of 0 and a standard deviation of one. PCA was conducted using the "princomp" function in R [89] to determine how environmental variables were correlated with algal parasite OTU richness prior to statistical analysis. A biplot was created to visualize the scores and loadings of the first two principal components.

Fungal community composition analysis was conducted on presence/absence of OTUs using the Jaccard distance metric [18, 95]. For the zooplankton data set we used the Bray-Curtis metric. To visualize the effect of site and month on zooplankton and fungal community composition we used unconstrained ordination by non-metric multidimensional scaling (NMDS) utilizing the "metaMDS" function in the vegan package [81]. The "dimcheckMDS" function from the package "goeveg" [105] was used to produce a stressplot to determine the optimal value of k, and trymax was set to 1000. To test for differences in community composition among months and sites, a nonparametric permutational multivariate ANOVA (PERMANOVA) [6] was performed using the "adonis2" function [81]. To test whether community composition was more variable by site or by month a beta dispersion test was performed using the "betadisper" function [6].

A generalized linear mixed effects model (GLMM) was used to correlate the available environmental variables with algal parasite OTU richness, with a random effect of station. Two error structures, Poisson and Negative Binomial, were compared by checking for model overdispersion. The model was fit using the R package "glmmTMB" version 1.1.8 [13]. Stepwise backwards model selection was conducted using the drop1 function and Chi-square test [119]. We also made a GLMM to predict algal parasite OTU richness based on month, following the same methods.

The final models were validated by plotting the residuals versus fitted values for each predictor variable including those discarded during model selection to look for evidence of non-independence, testing for overdispersion of residuals using the "testDispersion" function for the R package "DHARMa" version 0.4.6 [51], checking for multicollinearity among predictors, if appropriate, using the "check_collinearity" function for the R package "per-

formance" [71]. The "r2_nakagawa" function from the "performance" package was used to estimate the variance explained by the fixed and random effects components of the model.

2.2.4.2 Trophic linkage between algal parasites and zooplankton

The gut sequences were subset by taxa collected from (Calanoid, cladoceran, or decapod) for analysis. Lifestyles of fungi found in the gut were determined using the FungalTraits database [88]. Each OTU in our data set was assigned a 0 if it did not appear in a zooplankton gut, and a 1 if it was a diet item. We made a binomial model to predict the occurrence of fungal OTUs in zooplankton guts based on their phylum or lifestyle to determine if some taxa or lifestyles of fungi are more likely to be consumed. We turned the predictor variables into factors and used Chytridiomycota as the reference level for phylum and "algal parasite" as the reference level for lifestyle.

2.2.4.3 Algal parasite diversity and zooplankton abundance

We used an ANOVA test to look for differences in Zooplankton density across sites or months, followed by a Tukey post-hoc test in the case of a significant result ($p < 0.05$). Zooplankton density data were normalized by log-transformation which was confirmed by a Shapiro-wilk test.

Two intercept-only models to explain zooplankton density and log zooplankton density, respectively, were constructed and residuals were compared to check for normality. We then made a linear mixed effects model (LMM) to predict log zooplankton density using algal parasite OTU richness, with a random effect of station to account for random variation by station. Model selection was conducted using the methods described above.

We used a canonical redundancy analysis (partial RDA) on the zooplankton community data, with the environmental data and the fungal OTU table as explanatory variables, using the "capscale" function in the R package "vegan" [81]. This allowed us to use multiple regression to analyze a combination of variables that best account for the variation in the zooplankton community. We created two versions of the RDA, one using the environmen-

tal data with the fungal variance conditioned out, and one using the fungal data with the environmental variance conditioned out. The most important variables from each explanatory data set were chosen based on p-value during forward selection using the "forward.sel" function from the R package "packfor" [27]. Significance of variables in the model was confirmed with the "anova.cca" function from the vegan package [81], with 999 permutations. We determined the amount of variance explained by each explanatory data set as well as the amount of shared variance using the variance partitioning, using the "varpart" function in the vegan package [66, 81].

2.3 Results

2.3.1 Drivers of algal parasite diversity in Chesapeake Bay

Principal components analysis of water quality variables revealed substantial variation in environmental conditions among sampling stations during our sampling season (Fig. 2.2). The first principal component accounted for 40% of the variation in water quality and the second principal component accounted for another 22%. Most water quality variables were positively correlated with the PC1 axis, save only salinity and DO, which had strongly negative relationships with PC1. PC1 appears to be an axis between deeper, warmer, nutrient rich water in the upper Chesapeake and the higher salinity and higher oxygenation conditions towards the mouth of the Bay. PC2 was positively correlated with DO, DOP, chlorophyll concentration, TP, water temperature, TN, and DON, and had a negative relationship with salinity, ammonium, and depth. PC2 appears to represent the spectrum of chlorophyll and phosphorus concentrations from high concentrations at the river inputs such as Poquoson and James river to the relatively low concentrations observed at the Bay Mouth station. Some stations (e.g. Bay Mouth) are tightly clustered and distinct from other stations indicating stable and unique conditions throughout the sampling period, while others are more scattered and interspersed (e.g. Tangier, Mid-Bay South) indicating more variable and overlapping environmental conditions. Algal parasite richness was

negatively correlated with PC1 (-0.18) and positively correlated with PC2 (0.28).

The community of algal parasites in Chesapeake Bay changed significantly throughout the summer. We collected a total of 95 OTUS from the phylum Chytridiomycota of the 464 total OTUs (20%), and of those 24 (5.2%) were identified as algal parasites via the FungalTraits database. 21 of the Chytridiomycota OTUs were unable to be assigned a primary lifestyle, so it is likely a subset of those are also algal parasites, though they were not considered in this analysis. The other lifestyles composing the detected Chytridiomycota OTUs included animal endosymbionts (12 OTUs, 12.6%), litter saprotrophs (32 OTUs, 33.6%), pollen saprotrophs (6 OTUs, 6.3%), and for 21 OTUs (22.1%) the lifestyle was "unknown". We observed an average detection of 2.27 algal parasite OTUs in June, 1.41 in July, and 1.75 in August. PERMANOVA testing showed a significant effect of month ($F_{2,48}$ = 9.03, R2 = 0.21, p = 0.001) as well as of station ($F_{7,43}$ = 2.14, R2 = 0.19, p = 0.001). There were not significant differences in community dispersion among months ($F_{2,48}$ = 1.01, p = 0.36) or stations ($F_{7,43}$ = 1.1, p = 0.37), indicating that the differences found in the PERMANOVA were due to differences in Chytridiomycota community composition. Of the 95 Chytridiomycota fungal LSU OTUs recovered 24 OTUs (25%) were identified as algal parasites using the FungalTraits database.

The best model to explain algal parasite richness included only chlorophyll concentration (z = -2.8, p = 0.004). Algal parasite OTU richness was negatively correlated with increasing chlorophyll concentrations. The fixed effect of chlorophyll concentration explained 9.6% of the variance, while the random effect of station explained an additional 46.4% of the variance in algal parasite OTU richness, totaling 56% of the variance explained.

Another GLMM, to explain algal parasite OTU richness using only the fixed effect of "month" was made as well, with a random effect of station. Month alone explained 6.1% of the variance (June as reference; July z = -2.41, p = 0.015; August z = -1.55, p = 0.11) while the random effect of station explained an additional 33.9% of the variance in algal parasite OTU richness, totaling 40%.

2.3.2 Trophic linkage between algal parasites and zooplankton

All the fungal OTUs that were detected in zooplankton gut samples were also detected from the water samples. Of the 464 total fungal OTUs found in this study, 147 (31%) were present in the guts of zooplankton (Table: 2.1). The richest fungal phylum in the gut were the same for the three zooplankton taxa groups; Ascomycota was the richest taxa, followed by Basidiomycota, then Chytridiomycota. Mucoromycota was present in the guts of Calanoids and Cladocerans, but not in the Decapods. We only collected one OTU belonging to Zoopagomycota, but it was not present in the gut of any taxon sampled. The four richest fungal lifestyles were the same for all taxa though the order differed, plant pathogens, wood saprotrophs, soil saprotrophs, and litter saprotrophs. Chytridiomycota DNA was present in 3 of 23 (13%) of calanoid samples, 1 of 23 (4.3%) of cladoceran samples, and 3 of 20 (15%) of decapod samples. Two algal parasite OTUs were found in calanoid guts (OTU 29 and 741) in 2 of the 23 samples (8.7%), and only one in decapod guts (OTU 83) in 1 of the 20 samples (5%). All the algal parasite OTUs were from the genus Zygophylictis, though the OTUs in the calanoid gut were Zygophylictis melosirae, while the algal parasite in the decapod guts was Zygophylictis planktonica.

The likelihood of detection for each fungal phylum and lifestyle in zooplankton gut samples differed among zooplankton taxa. For the calanoid models, phylum explained 21% of the variance in OTU occurrence in the gut, with Ascomycota and Basidiomycota both more likely to be eaten than Chytridiomycota. Lifestyle explained 9.2% of the variance, and plant pathogens were significantly more likely to be eaten than algal parasites. For cladocerans the phylum model explained 26% of the variance, with Ascomycota and Basidiomycota both significantly more likely to be present in the gut. Lifestyle explained 11.4% of the variance in cladoceran diet, though no individual lifestyle tested as significant. For the decapods phylum explained 15% of the variance, Ascomycota and Basidiomycota were both significantly more likely to be consumed than Chytridiomycota. Lifestyle explained 6.6% of the variance in diet, though no lifestyle was significantly more likely to be

consumed than algal parasites.

2.3.3 Algal parasite diversity and zooplankton abundance

Average zooplankton density varied widely by both station and month sampled (Table 2.2 Figure 2.6). June had the lowest average density of zooplankton with a monthly average of 14,150 ± 6,075 ind/m3, which was significantly lower than densities in July and August (23,059 ± 12,385 ind/m3 and 22,870 ± 14,499 ind/m3 respectively; $p < 0.02$). Average density by station ranged from a low of 13,249 ± 5,601 ind/m3 at the Eastern shore station, to a high of 40,615 ± 17,409 ind/m3 at the Poquoson station. Additionally, the Poquoson station had a significantly greater average density ($p < 0.05$ in all comparisons) than every site other than the James River station (25,243 ± 17,317 ind/m3, $p = 0.2$). There was no clear trend in increasing or decreasing zooplankton density over the duration of sampling. Four stations (James, Bay Mouth, Eastern Shore, and Mid-Bay North) had their maximum density of zooplankton in July, three stations (Poquoson, Mid-Bay South, and Maryland showed increasing density over the study period, and one station (Tangier) had decreasing density from June through August.

There were no significant differences in zooplankton biomass among stations or months (ANOVA; $p > 0.05$ in all comparisons). Average biomass by station ranged from a low of 10,217 µg/m3 at the Bay mouth station to a high of 22,231 µg/m3 at the James River station. Average biomass by month was 37,608 ± 19,454 µg/m3 in June, 56,301 ± 48,138 µg/m3 in July, and 41,397 ± 21,131 µg/m3 in August. No stations showed a trend of increasing biomass over time. Two stations (Eastern Shore and Bay Mouth) showed decreasing biomass over the study period, one (Mid-Bay South) had a minimum of biomass in July, and the others (James River, Tangier, Poquoson, Maryland, and Mid-Bay North) had a peak of biomass in July. When considering results of zooplankton biomass, it is important to consider that biomass regressions were only available for copepods (calanoid and cyclopoid) and cladocerans – all other zooplankton taxa are excluded from the biomass analysis and including them could change the results.

Zooplankton species richness also varied both by station and by month. The average species richness and SD in June was 10.4 ± 1.8, in July was 11.5 ± 2.5, and in August was 12.2 ± 1.5. August had significantly greater species richness than in June (ANOVA, $F_{2,67} = 5.01$, $p < 0.006$). Richness varied more by station with a minimum of 9.8 (± 1.8 and 1.0, respectively) at both the Maryland station and Mid-Bay North, and a maximum of 13.4 ± 1.4 at the James River station. Average richness at the James River station was significantly greater than Maryland, Mid-Bay North, and Mid-Bay South (ANOVA, $F_{7,62} = 4.4$, $p < 0.05$ in all comparisons.

Zooplankton community composition varied significantly among the months of sampling. NMDS ordination (k = 3, stress = 0.18) showed clear separation of August samples from the overlapping June and July sample clouds along the second axis (Figure: 2.3A). A PERMANOVA test confirmed a significant effect of month on the composition of zooplankton ($F_{2,67} = 9.46$, R2 = 0.22, p = 0.001). There were no significant differences in multivariate dispersion among months ($F_{2,67} = 2.01$, p = 0.14), indicating that the significant PERMANOVA was due to differences in zooplankton composition and not differences in betadispersion among months. The zooplankton community composition also varied significantly by station ($F_{7,62} = 2.67$, R2 = 0.23, p = 0.001) and there were also significant differences in the variability of the communities at each station ($F_{7,62} = 7.55$, $p < 0.001$), with "Bay mouth" having the greatest variability, and "Maryland" and "Mid-Bay North" having the lowest.

Total zooplankton density did not have a significant relationship with algal parasite OTU richness (Table: 2.3). Individual models for the top 10 most common zooplankton taxa were also constructed to examine correlations between fungal richness and individual zooplankton taxa. Two taxa, pteropods and decapods, were significantly and positively correlated with algal parasite richness (Table 2.3).

RDA showed that six variables from the environmental data (chlorophyll concentration, TP, water temperature, DOP, bottom depth, and ammonium) were significantly correlated with zooplankton community composition (Fig. 2.4B, Table 2.4). Ten fungal OTUs from

the fungal community data set were significantly correlated with zooplankton community composition (Fig. 2.4A, Table 2.4). Four of the OTUs belonged to the phylum Ascomycota, three were from Chytridiomycota, two were from Basidiomycota, and one OTU belonged to Mucoromycota. Two of the three Chytridiomycota OTUs were identified as algal parasites while the other acts as a litter saprotroph.

Our RDA variation partitioning (Fig. 2.5) demonstrated that the aquatic fungal community composition alone explained a significant fraction of the variance in zooplankton community composition (adjusted $R^2 = 0.138$). Environmental variables also explained a significant fraction of the variation in zooplankton community composition (adjusted $R^2 = 0.098$). There was substantial overlap (adjust $R^2 = 0.206$) in the variance explained by each set of predictor variables.

2.4 Discussion

2.4.1 Drivers of algal parasite diversity in Chesapeake Bay

Current knowledge of aquatic fungal communities suggests a lower occurrence of Chytridiomycota in marine environments compared to freshwater settings. For instance, Gao et al. (2010) conducted a study characterizing mycoplankton diversity off the coast of Hawaii and reported no presence of Chytridiomycota in their samples. Instead, the fungal community they identified solely consisted of Dikarya [41]. Similarly, Yang et al. (2021) investigated fungal diversity along the Elbe River, spanning from freshwater to marine environments, and observed a decline in the relative abundance of Chytridiomycota from approximately 85% in freshwater sites to 0% in marine sites. Despite this, of the 913 OTUs they identified, 615 (67%) were classified under Chytridiomycota [115]. Another study analyzed mycoplankton communities in estuarine and marine sites in China and found Chytridiomycota ASVs to range from 0.02% to 1.3% across their samples [96]. In contrast, our study discovered that 95 of the 464 OTUs (20%) we identified belonged to the phylum Chytridiomycota and we did not find a significant effect of salinity in determining algal

parasite richness. The disparity between our findings and those of other studies on brackish and marine fungi might be attributed to differences in primer sets and taxonomic databases used for identification. Reynolds et al. (2022) found that LSU performed better at detecting early diverging fungal lineages than other common markers, such as ITS. Notably, Gao et al. (2010) did not detect any sequences from early diverging or classically aquatic fungal groups like Chytridiomycota or Zygomycota, which suggests that their findings may not fully represent the complete fungal community [41].

Seasonality in algal parasites is context dependent and likely depends on the specific phenologies of local host and parasite taxa. A study by Rasconi et al. (2012) found the greatest algal parasite abundance in the spring and autumn, with minima in summer and winter. By mid-June they observed parasite abundance dropping to its lowest levels and staying consistently low through the summer [91]. Our results are contrary to their findings. We observed the greatest average algal parasite OTU richness in June (2.27), a minimum in July which was significantly lower than June and August (1.41) and an increase in algal parasite richness in August (1.75) however they looked directly at algal parasite abundance while we only considered OTU richness and parasite abundance in the Chesapeake during summer could be low compared to other times of the year. A study done by Yang et al. (2021), which found a high prevalence of Chytridiomycota in freshwater and some brackish sites, conducted their sampling in August, which is when we found increasing richness in our algal parasites. Our NMDS of the Chytridiomycota community does show seasonality community composition. There were distinct communities in the early and late summer, with June being distinct from the communities observed in July and August. This change in Chytridiomycota community composition over the course of the summer is likely a result of the association of algal parasites with their hosts, as the community of phytoplankton changes over the summer and host-specific parasite populations track changes in host population.

The OTU richness of Chytridiomycota that were identified as algal parasites also changed significantly over time. On average, algal parasite richness was highest in June

and decreased later into the summer. Algal parasite richness may have a peak in June because of algal blooms typically occur during spring in the Chesapeake and it is possible that spring blooming taxa (more commonly diatoms and dinoflagellates) support a greater diversity of algal parasites than the taxa that bloom in the late summer, which are most commonly cyanobacteria [113]. However, while there is commonly a cyanobacterial bloom in the lower Chesapeake, where we found the greatest richness of algal parasite OTUs, HABs in the Chesapeake Bay occur most commonly far in the upper bay and in the Potomac and Susquehanna rivers [68, 113, 114] indicating that diatoms and dinoflagellates in the Chesapeake are possibly supporting a greater diversity of algal parasites. Of the 24 algal parasite OTUs we collected few were identified to the species level to be able to determine their host, however we collected 3 OTUs who's hosts could be identified. *Zygophylictis melosirae* was a common algal parasite species which has a broad range of hosts that includes many diatom taxa (*Asterionella* spp., *Melosira* spp., *Synedra* spp., among others), *Zygophylictis planktonica* which also parasitizes diatom taxa (*Synedra* spp., *Ulanaria* spp.), as well as *Zygophylictis asterionellae* which parasitizes *Asterionella* spp.

Previous research has shown that the diversity and composition of Chytridiomycota assemblages track spatial variation in environmental variables. For instance, a meta-analysis of publicly available 18S rRNA sequences in lakes found that 61% of their OTUs were found in only one lake [67]. Likewise, a survey of the aquatic fungal community along a transect from freshwater to marine water in the Delaware Bay found spatial changes that tracked primarily with changes in salinity along the transect [14]. Salinity is widely thought to be an important determinant of Chytridiomycota diversity [43]. McKindles et al. (2021) found that increasing conductivity, which is associated with higher levels of salinity, resulted in lower rates of chytrid infection in a community of cyanobacteria. Additionally, French (2024) found that overall fungal richness decreased significantly with salinity. However, these studies were in lower salinity environments – Sandusky Bay of Lake Erie [74] and the freshwater and tidal creeks of the Virginia peninsula [36]. Both have a markedly different relationship to salinity than an estuary such as Chesapeake Bay.

One potential explanation for the disparity between our study and the previously reported strong relationships with salinity is that chytrids in the Chesapeake Bay are more adapted to variable levels of salinity than their freshwater counterparts and therefore do not respond as strongly to spatial variation in salinity [4].

Our study revealed significant spatial variation in Chytridiomycota throughout Chesapeake Bay, similar to what has been observed among freshwater lakes, despite the inherent connectedness of localities within Chesapeake Bay and mixing of tidal flows. However, in our study salinity did not appear to account for the spatial variation. For instance, although stations closer to the mouth of the Bay tended to have greater algal parasite OTU richness than more inland sites salinity was not a significant predictor in our model of algal parasite OTU richness. In fact, two of the stations with the greatest average algal parasite OTU richness (Poquoson and James River) had very low average salinity (21.6 ppt, minimum was 18.6 ppt at the Maryland station in the upper bay), and the Bay Mouth station with the highest average salinity (28.8 ppt) had the second richest algal parasite community. Therefore, factors other than salinity must drive the significant spatial variability in algal parasite richness.

We had hypothesized that chlorophyll concentration would be an important factor driving algal parasite richness because parasite diversity typically increases with host diversity [62], and we assumed that chlorophyll concentration and phytoplankton diversity would be positively correlated. Additionally, many orders of chytrids are thought to be obligate aerobes who's growth rate is impaired by low oxygen concentrations [45], a condition that would be alleviated as a result of oxygen enrichment due to oxygenic photosynthesis. Surprisingly, we observed a significant negative relationship between the diversity of algal parasites and the concentration of chlorophyll. One possible explanation for this negative relationship is that the algal parasite densities and diversity lag behind their host densities similar to Lotka-Volterra predator-prey dynamics, and therefore the peak of phytoplankton density, and therefore chlorophyll concentration, has already peaked by the time we observe a greater richness of algal parasite OTUs. Another possibility is that peak chlorophyl

concentrations correspond to blooms of one or a few algal species and do not necessarily reflect high algal diversity.

In addition to salinity, water temperature is also thought to be an important driver of the distribution of Chytridiomycota, and marine fungi in general [43, 98]. Laboratory studies have shown that high temperatures can cause limitations in growth rate of chytrids. For instance, no chytrids are known to grow at temperatures above 45°C [12, 44]. One study found that chytrids growing on Planktothrix grew most effectively in the range of 21-22°C, though at temperatures between 17-24 °C infection rate of their hosts was $> 70\%$ [74]. We did not find a significant relationship between temperature and algal parasite richness in our study. The temperatures present in our study ranged from 20.6°C - 29°C, with the lower temperatures occurring in June when we observed the greatest richness of algal parasite OTUs. The average water temperature in July and August was very similar to each other (26.6 °C and 26.5 °C, respectively) despite the significant difference in the richness of algal parasite OTUs. One likely explanation is that chytrids in Chesapeake Bay are adapted to a wide range of temperatures and some other factor, such as seasonality of their phytoplankton hosts, is leading to the peaks in algal parasite OTU richness in June and August.

2.4.2 Trophic linkage between algal parasites and zooplankton

Our study was specifically interested in assessing zooplankton diet for the presence of fungi that are algal parasites to confirm a trophic connection between algal parasites and higher trophic levels in the planktonic food web of Chesapeake Bay. This connection is necessary for the mycoloop hypothesis to be plausible in the estuary. While fungi are thought to be common prey for zooplankton, to our knowledge, very few studies have directly assessed the fungal components of the gut contents of free-living zooplankton. A study done by Yeh et al. (2020) used molecular methods to assess the eukaryotic component of the gut contents of the copepod Calanus finmarchichus in Icelandic waters. Although the study was not focused on identifying the fungal component of the diet,

they incidentally found that fungal DNA made up roughly half of the DNA sequence reads [116], though they did not provide finer taxonomic assessment beyond kingdom Fungi. Another study, looking at copepod and cladoceran guts in the Baltic Sea, found that Ascomycota composed a large proportion of zooplankton diets (20% - >50%) from March through November. Our results confirmed previous work by showing that diverse fungi are consumed by zooplankton, with Ascomycota and Basidiomycota being the most frequently detected phyla. Additionally, some zooplankton, specifically calanoid copepods and decapods, had consumed Chytridiomycota fungi including taxa that are known to parasitize phytoplankton which provides some plausibility for the mycoloop hypothesis in our system. However, contrary to our expectations, we did not find evidence of algal parasite DNA in the guts of cladocerans. This was surprising because cladocerans are often used in laboratory studies intended to test predictions from the mycoloop hypothesis [3, 40]. Additionally, only 3 algal parasite OTUs of the 95 algal parasite OTUs observed in our study, were detected in the gut samples. Therefore, algal parasites are either not a primary diet component of the zooplankton in Chesapeake Bay, or zooplankton feed selectively on a few algal parasite taxa. It is also possible that a few phytoplankton dominated the community, which reduced the diversity of algal parasites that were present, or perhaps that the zooplankton present feed preferentially on microzooplankton, which would reduce the likelihood of consuming fungal sporangia attached to a phytoplankton cell. Additionally, due to the lack of chitinous cell wall and short lifespan of the zoospores, it is possible that they were quickly digested after ingestion. Importantly, the consumption of even small amounts of fungi may be important for supplementing zooplankton diet with polyunsaturated fatty acids and cholesterols [1, 42, 61].

2.4.3 Algal parasite diversity and zooplankton abundance

According to the mycoloop hypothesis, algal parasites supplement zooplankton diet, especially when the phytoplankton are dominated by inedible taxa or taxa with low nutritional quality. Consequently, we expect that increased infection rates of the phytoplankton by

algal parasites should stimulate zooplankton population growth and secondary production. Previous experimental work in laboratory conditions supports this prediction. For instance, Chytridiomycosis in phytoplankton causing fragmentation of phytoplankton filaments caused a 2x increase in average clearance rates of Daphnia [40]. In another example, Keratella rotifers fed an infected culture of the cyanobacteria Planktothrix, a poor quality food, demonstrated no fitness differences when compared to Keratella fed a high quality food, while Keratella kept in an uninfected culture swiftly decreased in density [39]. Thirdly, Daphnia kept in a culture of cyanobacteria infected by Chytrids showed greater fecundity, a larger body size, faster growth rate, and younger age of maturity when compared to Daphnia kept in an uninfected culture [3]. While our methods could not directly assess infection rates of algal parasites, they are effective for assessing taxonomic richness from environmental samples. We assumed that higher algal parasite diversity would correspond to higher infection rates because many algal parasites are host specific and therefore, we assessed algal parasite richness as a predictor of zooplankton density and predicted a positive relationship. In partial support of our prediction, *Pteropoda* and *Decapoda* zooplankton densities were significantly positively correlated with algal parasite OTU richness. However, there was no significant relationship between algal parasite richness and any other zooplankton taxon, or the total zooplankton abundance. These results suggest that the population growth of some zooplankton taxa may increase algal parasite diversity, while others may not. Based on our results, future work could use targeted quantitative approaches to explore relationships between the abundance or infection rates of algal parasites found in zooplankton gut and the densities and secondary production of zooplankton populations.

The trophic connection between algal parasites and zooplankton in the Chesapeake Bay may involve additional or alternative zooplankton taxa compared to previous assessments of this hypothesis. Congruent with our diet analysis that found no evidence of algal parasite DNA in cladoceran gut contents, there was also not a significant relationship between cladoceran density and algal parasite richness. Again, this result is surprising be-

cause cladocerans such as Daphnia are commonly used in experimental mycoloop studies [3, 40, 61]. Similarly, calanoid copepods did not show a significant relationship with algal parasite richness, despite previous research showing positive impacts for copepod fitness due to the mycoloop as well as evidence of chytrid biomass in their guts [40, 116]. Perhaps copepods and cladocerans, benefit from algal parasites more in lower productivity systems than in systems as productive as Chesapeake Bay [3, 39, 61] due to the increased abundance of their phytoplankton prey. However the most abundant copepod in Chesapeake Bay *Acartia tonsa* has been shown to display a preference for microzooplankton over phytoplankton in laboratory studies, which would reduce its likelihood of consuming fungal biomass attached to phytoplankton cells and receiving benefits that way (N. Millette, personal communication). We did find some economically important taxa with strong relationships to algal parasite richness, Pteropods and Decapods. Decapods are an important group in the Chesapeake, most notably for the blue crab *Callinectes sapidus* an iconic Chesapeake Bay species and a commercially important fishery for the states of Virginia and Maryland [79].

The role of environmental factors in influencing zooplankton community structure is well known [19, 22, 26, 84]. Given the benefits of algal parasitism on zooplankton fitness [3, 39, 58], as well as the varied correlations between algal parasite richness and different zooplankton taxa that we demonstrated, it is possible that the fungal community may also play a significant role in structuring zooplankton communities. Variation partitioning analysis in our study demonstrated that fungal effects account for significant variation in zooplankton community structure, even after environmental effects were removed. This result suggests that the correlation between fungal community structure and zooplankton community structure could reflect a direct causal relationship, not simply correlated variation do to similar responses to environment among zooplankton and fungi. Additional experimental work is needed to verify such causal relationships and underlying mechanisms.

The environmental variables that were identified as significant correlates of zooplankton community structure in our analysis agree with the current understanding of how the

environment impacts zooplankton communities, with one conspicuous exception. Salinity was not identified as a significant factor in structuring zooplankton communities in the RDA analysis. Prior research has found salinity to be an influential factor [19, 84] and our expectations were that this pattern would be conserved in estuarine conditions. Water temperature was the most important environmental variable in our analysis, agreeing with decades of other studies emphasizing the importance of temperature in structuring zooplankton communities [19, 55, 75]. Temperature in our RDA analysis was positively associated with both CAP axes in the direction of calanoid and cyclopoid copepods, indicating that higher temperatures tend to mean a greater representation of those groups in the community. Chlorophyll, TP, and DOP are all positively associated with the CAP1 axis in the same direction as copepods, cladocerans, and larvaceans, showing that more productive stations tended to have more of these groups. Concentrations of chlorophyll and phosphorus have both been shown to be significant determinants of zooplankton community structure in previous studies [84], though in a canonical analysis of zooplankton communities in the Gulf of Mexico chlorophyll did not play a significant role [19]. Depth has also been shown to be an important determinant of zooplankton community composition [84, 86]. Pepin et al. (2015) conducted a redundancy analysis to determine the effect of various environmental variables on the zooplankton community and found that depth contributed the most to structuring zooplankton communities in the north Atlantic.

To our knowledge, we are the first to show significant correlations between specific fungi/OTUs and zooplankton community composition. We found that the presence/absence of ten fungal OTUs (of 464, 2.1%) were significant predictors of zooplankton community composition. Four of the OTUs were from the phylum Ascomycota, three from Chytridiomycota, two from Basidiomycota, and one from Mucoromycota. Despite the taxonomic diversity in the significant OTUs the functional diversity is more limited. Six of the OTUs were saprotrophs including 3 litter saprotrophs, 1 wood, 1 soil, and 1 unspecified. Two OTUs were algal parasites, and for two the lifestyle was unknown. The saprotrophs are likely associated with the more productive stations like James River and Poquoson Flats,

where organic matter from their freshwater inputs provides aquatic saprotrophs with ample substrate for colonization. It is possible that these fungi are also directly consumed by the zooplankton as well, as happens when aquatic macroinvertebrates in streams consume leaves that have been colonized by fungi [15]. All the significant fungal OTUs, except for OTU 310 (unspecified saprotoph), are positively associated with the CAP2 axis, along with roughly half of our samples. This could indicate that only a portion of the communities we sampled are driven by fungi, and that these are likely to occur in productive areas that have ample organic matter for decomposition as well as a greater abundance of phytoplankton to parasitize, i.e. more available niche space to support a greater diversity of estuarine fungi. Both of the algal parasite OTUs were also associated with the CAP2 axis, in the same direction as nauplii, copepodites, and cyclopoids. A possible explanation for this relationship is that the mycoloop improves food quality by synthesizing PUFAs that are necessary for zooplankton reproduction [76, 99] and the consumption zoospores may increase cyclopoid fecundity and reproductive success through PUFA enrichment [1].

2.5 Figures and Tables

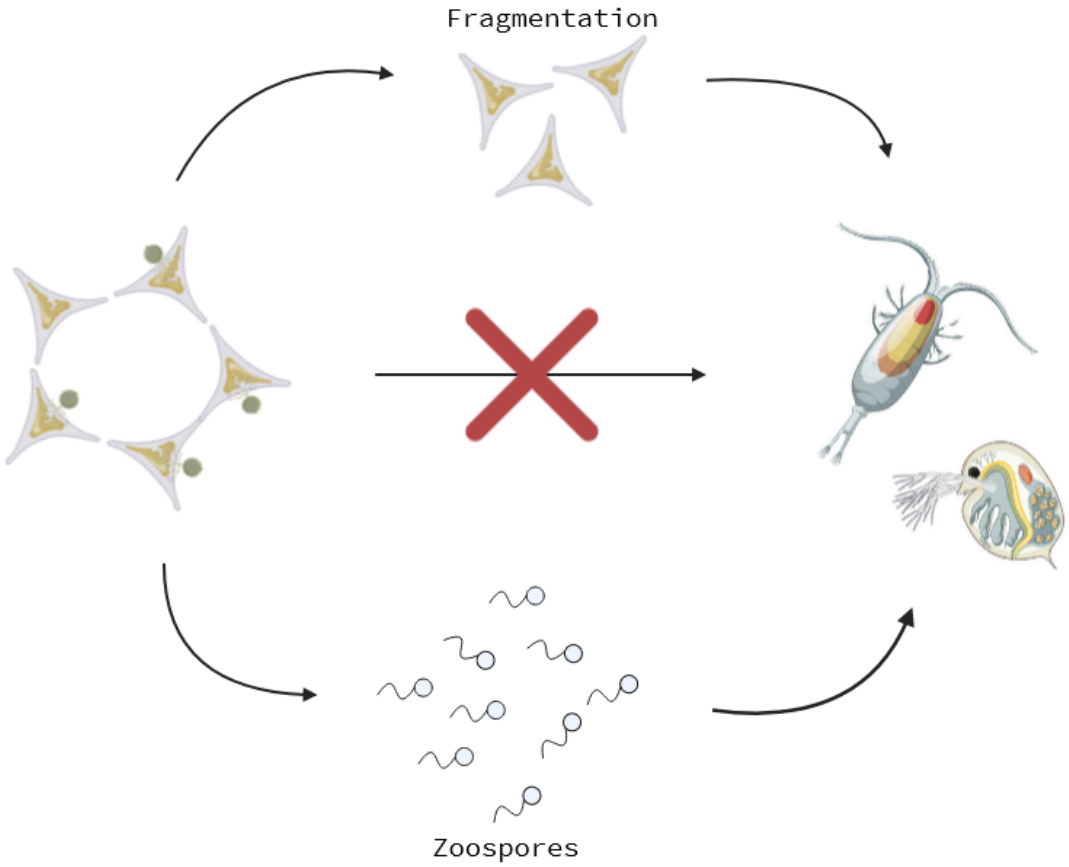

Figure 2.1: Diagram of the mycoloop. Parasitic fungi (Chytrids) can aid in trophic transfer to the zooplankton in two ways. They can fragment aggregations of phytoplankton as a by-product of infection, allowing direct consumption of phytoplankton cells. They can also transfer energy via consumption of their zoospores. Zoospores are excellent food for zooplankton in both size (2-5 µm) and nutrition (high concentrations of PUFAs).

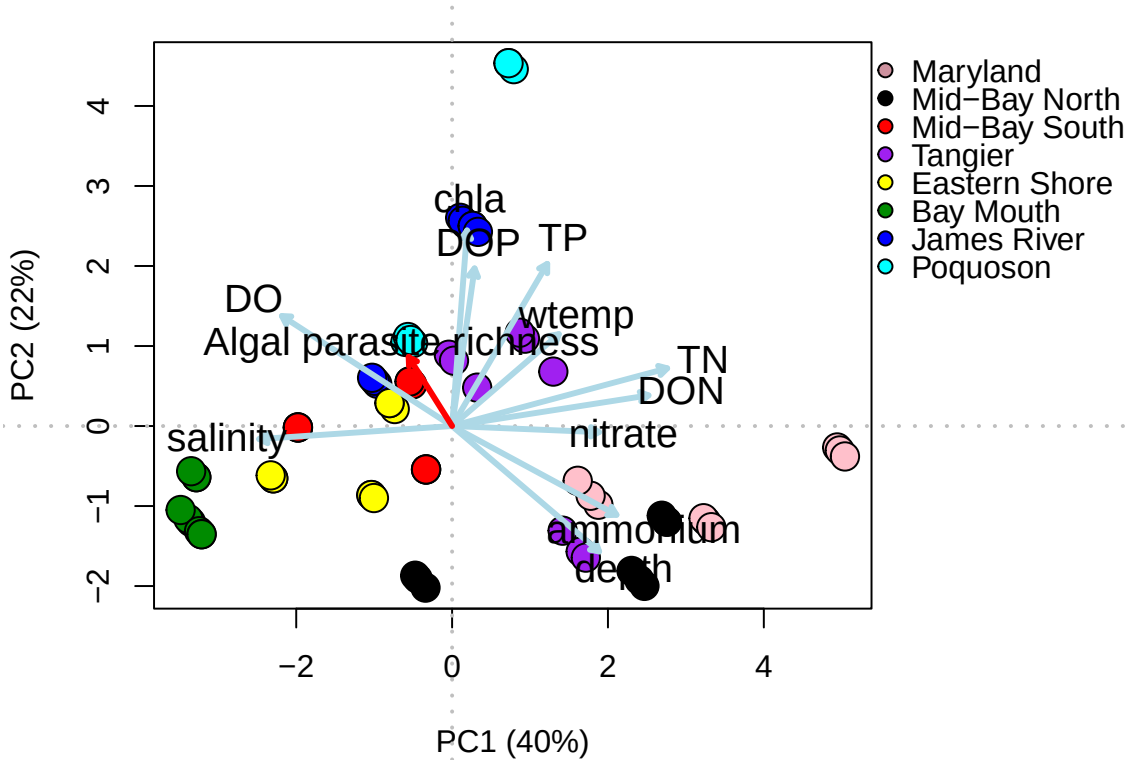

Figure 2.2: Principal components analysis (PCA) of the sampling sites, by environmental factors. Points are colored by the station at which they were collected. Blue arrows indicate the relative strength and direction of the correlation for each environmental variable. The red arrow indicates the relative strength and direction of the correlation with the OTU richness of algal parasites. PC1 accounted for 40% of the variance, and PC2 for 22%.

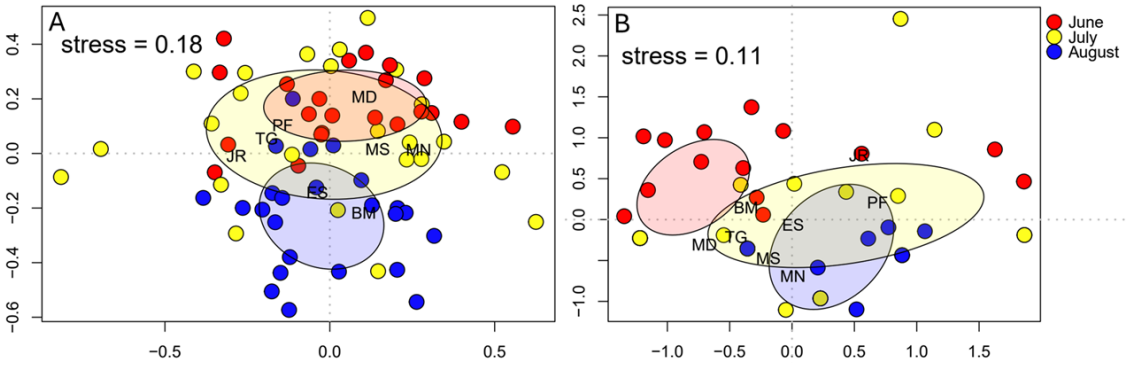

Figure 2.3: Non-metric multidimensional scaling (NMDS) ordination showing the effect of month on the zooplankton (panel A) and algal parasite (panel B) community composition. Each point represents the total community at a specific site (abundance for zooplankton, presence/absence for fungi). The ellipses indicate the standard deviation around the centroid for each grouping variable and are colored by month.

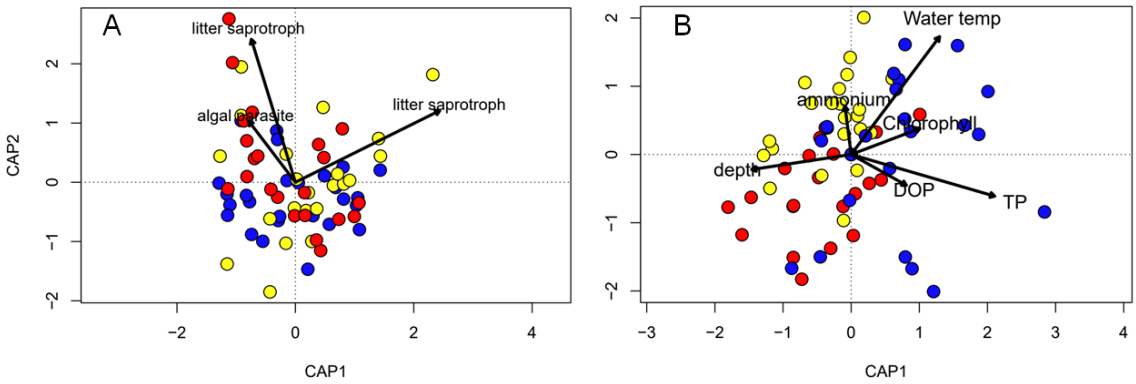

Figure 2.4: Plot of the redundancy analysis (RDA) of the zooplankton communities of 24 sampling locations sampled over 3 months showing the effects of the aquatic fungal community (A) and environmental variables (B). Arrows indicate variables that explain a significant portion of the zooplankton communities. Points represent the zooplankton community at a specific site, and are colored according to the month collected, red for June, yellow for July, and blue for August.

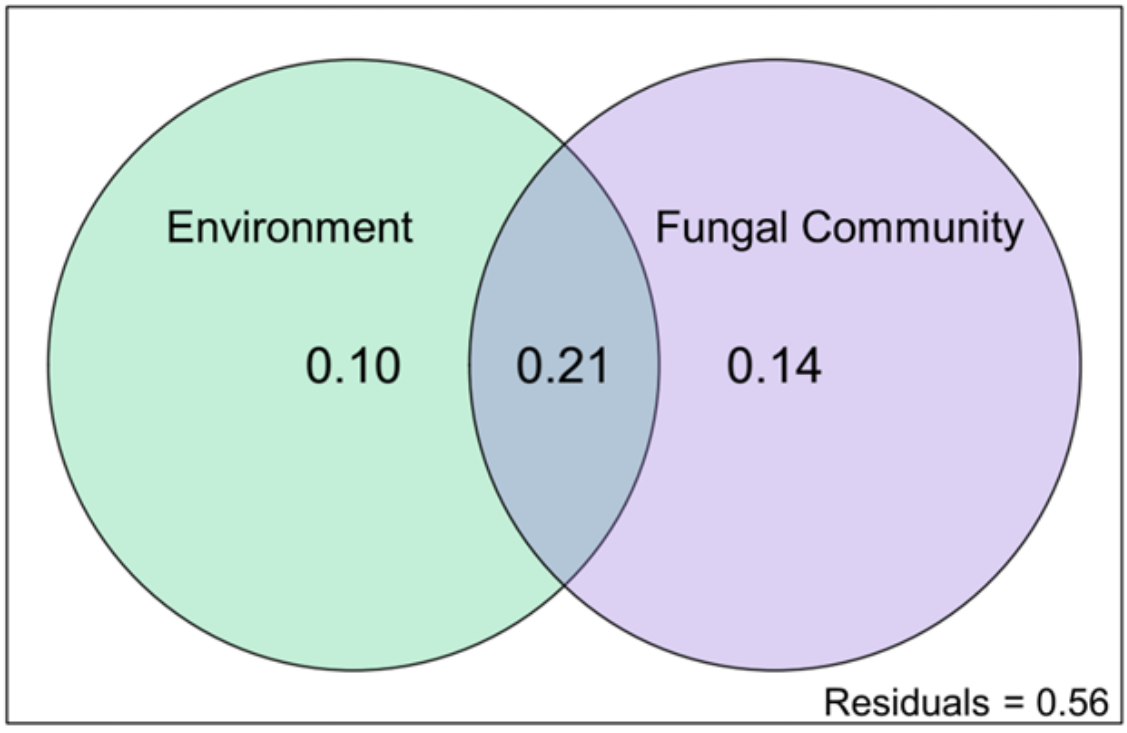

Figure 2.5: Venn diagram showing the partitioning of the variance of the environmental variables and the fungal community in structuring zooplankton community composition. The overlap represents variance that is explained by both components. Numbers indicate the adjusted R2 values.

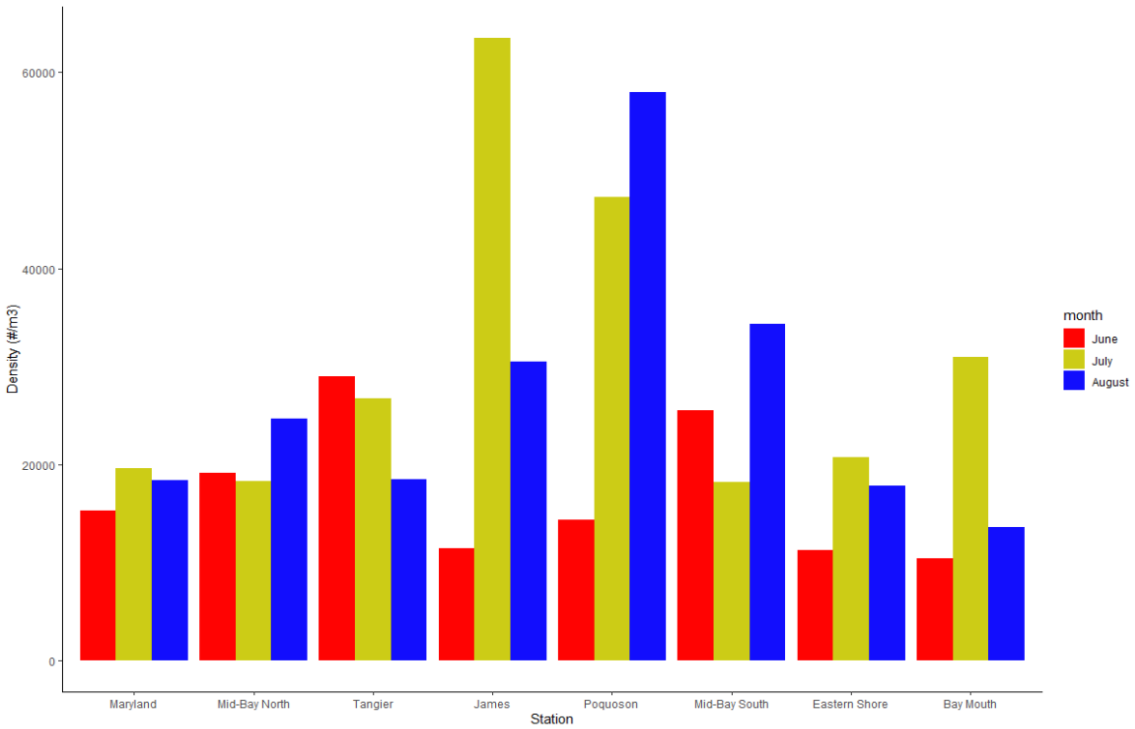

Figure 2.6: Box plots of density (individuals/m 3) of zooplankton estimated within each station in each month. Horizontal line = median, box = 25 and 75th percentile, whiskers = largest or smallest value no further than 1.5 * interquartile range from the box, solid points = outlier values.

Calanoid			
Fungal Lifestyle	Lifestyle richness	Phylum	Phylum richness
algal parasite	2	Ascomycota	49
animal parasite	1	Basidiomycota	24
foliar endophyte	1	Chytridiomycota	3
lichen parasite	1	Mucoromycota	1
lichenized	1	Unknown	1
litter saprotroph	6		
mycoparasite	1		
nectar saprotroph	1		
plant pathogen	11		
pollen saprotroph	0		
soil saprotroph	9		
unknown	29		
unspecificed saprotroph	4		
wood saprotroph	11		
Cladoceran			
Fungal Lifestyle	Lifestyle richness	Phylum	Phylum richness
algal parasite	0	Ascomycota	61
animal parasite	3	Basidiomycota	21
foliar endophyte	0	Chytridiomycota	1
lichen parasite	0	Mucoromycota	1
lichenized	1	Unknown	2
litter saprotroph	6		
mycoparasite	1		
nectar saprotroph	0		
plant pathogen	12		
pollen saprotroph	0		
soil saprotroph	8		
unknown	41		
unspecificed saprotroph	2		
wood saprotroph	12		
Decapods			
Fungal Lifestyle	Lifestyle richness	Phylum	Phylum richness
algal parasite	1	Ascomycota	31
animal parasite	1	Basidiomycota	10
foliar endophyte	1	Chytridiomycota	3
lichen parasite	1	Mucoromycota	0
lichenized	0	Unknown	2
litter saprotroph	6		
mycoparasite	0		
nectar saprotroph	0		
plant pathogen	5		
pollen saprotroph	1		
soil saprotroph	3		
unknown	20		
unspecificed saprotroph	2		
wood saprotroph	5		

Table 2.1: Breakdown of the OTUs present in the guts of the 3 taxa analyzed. OTU richness is presented in the form of fungal lifestyle as well as taxonomic group.

Zooplankton Summary Statistics

Station	Month	Density (#/m3)	*Biomass (µg/m3)	Species Richness	Shannon Richness
Maryland	June	13,271.2	20,348.5	8.3	1.20
Maryland	July	15,470.0	37,559.5	8.6	1.41
Maryland	August	16,762.8	51,490.9	12.3	1.66
Mid-Bay North	June	16,133.4	35,877.4	9.3	1.32
Mid-Bay North	July	13,625.0	42,155.1	10	1.40
Mid-Bay North	August	14,532.0	40,586.5	10	1.71
Poquoson	June	14,340.5	26,002.6	14	1.84
Poquoson	July	34,136.9	49,064.4	10	1.60
Poquoson	August	55,852.5	67,784.1	12.3	1.92
Tangier	June	24,264.0	23,368.8	9.6	1.31
Tangier	July	20,545.3	72,984.7	13	1.33
Tangier	August	16,832.2	29,818.9	13	1.87
James	June	10,379.1	29,675.5	12.3	1.66
James	July	40,964.6	103,010.1	14.3	1.42
James	August	24,386.0	59,985.9	13.6	1.99
Mid-Bay South	June	17,993.2	65,206.3	9	1.58
Mid-Bay South	July	15,486.6	24,224.1	10.6	1.36
Mid-Bay South	August	28,398.3	47,439.8	12	1.82
Eastern Shore	June	8,218.1	38,888.4	11	1.21
Eastern Shore	July	16,213.5	31,078.1	13.3	1.59
Eastern Shore	August	15,315.0	24,916.8	12	1.79
Bay Mouth	June	8,727.6	53,763.7	11.6	1.41
Bay Mouth	July	28,033.5	90,339.2	11.6	1.26
Bay Mouth	August	10,878.6	9,157.4	12.3	1.92

Table 2.2: Zooplankton summary statistics for each site and each month, including average density (#/m3), biomass (µg/m3), species richness (#), and Shannon diversity. *Biomass values only include copepods and cladocerans.

Taxa	t value	p-value	adj. R2
All zooplankton	-0.59	0.550	0.9%
Copepodites	-0.36	0.720	1.2%
Calanoids	0.98	0.330	0.0%
Cyclopoids	-1.3	0.190	1.1%
Nauplius	0.96	0.340	1.1%
Cladocerans	-1.25	0.210	0.8%
Larvaceans	-1.3	0.180	1.1%
Pteropods	**2.99**	**0.003**	**10.3%**
Veligers	1.78	0.070	3.1%
Polychaetes	1.77	0.080	3.0%
Hydrozoans	1.42	0.160	1.4%
Decapods	**2.51**	**0.010**	**7.1%**

Table 2.3: Model results to explain zooplankton density using algal parasite richness for the full zooplankton community model as well as each individual taxon a model was made for. Models with significant effects have been bolded.

LSU Fungal RDA				
OTUs	Df	SumOfSqs	F	p
OTU 10; Ascomycota, Litter saprotroph	1	0.51	6.54	0.001
OTU 29; Chytridiomycota; Algal parasite	1	0.06	0.83	0.59
OTU 75; Chytridiomycota; Algal parasite	1	0.19	2.51	0.019
OTU 156; Ascomycota; Unknown	1	0.14	1.84	0.09
OTU 278; Chytridiomycota; Litter saprotroph	1	0.06	0.79	0.56
OTU 291: Mucoromycota; Soil saprotroph	1	0.07	0.9	0.46
OTU 310; Basidiomycota; Saprotroph	1	0.06	0.74	0.68
OTU 363; Ascomycota; Unknown	1	0.12	1.53	0.15
OTU 657; Basidiomycota; Wood saprotroph	1	0.12	1.58	0.15
OTU 766; Ascomycota; Litter saprotroph	1	0.31	3.89	0.002
LSU Environmental RDA				
Environmental vars	Df	SumOfSqs	F	p
Chlorophyll	1	0.18	2.29	0.028
Total Phosphorus	1	0.22	2.82	0.006
Water temperature	1	0.23	3.03	0.004
DOP	1	0.14	1.86	0.057
Bottom depth	1	0.21	2.68	0.005
Ammonium	1	0.08	1.13	0.3

Table 2.4: Results of the permutation tests of the RDA analysis for relationships between zooplankton communities and the fungal communities or environmental factors at 70 sites in Chesapeake Bay. Results are based on 999 permutations.

Chapter 3

Conclusion

Primer choice has a major influence on fungal community inferences from metabarcoding, especially withing zoosporic groups such as Chytridiomycota and Mucoromycota. We found that the LSU primer set provides a more complete picture of the taxonomic diversity of our system, however both primer sets perform similarly when performing community analysis. Future studies would benefit from the inclusion of multi-marker methodologies to ensure a more complete survey of fungal diversity, and to leverage the spotty completion of the respective reference databases. We found that salinity, DO, and chlorophyll concentration were significant drivers of fungal OTU richness in the Chesapeake, and environmental and physical factors (water temperature, DO, salinity, chlorophyll concentration, TP, DON, bottom depth) are significantly correlated with the composition of the fungal community.

Our results provide some support for the Mycoloop hypothesis in Chesapeake Bay. We found that algal parasite OTU richness, and a putative greater biovolume of algal parasites, was correlated with an increasing abundance of some groups of zooplankton. This is the first evidence of increased zooplankton density because of algal parasite diversity in an estuarine system, or in a system as productive as the Chesapeake. We found differential responses by different zooplankton groups, though all significant relationships were positive. We determined that the aquatic fungal community plays an important role in structuring zooplankton community composition and identified ten fungal OTUs from two

distinct lifestyles that have the most influence. Two of these OTUs were algal parasites. These two OTUs demonstrate the importance of understanding how parasites interact with and structure biological communities, especially in aquatic systems. More research must be done to understand how algal parasite influence zooplankton communities and improve trophic transfer. This study improves our understanding of the functional roles of fungi in estuarine systems and supports the importance of the mycoloop hypothesis in the lower food web of Chesapeake Bay.

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