12-10-2022

# Next-generation sequencing shows increasing temperatures affect stream fungal communities

Regan Hodgson
*Coastal Carolina University*

**Next-generation sequencing shows increasing temperatures affect stream fungal communities**

By

Regan Hodgson

---

Submitted in Partial Fulfillment of the

Requirements for the Degree of Master of Science in

Coastal Marine and Wetland Studies in the

School of the Coastal Environment

Coastal Carolina University

2022

| | |
|---|---|
| Dr. Vladislav Gulis<br>Major Advisor | Dr. Chad L. Leverette<br>Dean, Gupta College of Science |
| Dr. John J. Hutchens<br>Committee Member | Dr. Erin Hackett<br>CMWS Graduate Programs Director |
| Dr. Megan E. Cevasco<br>Committee Member | |

**Abstract**

Under climate change predictions, surface air temperature is expected to continue to rise and will likely affect functioning of stream ecosystems and microbial communities. Aquatic hyphomycetes are key fungal decomposers of plant litter in headwater streams controlling carbon, energy, and nutrient flows to higher trophic levels. This project addressed the effects of temperature on stream fungal communities in two recently conducted experiments (microcosm and streamside channel studies) using Illumina sequencing. The objectives were to determine (1) if temperature affects community structure of stream fungi based on relative abundances of ITS2 rDNA sequences, (2) if the effects of temperature on aquatic fungal communities vary for substrates of different carbon quality, and (3) if next-generation sequencing provides similar insights to those obtained earlier based on microscopic approach (spore counts). The microcosm experiment with natural stream microbial assemblages, five temperature treatments (4, 8, 12, 16, 20˚C) and four sampling dates showed that temperature had a significant effect on fungal community structure as some species increased or decreased their relative abundances. The streamside channel experiment (ambient, +2˚C and +4˚C treatments) with wood and two types of leaf litter and two sampling dates showed no significant effect of temperature on fungal community structure regardless of substrate type, however, litter type did control community structure. Thus, larger shifts in temperature (microcosms) did affect fungal communities while smaller increases (streamside channels) did not. Illumina sequencing showed much higher fungal diversity (e.g., 330 species in channels) than previously recorded by spore counts, however, aquatic hyphomycetes dominated fungal communities regardless of the approach. Temperature

effects may have important implications for stream fungal communities and ecosystem functioning under climate change scenarios.

## TABLE OF CONTENTS

## List of Tables

individual species) associated with plant litter in streamside channels. Note that the list does not include all species of aquatic hyphomycetes detected and does not include representatives from any other groups of fungi. Column labels designate temperature treatment (0 = ambient, 2 = +2°C, 4 = +4°C), substrate type (M = maple leaf litter, R = rhododendron leaf litter, V = wood veneers) and sampling day (21 or 63).

## List of Figures

incubation temperature T (4, 8, 12, 16 or 20 °C) and sampling day D (6, 12, 18, 24), initial fungal community after 4-day stream colonization is also shown.

Figure 6: Ordination of streamside channel samples from different substrates, temperature treatments and sampling dates by Principal Coordinate Analysis (PCoA) based on fungal community structure (relative abundances of fungal species were used to calculate Bray-Curtis dissimilarity indices). Temperature treatments are color coded. Shapes represent sampling day 21 (circles) and day 63 (squares).

Figure 7: Mean fungal species richness from streamside channel experiment across all temperature treatments. Bars represent means +1 SE.

Figure 8: Relative abundances of dominant fungal species in streamside channel samples from different substrates, temperature treatments and sampling dates. Sample codes (e.g., 0M21) are as follows: 0=ambient temperature, 2=+2°C treatment, 4=+4°C treatment; M=maple leaf litter, R=rhododendron leaf litter, V=wood veneers; 21 or 63 denote sampling day.

## Introduction

Although climate change is now a well-studied field (IPCC 2022), the effects of increasing temperatures on aquatic microbial communities and functioning of stream ecosystems remain poorly understood. Climate change predictions include increases in extreme high temperatures, decreases in extreme low temperatures, increases in drought periods, and intense rainfall (Easterling et al. 2000; Jentsch et al. 2007). Understanding the effects of these conditions on microbial communities and their activity may help predict the future responses of stream ecosystems to climate change.

Aquatic hyphomycetes (also referred to as Ingoldian fungi or freshwater hyphomycetes) are microorganisms that are characterized by their ability to sporulate underwater and to thrive on submerged decaying organic matter in freshwater systems (Krauss et al. 2011; Barros & Sahadevan 2022). These fungi release abundant amounts of asexual spores (conidia) that are dispersed until attachment to a substratum *via* mucilage secretion. Traditionally, aquatic hyphomycetes are identified based on the morphology of their tetraradial and sigmoidal conidia. In a recent review, Duarte et al. (2016) noted 335 different aquatic fungal morphospecies described between 1942 and 2014. Biogeographically, aquatic hyphomycetes are found around the world with most studies biased towards temperate regions (Duarte et al. 2016).

Because of their size, streams are strongly influenced by riparian vegetation, nutrient flow, air temperature changes, seasons, and other biotic and abiotic factors. Riparian vegetation has the largest impact because it determines the input of plant litter (i.e., carbon and energy) and the shade-coverage (light and temperature) (Allan & Castillo 2007). The detritus-based food webs found in forested streams receive much of their carbon and energy from the input of allochthonous organic matter from the riparian vegetation. Aquatic hyphomycetes are important colonizers of the submerged plant litter and drive the microbial decomposition that transfers nutrients to higher trophic levels, such as stream macroinvertebrates (Gessner et al. 2007; Kuehn 2016). Aquatic fungi play an important role in energy flow within streams as they dominate microbial biomass on plant litter (leaves and likely wood as well) (Gulis et al. 2019), often accounting for 95-99.9% of total microbial biomass (Gessner et al. 2007; Krauss et al. 2011). Fungi are the dominant decomposers in streams, likely due to their ability to remain active at low temperatures that may be unsuitable for bacteria (Godfrey 1983). Gulis et al. (2008) found that fungal biomass, growth rate, and production are generally lower on wood than on leaves in the streams used for this study (Coweeta Hydrologic Laboratory, NC), although fungi remain important wood processors.

Stream fungal communities and their activity are largely controlled by the nature of the substrate (leaf litter type, wood), stream water chemistry, and temperature (Gulis et al. 2019). In reference to fungal activity, several studies suggest that an increase in stream water temperature may stimulate fungal metabolic activity and decomposition (Suberkropp 1984; Sridhar & Bärlocher 1993) as well as increase growth and sporulation rates up to their thermal optimum (Fernandes et al. 2009; Geraldes et al. 2012). Optimal

temperatures for growth of freshwater fungi are between 15-25˚C (Suberkropp 1998; Yuen et al. 1998; Ferreira et al. 2014). Some aquatic hyphomycetes have greater growth and sporulation rates at lower temperatures, whereas other species might experience this at higher temperatures (Ferreira et al. 2014). For example, in studies where the seasonal temperatures of a stream varied (5-20˚C), different fungal species assumed dominance depending on the season, likely due to changes in temperature or substrate availability (Suberkropp 1984). Duarte et al. (2016) found that structural similarities among aquatic fungal communities typically decreased with geographic/latitudinal distance unless they were in comparable climatic zones, in which case, similarities occurred between communities even though they were geographically distant.

In a process described as conditioning, aquatic fungi break down complex plant polymers (e.g., cellulose) and convert some leaf litter and wood carbon into their own biomass (Suberkropp & Klug 1980). Through this, fungi improve the palatability and nutritional quality of plant litter by increasing proteins, lipids, and carbohydrates, transforming complex polymers into labile molecules, and increasing the nitrogen and phosphorous concentrations (Suberkropp & Klug 1980; Mas-Martí et al. 2015; Bärlocher 2016; Arias-Real et al. 2018). Experimental evidence indicates that detritus-feeding macroinvertebrates known as shredders are more likely to feed on leaves colonized by fungi that influence shredder growth rates (Suberkropp 1992; Graça 1993) and fecundity (Graça et al. 1993). Because there is a trophic link between shredders and microbial decomposers, temperature induced shifts in fungal dominance patterns may also alter shredder feeding patterns. Shredders not only show a preference for fungi-colonized substrates (Suberkropp 1992), but they can also discriminate between different fungal

species colonizing leaf litter (Cornut et al. 2015) and preferentially feed on more palatable fungi (Bärlocher & Kendrick 1973; Bärlocher 1985; Arsuffi & Suberkropp 1986).

Identification of aquatic hyphomycetes to the species level historically depended on conidial morphology and, therefore, much of what is known today about their diversity and abundance is based on studies that relied on inducing sporulation followed by microscopy to count and identify spores. However, the two common morphologies (tetraradial and sigmoidal) are thought to have evolved convergently (Duarte et al. 2016) resulting in similarities among potentially different species. In addition, sporulation rates and the amount of carbon channeled into reproduction varies among species so inferences about fungal community structure based on released spores may not necessarily reflect relative abundances of mycelial biomass of different species within plant litter (Seena et al. 2010). Hence, studies relying on spore counts can be problematic due to possible errors caused by identification based on morphologies, favoring prolific spore producers and overlooking cryptic species (Duarte et al. 2016).

With recent advances in next-generation sequencing technology, fungal communities are becoming better understood. Metabarcoding, the parallel identification of organisms from different taxa found in the same environment *via* DNA marker genes, was introduced about a decade ago (Pompanon et al. 2011; Riaz et al. 2011) and based on identification of short but taxonomically informative DNA sequences (Taberlet et al. 2018). The ITS region of rDNA is commonly used to identify fungal taxa, often to species level, and was selected as the universal genetic barcode for fungi (Schoch et al. 2012). The Illumina sequencing platform can provide millions of reads of the amplified

DNA sequences for downstream applications. Together with the publicly available UNITE database (Nilsson et al. 2018) that combines reference fungal ITS sequences with taxonomic information, next-generation sequencing allows researchers to map environmental sequences to fungal species (or sometimes only higher-level taxa) and to gain insight into the diversity and community structure of fungi from environmental samples. Over the last few years, Illumina sequencing has been used to study fungal communities in terrestrial (e.g., Panneerselvam et al. 2020), deep-sea (Zhang et al. 2016; Yang et al. 2020) and some freshwater (de Souza et al. 2022), including stream (Seena et al. 2019), environments. Illumina metabarcoding of complex fungal communities emerges as a feasible and informative approach and likely gives an accurate depiction of community structure (Schmidt et al. 2013; Siddique & Unterseher 2016; de Souza et al. 2022). Next-generation sequencing promises to improve our ability to compare fungal communities from different environments providing unparalleled details including detection of rare species that may otherwise be overlooked.

This study focused on fungi from headwater streams at the Coweeta Hydrologic Laboratory in North Carolina. Litter-associated fungi from these streams have been extensively studied with respect to the effects of nutrient enrichment (Gulis & Suberkropp 2004, Gulis et al. 2008), carbon flow (Gulis & Suberkropp 2003a), and interactions with bacteria (Gulis & Suberkropp 2003b). For stream fungi, increasing temperatures could mean changes in community structure and dominance patterns that may lead to changes in fungal activity, plant litter decomposition, and ecosystem functioning either directly or *via* cascading effects involving modified shredder feeding. The objectives of this study were to determine (1) if temperature affects community

structure of stream fungi based on relative abundances of ITS2 rDNA sequences, (2) if the effects of temperature on aquatic fungal communities vary for substrates of different carbon quality (species of leaf litter vs. wood) (streamside channel experiment), and (3) if next-generation sequencing provides similar insights to those obtained earlier based on microscopic approach (spore counts).

## Materials and methods

*Microcosm Experiment*

Hunter Pates, Coastal Carolina University CMWS graduate (2019), carried out an experiment in microcosms simulating stream conditions. *Liriodendron tulipifera* (tulip poplar) leaf disks were placed in litter bags (in groups of 45) and colonized by a natural fungal assemblage for five days in a headwater stream (watershed 5a) at the Coweeta Hydrologic Laboratory, NC in November 2017 (Pates 2019). Leaf disks were then incubated at five different temperatures (4, 8, 12, 16 and 20˚C) in laboratory microcosms for 24 days (Figure 1). From this study, replicate samples (3) were preserved (frozen at -20°C) for DNA extraction from all five incubation temperatures and all sampling days (6, 12, 18, and 24) and from leaf disks colonized in a stream (i.e. initial sample), totaling 63 samples. His study also counted and identified aquatic hyphomycete conidia.

*Streamside Channel Experiment*

Kaity Ackerman, Coastal Carolina University CMWS graduate (2020), used twenty artificial streamside channels set up at the Coweeta Hydrologic Laboratory to simulate four different temperature increases (+1, +2, +3 and +4˚C) (Ackerman 2020) (Figure 2). From this study, replicate samples (3) from three substrates (*Rhododendron maximum* and *Acer rubrum* leaf litter, and wood veneers) at three temperature treatments (ambient, +2˚C and +4˚C) and two sampling days (21, 63 days) were preserved for DNA

extraction, totaling 54 samples. Aquatic hyphomycete conidia were counted and identified from this study by Beasley-Polko (2021).

*Fungal DNA Extraction*

DNA from all plant litter samples was extracted using Qiagen DNeasy PowerSoil kit generally following manufacturer's protocol. However, DNA extraction was improved by adding 2 stainless steel 3.4-mm beads to each 2-ml microcentrifuge screw-cap extraction tube that resulted in more efficient homogenization of tough plant material during the bead-beating step of DNA extraction. Samples were also heated to 60 °C just before the extraction to aid in lysing fungal cells.

*Primers and PCR amplification*

The primers chosen for this study targeted the ITS2 region of fungal rDNA and thus allowed the use of the UNITE database that covers the entire ITS region (ITS1 + ITS2). The primers designed by V. Gulis (unpublished) are fungi-specific and were successfully used in an earlier unrelated project. These primers are based on fungal ITS3 and ITS4 primer pair (White et al. 1990), however, they also included Illumina-specific adaptors, linkers and pads as described by Caporaso et al. (2011, 2012) for 16S rDNA primers used in bacteria. In addition, ITS4-based reverse primers were barcoded using Golay error-correcting 12-bp barcodes (Caporaso et al. 2012) to allow for multiplexing (Table 1). Each primer pair (i.e. a single forward ITS3-based primer and each of the 39 reverse ITS4-based barcoded primers used in this study) were checked using Primer Prospector software (Walters et al. 2011). Each set of three leaf litter or wood replicates was assigned a unique reverse ITS4-based barcoded primer. Fungal DNA extracted from environmental samples was amplified by the Polymerase Chain Reaction (PCR) using

GoTaq G2 Hot Start Colorless Master Mix (Promega) and MJ Mini thermocycler (Bio-Rad). The amplification program included initial denaturation at 95˚C for 2 min followed by 29 cycles of 95˚C for 30 s, 55˚C for 40 s, 72˚C for 1 min, and a final extension at 72˚C for 5 min. PCR products (~450 bp) stained with SYBR Gold were checked by electrophoresis on a 1% agarose gels using Bio-Rad Gel Doc XR+ gel documentation system.

*Library Assembly and Purification*

Samples were amplified using 39 unique barcoded primers, allowing them to be pooled into a single library. For the library (39 barcodes × 3 replicates or 117 amplified DNA samples), DNA concentrations in all PCR products were quantified using QuantiFluor ONE dsDNA System using QuantiFluor-P fluorometer (Promega). To assemble DNA library, the lowest concentration (ng/μL) determined the amount of DNA to add for each PCR product (90 ng). The volumes for each PCR product added to the library were calculated and the library assembled. Two subsamples of 50 μL each were purified using E.Z.N.A. Cycle Pure Kit (Omega Bio-Tek) and eluted in 50 μL of 10mM Tris buffer. DNA concentration in the purified library sent for sequencing was ~21 ng/μL as determined fluorometrically.

*Illumina Sequencing*

Next-generation sequencing was performed using Illumina MiSeq platform with MiSeq v2 Nano 250bp PE kit. Paired-end sequencing was performed by Duke Center for Genomic and Computational Biology at Duke University that included quality control of the library, dilution and adding PhiX DNA according to the normal protocol for environmental metagenomics on Illumina platform. The resulting raw fastq files from

Illumina sequencing (read 1, read 2 and index read) were imported into QIIME 2 (Quantitative Insights into Microbial Ecology) software (Caporaso et al. 2010) for bioinformatics analyses.

*Data Analyses*

All sequence bioinformatics analyses were performed using QIIME 2 version 2022.2 (Bolyen et al. 2019, https://qiime2.org/) since it includes multiple statistical plugins based on R (e.g., vegan package for R, Oksanen et al. 2018). Paired-end sequences were assembled, denoised, and filtered using the qiime2-demux and q2-dada2 plugins (Callahan et al. 2016). Chimeras were removed by trimming primers and adjusting the parameter (set to 2) for minimum abundance of potential parents being tested as chimeric. Feature tables were created by trimming all features with a frequency lower than 10, retaining the remaining features for statistical analyses. Taxonomic assignments were determined for amplicon sequence variants (ASVs; ≈ operational taxonomic unit, OTU; ≈ species) by using the qiime2-feature-classifer (Bokulich et al. 2018) classify-sklearn against the customized UNITE ITS rDNA database 8.2 (Nilsson et al. 2018, https://unite.ut.ee). The database was modified by V. Gulis to remove or edit ~70 sequences of aquatic fungi and to include 186 additional reference sequences of aquatic hyphomycetes that were either omitted from the UNITE database or are unpublished. Sequences of 53 species from GenBank and 99 species of aquatic fungi from VG culture collection were added, including dozens of sequences from fungal species isolated from Coweeta streams where this study was performed. The new classifier was then used to identify taxa found in our samples.

ASVs were aligned with mafft (Katoh et al. 2002) and constructed a phylogeny tree with fasttree2 using the q2-phylogeny plugin (Price et al. 2010). Using q2-diversity, samples were rarefied to 6490 (microcosms) and 4294 (streamside channels) sequences per sample to generate alpha-diversity metrics and beta diversity metrics (Bray-Curtis dissimilarity).

Data were visualized using interactive taxa barplots to explore relative abundances at different taxonomic levels within and among samples. We used Bray-Curtis similarity matrices for ordinations and parametric analyses. The Principal Coordinate Analysis (PCoA) was used to visualize similarities among samples through their plotted ordinations (Legendre & Legendre 2012). Because several variables in addition to temperature needed to be considered, a Permutational Multivariate Analysis of Variance (PERMANOVA) was used (Adonis package, Anderson 2001; Oksanen et al. 2018). For microcosm experiment, we used (temperature+day) model, while for the more complex design of the streamside channel experiment, we used (substrate+temperature+day+temperature*substrate) model.

We also compared our DNA-based data on fungal community structure with previously obtained data from the same sets of samples based on microscopic approach (spore counts). Shannon-Wiener diversity index (H) and evenness (E) were calculated based on the relative abundances of individual taxa for the microcosm fungal community (Magurran 1988).

## Results

### *Illumina Sequencing Output*

For the microcosm experiment, a total of 230,023 fungal sequences distributed among 76 species were retained after filtering. Two species of aquatic hyphomycetes, *Articulospora tetracladia* and *Anguillospora filiformis*, appeared in all samples and dominated fungal communities with 66,044 and 51,248 sequences, respectively. From the streamside channels, 135,484 fungal sequences representing 330 species were retained after filtering. *A. tetracladia* and *A. filiformis* were again the two species with the highest number of sequences (28,317 and 12,354, respectively) and appeared in all samples. Fungal communities from the streamside channel experiment were considerably more diverse than those from the microcosms.

### *Microcosm Experiment*

Temperature had a significant effect on fungal community structure in microcosms (PERMANOVA, $F_{4,19} = 4.21$, $p = 0.001$) while sampling day did not ($F_{4,19} = 1.96$, $p = 0.057$). The three samples with the highest temperature and longest incubation periods (T20D12, T20D18, T20D24) were the most dissimilar from other samples as apparent from PCoA ordinations based on Bray-Curtis dissimilarity matrix (Figure 3). In general, samples were arranged along axis 1 from the lowest temperature treatments on the left to the highest temperature treatments on the right. The highest number of species was observed in the initial sample, i.e. in a sample corresponding to day 0 of microcosm

experiment, after four days of in-stream colonization by natural fungal assemblage, while average numbers of species detected at different temperatures in the microcosm experiment were generally lower (Figure 4).

Relative abundances of fungal taxa at the species level across experimental treatments are illustrated in Figure 5. Interestingly, in most samples, >95% of fungal species belonged to the order Helotiales (Leotiomycetes, Ascomycota). Among the dominant taxa, 8 out of 10 species were aquatic hyphomycetes. Several dominant species of aquatic hyphomycetes showed altered relative abundances with temperature. For example, contribution of *A. tetracladia* increased the most at the highest temperature (20˚C), in some cases exceeding 50% of sequences (T20D12 sample, Figure 5). On the other hand, the relative abundance of *Fontanospora alternibrachiata* decreased with increasing temperatures, especially at later stages of decomposition.

Spore counts from all microcosm samples detected 11 species of aquatic hyphomycetes (Table 2, data from Pates 2019). Eight of those species were also detected by Illumina sequencing. DNA sequences from these eight aquatic hyphomycetes accounted for 79.1% to 86.7% of all fungal sequences in our study (Table 3). Both tables feature *A. filiformis, A. tetracladia,* and *F. alternibrachaiata* as top dominant species. At the same time, *Alatospora acuminata* was a dominant species based on microscopy whereas it showed a very low relative abundances from sequencing dataset (often <0.1%). Other fungal species (including additional species of aquatic hyphomycetes) were detected using molecular approach (not listed in Table 3) with cumulative numbers of fungal species ranging from 28 to 43 depending on temperature treatment. However,

these additional species were rare and represented by relatively few sequences compared to dominant aquatic hyphomycetes detected by both approaches.

*Streamside Channel Experiment*

Temperature did not have a significant effect on fungal community structure in the streamside channels (PERMANOVA, $F_{2,17} = 0.621$, $p = 0.801$). The effects of substrate type and sampling day on fungal communities were both significant ($F_{2,17} = 9.05$, $p = 0.001$ and $F_{1,17} = 2.86$, $p = 0.038$) while the interaction term (temperature*substrate) had no significant effect ($F_{4,17} = 0.514$, $p = 0.961$). Wood and leaf litter samples were clearly separated on a PCoA ordination plot (Figure 6). Maple and rhododendron leaves were generally distinct early in the experiment (day 21) but fungal communities on leaf litter converged by day 63. A total of 330 fungal species were detected from all streamside channel samples. The average number of observed species was comparable among samples regardless if they were grouped by temperature (Figure 7) or substrate (not shown).

Relative abundances of fungal taxa at the species level across experimental treatments are shown on Figure 8. As with microcosms, the top four species in the list are aquatic hyphomycetes, however, the patterns are more complex. *A. tetracladia* was the most abundant species on leaf litter, especially later in the experiment (day 63). *Phyllosticta philoprina*, a terrestrial leaf-associated species, only appeared on leaf litter samples with high abundance on day 21 and was later replaced by species of aquatic hyphomycetes such as *A. tetracladia, A. filiformis, T. elegans* and others. Two species, namely *Casaresia sphagnorum* and *Psychrophila olivacea* dominated fungal communities on wood.

Spore counts detected at least 14 species of aquatic hyphomycetes across all streamside channel samples (wood veneers from day 21 were not included in this analysis) (Table 4). Nine of those species were also detected by Illumina sequencing. DNA sequences from these species of aquatic hyphomycetes accounted for 47.6% to 75.7% of all fungal sequences on maple leaf litter, 11.6% to 82.6% on rhododendron, and 29.1% to 51.1% on wood samples (Table 5). Both tables show *A. filiformis* and *Tetracahetum elegans* as top dominant species. On the other hand, *Flagellospra curvula,* a dominant species based on microscopy, showed low relative abundance based on DNA. Many additional fungal species (including more species of aquatic hyphomycetes) were detected using molecular approach (not listed in Table 5) with a cumulative number of fungal species ranging from 40 to 108 depending on treatment. The two most abundant species of aquatic fungi on wood based on DNA sequences (*C. sphagnorum* and *P. olivacea,* Figure 8) were not detected by spore counts, because the latter produces no spores at all while *C. sphagnorum* produces by far the largest but relatively few spores.

## Discussion

Since 1860, Earth's surface air temperature has increased by about 1˚C (IPCC 2022). Climate change can alter stream ecosystems *via* higher temperatures (direct effects on metabolic rates, changes in community structure, decreases in oxygen availability), longer drought periods (alters flow), and increased storms (introduces unwanted runoff). Increases in temperatures are predicted to be greater at higher latitudes and elevations (Canhoto et al. 2016), such as the mountain streams at Coweeta Hydrologic Laboratory used in this study. As aquatic hyphomycetes are important decomposers of plant litter in streams, changes in temperature and carbon cycling may have important consequences in these systems.

Results from both the microcosm and the streamside channel experiments revealed driving forces controlling the structure of stream fungal communities, with temperature having a significant effect in microcosms and substrate and time shaping fungal communities in the streamside channels. Overall, it appeared that large shifts in temperature (such as in microcosms) did affect aquatic fungi while smaller increases (up to 4˚C in channels) did not. With a controlled experiment in the microcosms, we saw large increases in relative abundance for some dominant species (e.g., *Articulospora tetracladia,* 27.3 to 42.0%) and large decreases for other dominant species (e.g., *Fontanospora alternibrachiata,* 31.6 to 9.1%) as temperature increased from 4˚C to 20˚C. Species richness stayed the same across temperature treatments in both

experiments. Despite the inherent variability of a field experiment, the streamside channels produced results that showed distinct communities on different substrates changing over time, suggesting ecological succession. Streamside channels tended to have a much higher species richness across samples (330 compared to 76 in microcosms). However, both experiments detected species not previously observed by spore counts, demonstrating the capability of detecting rare and cryptic species with next-generation sequencing.

The lower taxa richness found in microcosms *vs.* streamside channels could be explained by several factors. First, leaf disks for microcosm experiment were incubated in the stream to be colonized by natural microbial assemblage for only four days in November, thus, the resulting fungal communities in the microcosms have all originated from taxa present in the stream during those 4 days, essentially a snapshot in time. On the other hand, plant litter in streamside channels was constantly bombarded by spores carried by stream water during the whole experiment (>two months). Second, only tulip poplar leaf litter was used in microcosms, while two types of leaf litter (maple and rhododendron) and wood were deployed in streamside channels. Third, while leaf disks for microcosms were sterilized, in streamside channels, we used autumn-shed, non-sterilized leaf litter initially carrying terrestrial leaf-associated fungi. Indeed, during early stages of decomposition (day 21) in channels, fungal diversity was high and terrestrial fungi were still prominent, especially on slowly decomposing rhododendron leaf litter, while they were later (day 63) largely replaced by aquatic hyphomycetes (Table 4, Figure 8). By day 63, fungal communities on maple and rhododendron leaf litter tended to converge and were dominated by aquatic hyphomycetes (Figure 6).

Colder temperatures, such as 4˚C, have been known to cause slower rates of sporulation and growth in aquatic hyphomycetes (Nikolcheva & Bärlocher 2005; Fernandes et al. 2009). On the other hand, increasing temperatures to around 16-18°C stimulated growth rate, respiration, and sporulation of aquatic fungi (Pates 2019). In general, different temperature optima for growth and sporulation of individual species may help explain shifts in community structure of aquatic fungi as temperature changes. This has been observed in previous studies where certain warm-water species appeared more frequently at higher temperatures (Ferreira & Chauvet 2011) or seasonally common species, such as *Tetracladium marchalianum* during summer, became more dominant when the mean temperature was increased (Dang et al. 2009). Fernandes et al. (2009) reported similar findings that different species were dominant according to their optimal growth and/or sporulation temperatures. They also found that freezing completely inhibited the sporulation of one previously dominant warm-water species (*Lunulospora curvula*). Duarte et al. (2013) found a highly significant effect of temperature on *Articulospora tetracladia,* which agrees with the increased relative abundance we saw in microcosm experiment at 20˚C (Figure 5). *A. tetracladia*, a species with an optimal temperature around 20˚C (Chauvet & Suberkropp 1998) is common or dominant in many temperate stream systems (Fernandes et al. 2009). Nevertheless, *A. tetracladia* can survive under cold conditions; Fernandes et al. (2009) reported that it was able to continue to grow after being frozen. Some aquatic hyphomycetes are known to have high temperature optima, found predominantly in summer months in temperate streams or are reported from tropical areas (Ferreira et al. 2014). Thus, increasing temperatures may produce shifts in fungal communities of temperate streams to become more similar to

those found in summer or in subtropical systems. But even in colder environments, the effect of temperature increases on fungal communities can be detectable. Dang et al. (2009) found that oscillating temperatures had no effect on community structure of aquatic fungi but that there was a consistent shift in the relative abundances of species when the mean temperature increased from 3˚C (ambient) to 8˚C (warming scenario).

Based on relative abundances of fungal species assessed from spore counts, Ferreira & Chauvet (2011) found that temperature was the leading factor (compared to litter quality) affecting community structure. Conversely, based on molecular data from our experiment in streamside channels, we found no effect of temperature on fungal community structure while the effect of substrate was highly significant. This discrepancy can be explained by more narrow temperature range in our study (just 4°C) and the inclusion of wood in addition to leaf litter types in our experiment. Wood fungal communities are known to be distinct from those on leaf litter (e.g., Gulis 2001). In our study, *Casaresia sphagnorum* and *Psychrophila olivacea* dominated fungal communities on wood, while they showed much lower abundances or were absent from leaf litter samples. Compared to leaves, wood typically has a slower decomposition rate as fungal biomass, growth rate, and production are known to be lower on wood in the Coweeta streams (Gulis et al. 2008). Wood veneers and rhododendron leaves have a higher lignin content and a higher carbon to nitrogen ratio, making their breakdown and decomposition slower as well as affecting associated fungal communities.

Next-generation sequencing proved to be an effective tool for analyzing aquatic fungal communities. In this study, Illumina sequencing found over six times the number of taxa in microcosms and 22 times the number of taxa in streamside channels compared

to data obtained earlier from the same experiments using microscopy (spore counts). This agrees with an earlier pioneering study that used 454 sequencing and found twice as many taxa as traditional spore counts (Duarte et al. 2015). However, counting spores seems to be a reliable method to get an insight into patterns of dominant species of litter-associated fungi. Aquatic hyphomycetes recorded from spores in our microcosms also accounted for at least 79% of all fungal sequences detected by Illumina, depending on temperature treatment (Table 2), and contributed on average 53% in streamside channels (Table 4). Duarte et al. (2015) also found congruency among approaches for diversity and abundance patterns. At the same time, in our study, at least three species of aquatic hyphomycetes identified from spores were not detected by sequencing, suggesting issues with either amplification or bioinformatics pipeline. While Illumina sequencing showed much higher fungal diversity (e.g., 330 species in channels) than recorded by spore counts, both methodological approaches described patterns of dominant species in a similar way.

Aquatic hyphomycetes dominated fungal communities in our experiments. Only dominant species (8-9) of aquatic hyphomycetes comprised 79-87% of all fungal sequences in microcosms and >62% on leaf litter after two months in streamside channels (these are underestimates as not all detected aquatic hyphomycetes are included in these values or listed in Tables 2 and 4). In comparison, a study of leaf-associated stream fungi from around the world by Seena et al. (2019) using Illumina sequencing found aquatic hyphomycetes to account for only 17% of sequences across samples from all countries (the highest being in samples from Europe, e.g., 48% from France). More accurate insights from our study are due to the use of an updated and amended UNITE

database for taxonomic identification. Seena et al. (2019) used a 2017 version of UNITE that simply did not include information about many, even common, species of aquatic hyphomycetes, while our in-house amended database contained sequence data for >100 additional species (see Methods). Seena et al. (2019) likely also experienced technical issues such as deterioration of samples while they were shipped across the world. Hayer et al. (2021) using Illumina sequencing did not find aquatic hyphomycetes dominating fungal community in microcosm experiment due to gross technical errors with the experiment setup that prevented establishing of any meaningful aquatic fungal community, thus, it does not warrant further discussion.

In conclusion, it appeared that relatively small temperature shifts (up to +4°C as achieved in streamside channels) had little effect on stream fungal communities regardless of the substrate type, while large temperature changes (as seen in the microcosm experiment) resulted in significant changes in fungal community structure. Next-generation sequencing paired with an updated UNITE database provided similar but more detailed insight into fungal community structure compared to that from a traditional microscopic approach. However, our relatively short-term experiments (1-2 months) did not provide the insight into longer-term effects of chronic temperature increases since they did not encompass seasonal temperature fluctuations and known seasonal changes in fungal communities (driven not only by temperature but also seasonal substrate availability). Long-term multidisciplinary studies in streams are critical as we may learn about temperature effects on natural fungal communities and their activities, as well as about cascading effects on shredding invertebrates and carbon flow. These temperature

effects may have important implications for stream fungal communities and ecosystem functioning under climate change scenarios.

Table 1. Primers used in this study to amplify fungal ITS2 rDNA region (including 2 out of 39 examples of barcoded primers) as well as custom primers for Illumina sequencing.

| **Primer name** | **12-bp Golay barcode** | **Complete primer sequence (5' to 3')** | **Notes** |
|---|---|---|---|
| ITS3F | | AATGATACGGCGACCACCG AGATCTACACTATGGTAAT TAAGCATCGATGAAGAACG CAGC | forward primer used for all samples, not barcoded |
| ITS4Rbc1 | ACGAGACTGATT | CAAGCAGAAGACGGCATAC GAGATACGAGACTGATTAG TCAGTCAGAATCCTCCGCT TATTGATATGC | reverse, barcoded primer 1 |
| ITS4Rbc2 | GCTGTACGGATT | CAAGCAGAAGACGGCATAC GAGATGCTGTACGGATTAG TCAGTCAGAATCCTCCGCT TATTGATATGC | reverse, barcoded primer 2 |
| … | … | … | … |
| Gulis_R1 | | TATGGTAATTAAGCATCGA TGAAGAACGCAGC | Illumina Read1 custom primer |
| Gulis_R2 | | AGTCAGTCAGAATCCTCCG CTTATTGATATGC | Illumina Read2 custom primer |
| Gulis_Index | | GCATATCAATAAGCGGAGG ATTCTGACTGACT | Illumina Index custom primer |

Table 2. Mean relative abundances (%) of aquatic hyphomycetes based on microscopy (spore counts) associated with leaf litter incubated at different temperatures throughout the experiment in laboratory microcosms (days 0-28). Data from Pates (2019).

| **Species** | **Temperature** | | | | |
|---|---|---|---|---|---|
| | **4°C** | **8°C** | **12°C** | **16°C** | **20°C** |
| *Articulospora tetracladia* | 59.3 | 35.8 | 21.8 | 23.0 | 32.6 |
| *Fontanospora alternibrachiata* | 18.3 | 18.0 | 21.9 | 13.7 | 11.0 |
| *Anguillospora filiformis* | 3.5 | 12.5 | 21.9 | 16.6 | 17.1 |
| *Alatospora acuminata* | 3.4 | 11.7 | 13.8 | 19.2 | 18.7 |
| *Flagellospora curvula* | 4.8 | 15.0 | 11.1 | 16.9 | 11.0 |
| *Flagellospora* sp. 1 | 3.3 | 1.9 | 6.3 | 5.5 | 7.7 |
| *Tetrachaetum elegans* | 4.7 | 2.5 | 1.4 | 2.4 | 1.1 |
| *Lemonniera pseudofloscula* | 2.5 | 2.5 | 1.1 | 2.5 | 0.8 |
| *Culicidospora aquatica* | 0.3 | 0.2 | <0.1 | 0.1 | - |
| *Lunulospora curvula* | - | - | 0.6 | <0.1 | <0.1 |
| *Tricladium chaetocladium* | <0.1 | <0.1 | <0.1 | - | <0.1 |
| **Total (%)** | **100** | **100** | **100** | **100** | **100** |
| Total no. of species | 10 | 10 | 11 | 10 | 10 |
| Diversity (H) | 1.36 | 1.74 | 1.83 | 1.88 | 1.75 |
| Evenness (E) | 0.62 | 0.79 | 0.83 | 0.85 | 0.84 |

Table 3. Mean relative abundances (%) of dominant species of aquatic hyphomycetes calculated from DNA sequencing data (i.e. based on the number of sequences of individual species) associated with leaf litter incubated at different temperatures throughout the experiment in laboratory microcosms (days 0-24). Note that the list does not include all species of aquatic hyphomycetes detected and does not include representatives from any other groups of fungi.

| **Species** | **Temperature** | | | | |
|---|---|---|---|---|---|
| | **4°C** | **8°C** | **12°C** | **16°C** | **20°C** |
| *Articulospora tetracladia* | 27.2 | 24.7 | 27.8 | 27.0 | 42.0 |
| *Anguillospora filiformis* | 13.8 | 17.1 | 21.8 | 33.6 | 25.0 |
| *Fontanospora alternibrachiata* | 31.6 | 28.7 | 21.9 | 10.5 | 9.12 |
| *Tetrachaetum elegans* | 8.1 | 7.4 | 11.0 | 5.4 | 6.4 |
| *Lemonniera pseudofloscula* | 3.6 | 3.5 | 3.9 | 2.5 | 1.6 |
| *Culicidospora aquatica* | 0.1 | 0.1 | - | - | 0.1 |
| *Flagellospora curvula* | 0.1 | 0.1 | 0.4 | 0.1 | - |
| *Alatospora acuminata* | - | - | - | - | 0.3 |
| *Flagellospora* sp. 1 | - | - | - | - | - |
| *Lunulospora curvula* | - | - | - | - | - |
| *Tricladium chaetocladium* | - | - | - | - | - |
| **Contribution of dominant aquatic hyphomycetes (%)** | **84.6** | **81.7** | **86.7** | **79.1** | **84.2** |
| Cumulative no. of fungal species | 35 | 32 | 28 | 40 | 43 |
| Diversity (H) | 1.86 | 1.95 | 0.61 | 0.64 | 0.77 |
| Evenness (E) | 0.52 | 0.56 | 0.18 | 0.17 | 0.20 |

* Cumulative number of fungal species, diversity and evenness estimates are based on all fungal species detected based on DNA (not shown in the list).

Table 4. Mean relative abundances (%) of aquatic hyphomycetes based on microscopy (spore counts) associated with plant litter in streamside channel experiment (data from Beasley-Polko 2021). Column labels designate temperature treatment (0 = ambient, 2 = +2℃, 4 = +4℃), substrate type (M = maple leaf litter, R = rhododendron leaf litter, V = wood veneers) and sampling day (21 or 63).

| **Species** | **0M21** | **2M21** | **4M21** | **0M63** | **2M63** | **4M63** | **0R21** | **2R21** | **4R21** | **0R63** | **2R63** | **4R63** | **0V63** | **2V63** | **4V63** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Anguillospora filiformis* | 30.4 | 12.0 | 30.2 | 38.0 | 35.3 | 37.0 | 1.6 | 8.1 | 4.6 | 43.0 | 27.2 | 24.5 | 42.5 | 36.8 | 19.4 |
| *Flagellospora curvula* | 25.8 | 20.5 | 13.5 | 3.1 | 5.3 | 4.7 | 16.2 | 6.8 | 12.7 | 8.7 | 2.9 | 2.2 | 1.3 | 9.80 | 14.6 |
| *Tetrachaetum elegans* | 14.9 | 19.5 | 24.8 | 0.8 | 2.7 | 4.9 | 0.9 | 5.6 | 3.1 | 5.4 | 19.6 | 8.7 | - | 16.1 | 6.3 |
| *Flagellospora* sp. 1 | 9.5 | 20.8 | 9.3 | 19.5 | 6.1 | 5.6 | - | 1.8 | 6.5 | 4.8 | 1.9 | 2.4 | - | 7.1 | 8.7 |
| *Tricladium chaetocladium* | 7.9 | 6.0 | 5.8 | 20.8 | 13.7 | 11.6 | 0.9 | 2.1 | 0.5 | 12.6 | 8.7 | 1.8 | 55.0 | 19.9 | 9.4 |
| *Alatospora acuminata* | 4.6 | 10.0 | 3.9 | 1.4 | 1.4 | 1.0 | - | - | 1.8 | 0.2 | 1.6 | - | - | - | - |
| *Articulospora tetracladia* | 2.8 | 4.2 | 0.9 | 12.1 | 26.1 | 19.9 | - | 0.4 | 0.8 | 1.9 | 7.0 | 13.5 | - | - | 16.7 |
| *Culicidospora aquatica* | 2.7 | 4.8 | 2.1 | 1.2 | 0.3 | - | - | 0.4 | 11.8 | 3.3 | 3.7 | 0.2 | - | 6.3 | - |
| *Heliscus lugdunensis* | 1.2 | 1.0 | 0.3 | <0.1 | 0.1 | 0.8 | 80.4 | 74.6 | 58.2 | 2.7 | 0.7 | 0.4 | 1.3 | - | - |
| *Lunulospora curvula* | - | 0.8 | 7.5 | 2.5 | 8.4 | 14.4 | - | - | - | 16.6 | 26.3 | 46.2 | - | 4.2 | 25.0 |
| *Lemoniera aquatica* | 0.2 | 0.6 | 1.8 | - | - | - | - | - | - | - | - | - | - | - | - |
| *Casaresia sphaguorum* | - | - | - | 0.1 | 0.1 | - | - | - | - | 1.0 | 0.4 | 0.2 | - | - | - |
| *Variocladium giganteum* | - | - | - | 0.3 | 0.1 | <0.1 | - | - | - | - | - | - | - | - | - |
| *Lemonniera pseudofloscula* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | <0.1 |
| Unidentified tetraradiate | <0.1 | - | - | - | 0.2 | - | - | 0.4 | 0.2 | - | - | - | - | - | - |
| **Total (%)** | **100** | **100** | **100** | **100** | **100** | **100** | **100** | **100** | **100** | **100** | **100** | **100** | **100** | **100** | **100** |
| Total no. of species | 11 | 11 | 11 | 12 | 13 | 10 | 5 | 9 | 10 | 11 | 11 | 10 | 4 | 7 | 7 |

Table 5. Relative abundances (%) of dominant species of aquatic hyphomycetes calculated from DNA sequencing data (i.e. based on the number of sequences of individual species) associated with plant litter in streamside channels. Note that the list does not include all species of aquatic hyphomycetes detected and does not include representatives from any other groups of fungi. Column labels designate temperature treatment (0 = ambient, 2 = +2°C, 4 = +4°C), substrate type (M = maple leaf litter, R = rhododendron leaf litter, V = wood veneers) and sampling day (21 or 63).

| **Species** | **0M21** | **2M21** | **4M21** | **0M63** | **2M63** | **4M63** | **0R21** | **2R21** | **4R21** | **0R63** | **2R63** | **4R63** | **0V63** | **2V63** | **4V63** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Articulospora tetracladia* | 26.1 | 23.2 | 23.5 | 29.1 | 41.5 | 32.5 | 7.8 | 12.5 | 18.2 | 35.5 | 50.0 | 31.1 | 1.1 | 11.9 | 1.2 |
| *Anguillospora filiformis* | 12.0 | 12.9 | 7.8 | 14.4 | 21.4 | 26.7 | 1.9 | 7.0 | 5.3 | 15.6 | 13.4 | 18.1 | 0.5 | 0.7 | 0.6 |
| *Tetrachaetum elegans* | 8.8 | 14.9 | 8.5 | 7.5 | 4.3 | 8.6 | 0.9 | 5.4 | 5.5 | 15.8 | 11.8 | 9.1 | 0.4 | 0.8 | 0.3 |
| *Lemonniera pseudofloscula* | 3.2 | 6.8 | 6.1 | 11.4 | 2.6 | 0.6 | 1.1 | 1.5 | 1.2 | 6.9 | 2.3 | 12.3 | - | 1.1 | 0.2 |
| *Flagellospora curvula* | 1.9 | - | 1.7 | - | - | - | - | - | - | - | - | 0.8 | - | - | 0.1 |
| *Alatospora acuminata* | - | - | - | - | - | 7.4 | - | - | - | - | - | - | - | - | - |
| *Casaresia sphagnorum* | - | - | - | - | 1.9 | - | - | - | - | 4.2 | - | - | 39.1 | 14.6 | 48.8 |
| *Culicidospora aquatica* | - | 3.3 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Flagellospora* sp.1 | - | - | - | - | - | - | - | - | - | 4.5 | 4.1 | - | - | - | - |
| *Heliscus lugdunensis* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Lemonniera aquatica* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Lunulospora curvula* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Tricladium chaetocladium* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Variocladium giganteum* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Contribution of dominant aquatic hyphomycetes (%)** | **52.0** | **61.2** | **47.6** | **62.3** | **71.6** | **75.7** | **11.6** | **26.3** | **30.1** | **82.6** | **81.7** | **71.3** | **41.0** | **29.1** | **51.1** |
| Cumulative no. of fungal species | 75 | 67 | 69 | 66 | 61 | 40 | 108 | 79 | 78 | 62 | 38 | 57 | 48 | 52 | 46 |

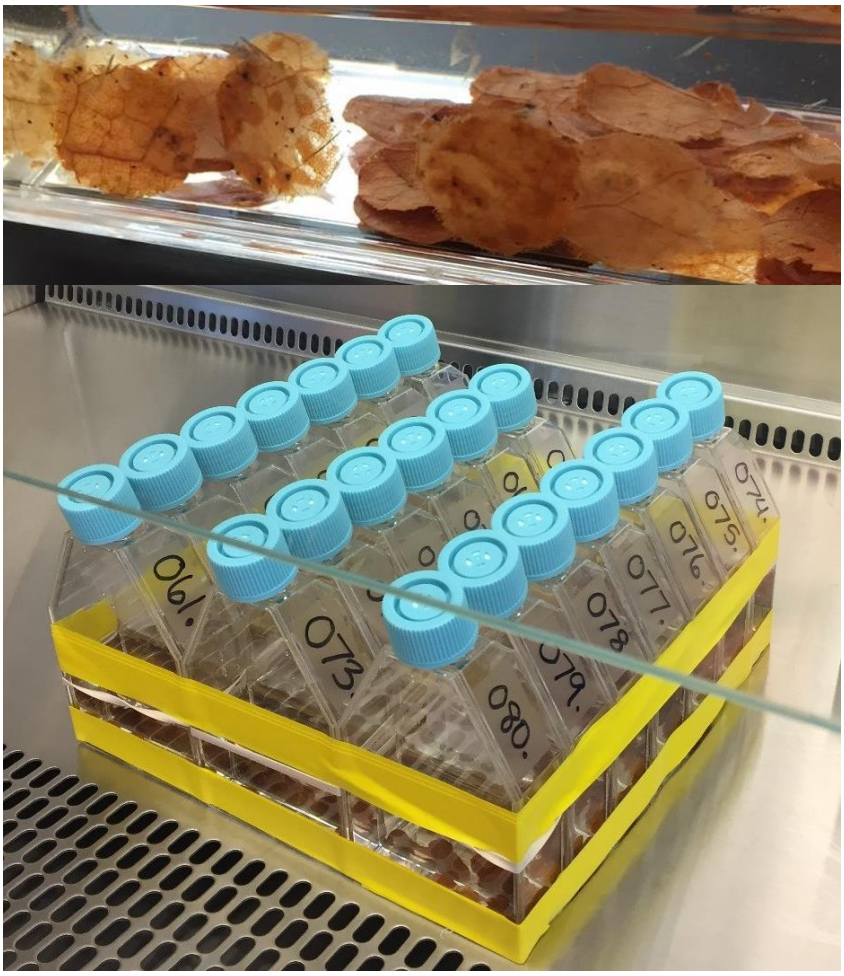


**Figure 1.** Laboratory microcosms (tissue culture flasks) containing naturally colonized tulip poplar leaf disks with nutrient solution that were incubated at 5 temperature treatments (4-20°C). Inset shows close-up of the bottom of the flask with decomposing leaf disks colonized by stream fungi.

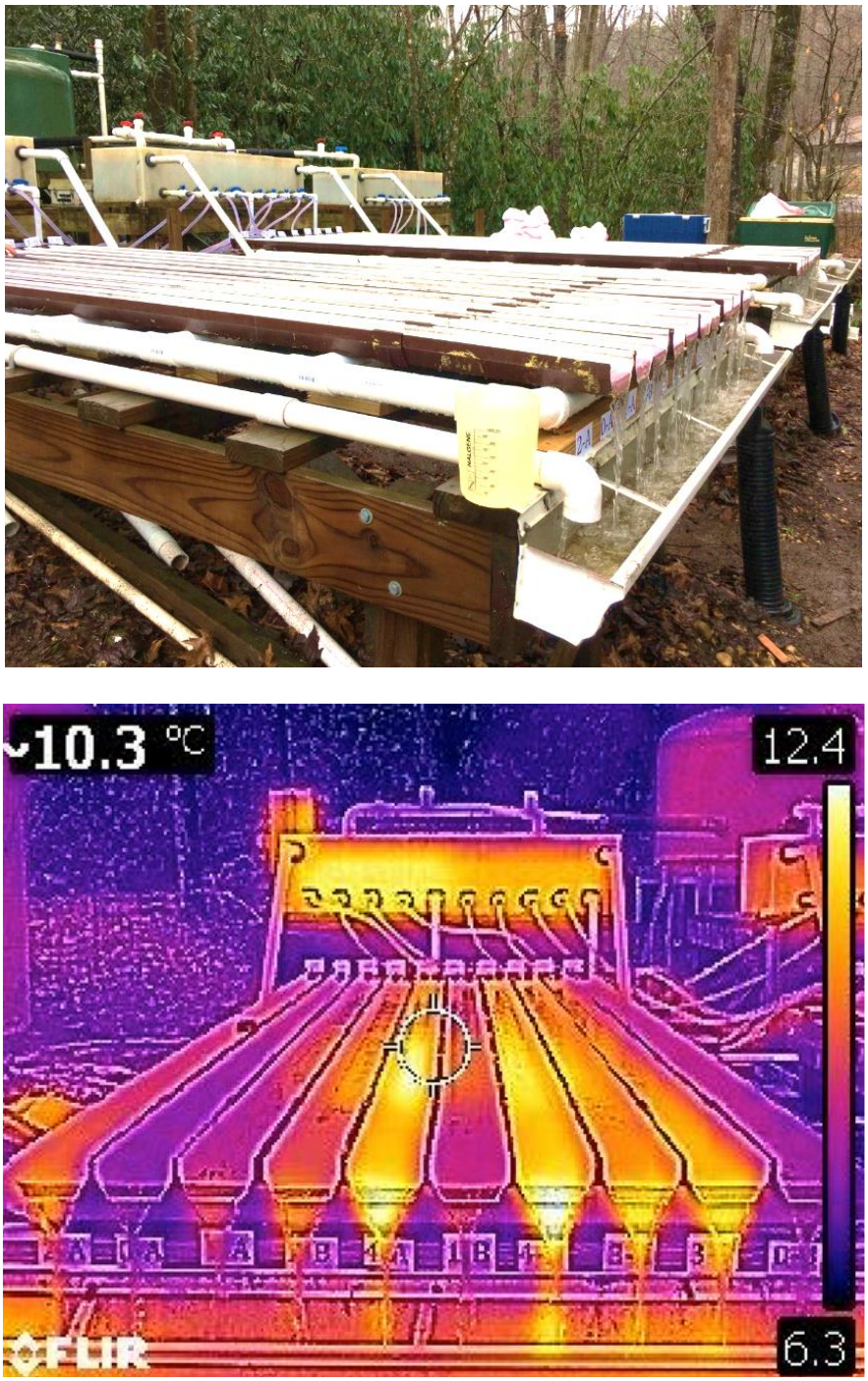


**Figure 2.** *Top panel*: Experimental set up with 20 streamside channels (5 temperature treatments × 4 replicates). *Bottom panel*: 10 of the streamside channels (two replicates of each temperature treatment: 0 (ambient control), +1, +2, +3, and +4°C) showing ±1°C differences in temperature (thermal imaging); photo credit: J. Benstead, University of Alabama.

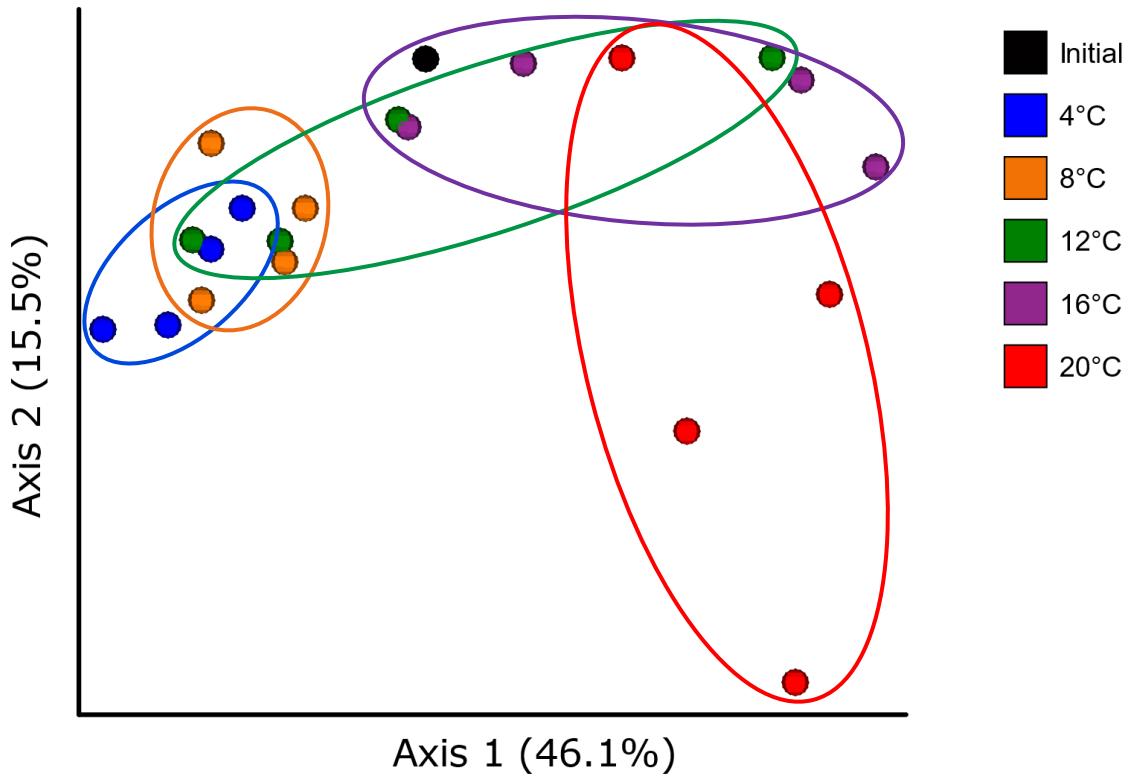


**Figure 3.** Ordination of microcosm samples from different temperature treatments and sampling dates by Principal Coordinate Analysis (PCoA) based on fungal community structure (relative abundances of fungal species were used to calculate Bray-Curtis dissimilarity indices). Temperature treatments are color coded. Initial fungal community after 4-day stream colonization is also shown.

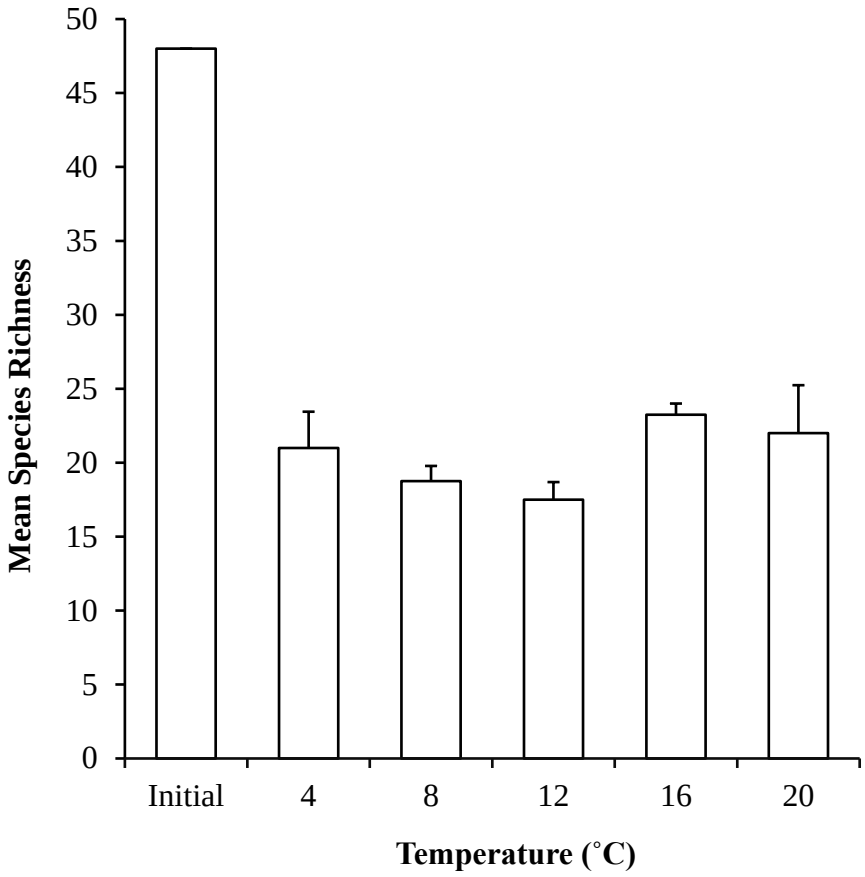


**Figure 4.** Mean fungal species richness from the microcosm experiment across temperature treatments. Bars represent means +1 SE.

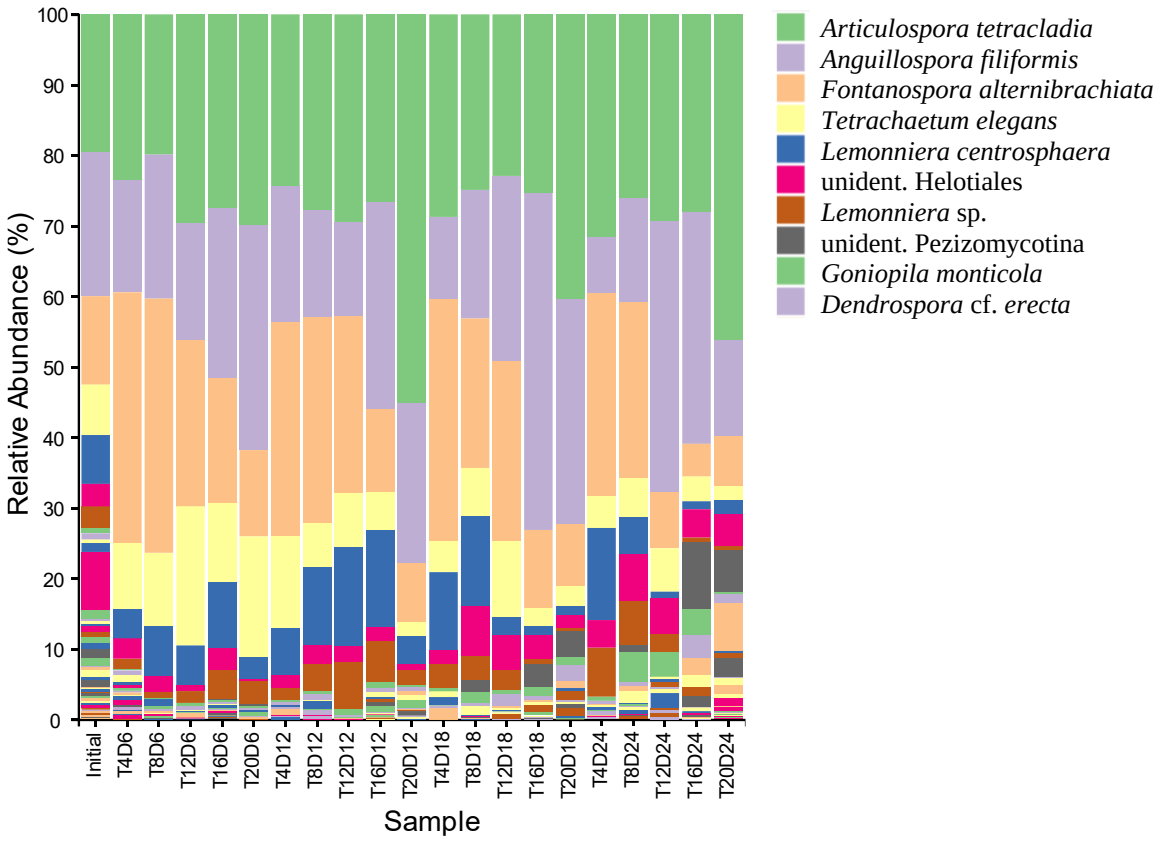


**Figure 5.** Relative abundances of dominant fungal species in microcosm samples from different temperature treatments and sampling dates. Sample codes (e.g., T4D6) denote incubation temperature T (4, 8, 12, 16 or 20 °C) and sampling day D (6, 12, 18, 24), initial fungal community after 4-day stream colonization is also shown.

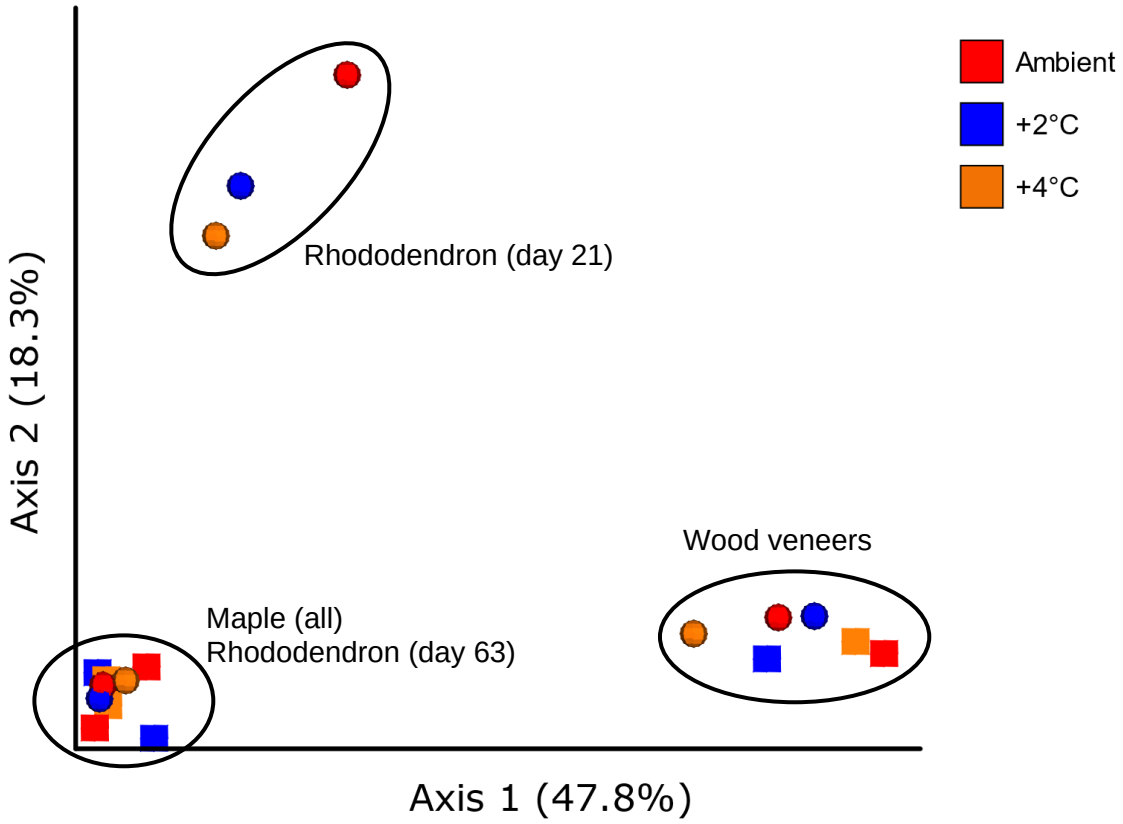


**Figure 6.** Ordination of streamside channel samples from different substrates, temperature treatments and sampling dates by Principal Coordinate Analysis (PCoA) based on fungal community structure (relative abundances of fungal species were used to calculate Bray-Curtis dissimilarity indices). Temperature treatments are color coded. Shapes represent sampling day 21 (circles) and day 63 (squares).

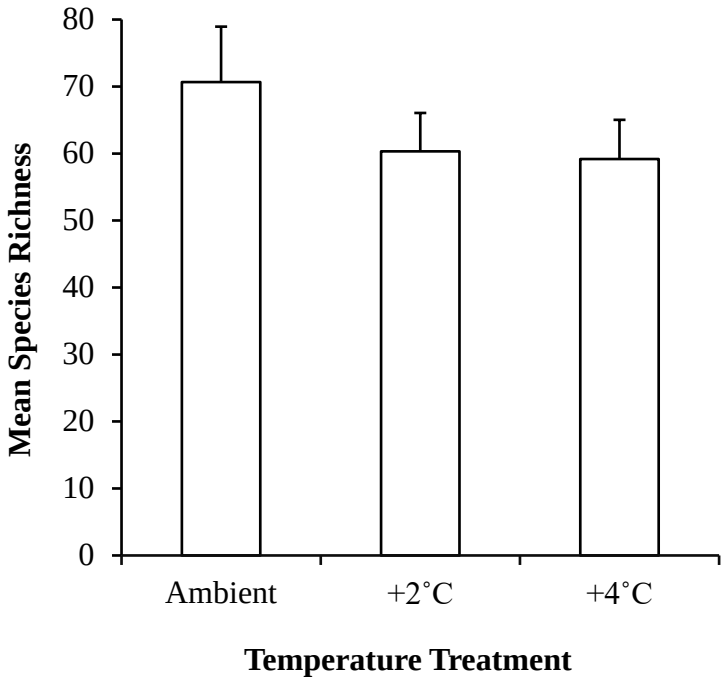


**Figure 7.** Mean fungal species richness from streamside channel experiment across all temperature treatments. Bars represent means +1 SE.

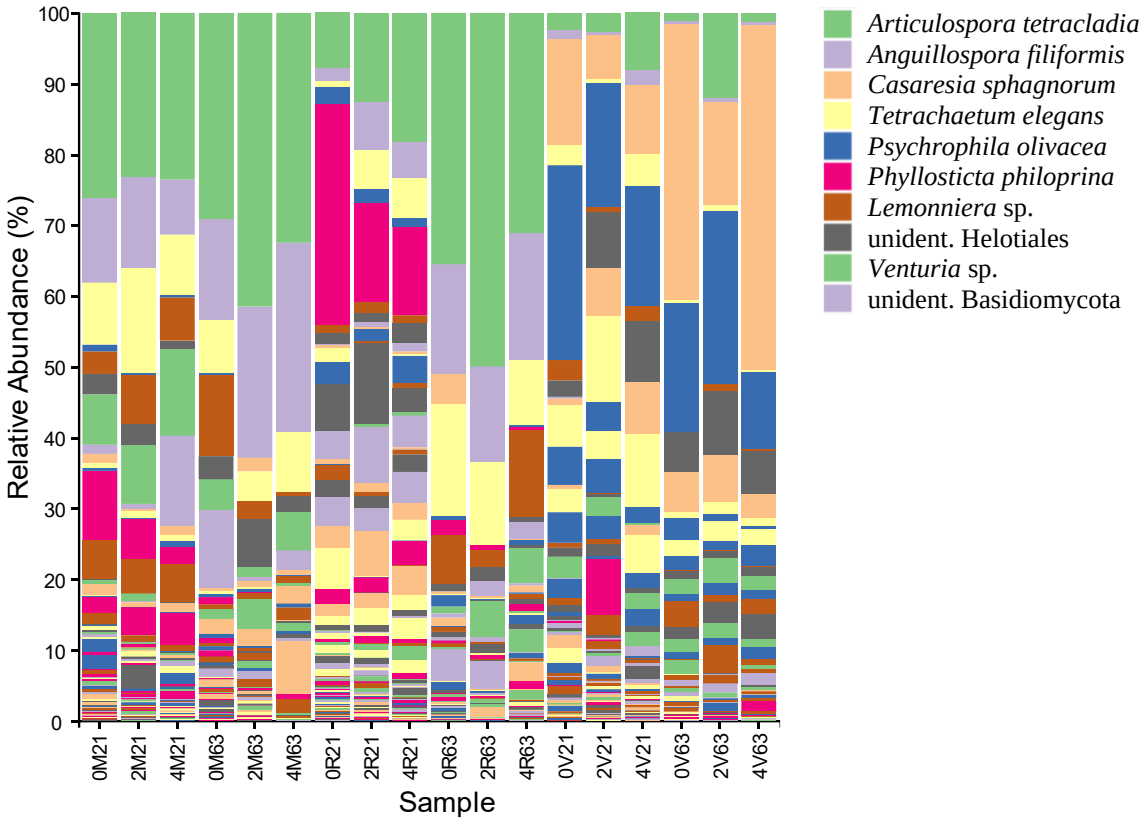


**Figure 8.** Relative abundances of dominant fungal species in streamside channel samples from different substrates, temperature treatments and sampling dates. Sample codes (e.g., 0M21) are as follows: 0=ambient temperature, 2=+2°C treatment, 4=+4°C treatment; M=maple leaf litter, R=rhododendron leaf litter, V=wood veneers; 21 or 63 denote sampling day.

**References**

Ackerman K. (2020). Effects of warming on decomposition of submerged plant litter and associated microorganisms in streamside channels. MS thesis. Coastal Carolina University.

Allan J.D. & Castillo M.M. (2007). Stream ecology: structure and function of running waters. 2nd edn. Springer, Dordrecht.

Anderson M.J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecology,* 26:32-46.

Arias-Real R., Menéndez M., Abril M., Oliva F. & Muñoz I. (2018). Quality and quantity of leaf litter: both are important for feeding preferences and growth of an aquatic shredder. *PloS ONE,* 13(12):e0208272.

Arsuffi T.L. & Suberkropp K. (1986). Growth of two stream caddisflies (Trichoptera) on leaves colonized by different fungal species. *Journal of the North American Benthological Society,* 5:297-305.

Bärlocher F. (1985). The role of fungi in the nutrition of stream invertebrates. *The Botanical Journal of Linnean Society,* 91:83-94.

Bärlocher F. (2016). Aquatic hyphomycetes in a changing environment. *Fungal Ecology,* 19:14-27.

Bärlocher F. & Kendrick B. (1973). Fungi in the diet of *Gammarus pseudolimnaeus* (Amphipoda). *Oikos,* 24:295-300.

Barros J. & Seena S. (2022). Fungi in freshwaters: prioritising aquatic hyphomycetes in conservation goals. *Water,* 14:605.

Beasley-Polko K. (2021). Warming stimulates sporulation rates and alters community structure of litter-associated fungi in streamside channel experiment. Honor's thesis. Coastal Carolina University.

Bokulich N.A., Dillon M.R., Zhang Y., Rideout J.R., Bolyen E., Li H., Albert P.S. & Caporaso J.G. (2018). Q2 longitudinal: longitudinal and paired-sample analyses of microbiome data. *mSystems,* 3:e00219-18.

Bolyen E., Rideout J.R., Dillon M.R., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology,* 37:852-857.

Callahan B.J., McMurdie P.J., Rosen M.J., Han A.W., Johnson A.J.A. & Holmes S.P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nature Methods,* 13:581-583.

Canhoto C., Gonçalves A.L. & Bärlocher F. (2016). Biology and ecological functions of aquatic hyphomycetes in a warming climate. *Fungal Ecology,* 19:201-218.

Caporaso J.G., Kuczynski J., Stombaugh J., Bittinger K., Bushman F.D., Costello E.K., Fierer N., Pena A.G., Goodrich J.K., Gordon J.I., Huttley G.A., Kelley S.T., Knights D., Koenig J.E., Ley R.E., Lozupone C.A., McDonald D., Muegge B.D., Pirrung M., Reeder J., Sevinsky J.R., Tumbaugh P.J., Walters W.A., Widmann J.,

Yatsunenko T., Zaneveld J. & Knight R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods,* 7:335-336.

Caporaso J.G., Lauber C.L., Walters W.A., Berg-Lyons D., Huntley J., Fierer N., Owens S.M., Betley J., Fraser L., Bauer M., Gormley N., Gilbert J.A., Smith G. & Knight R. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal*, 6:1621-1624.

Caporaso J.G., Lauber C.L., Walters W.A., Berg-Lyons D., Lozupone C.A., Turnbaugh P.J., Fierer N. & Knight R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences*, 108:4516-4522.

Chauvet E. & Suberkropp K. (1998). Temperatures and sporulation of aquatic hyphomycetes. *Applied and Environmental Microbiology*, 64:1522-1525.

Cornut J., Ferreira V., Gonçalves A.L., Chauvet E. & Canhoto C. (2015). Fungal alteration of the elemental composition of leaf litter affects shredder feeding activity. *Freshwater Biology,* 60:1755-1771.

Dang K., Schindler M., Chauvet E. & Gessner M.O. (2009). Temperature oscillation coupled with fungal community shifts can modulate warming effects on litter decomposition. *Ecology,* 90:122-131.

Duarte S., Bärlocher F., Cássio F. & Pascoal C. (2015). Stream-dwelling fungal decomposer communities along a gradient of eutrophication unraveled by 454 pyrosequencing. *Fungal Diversity,* 70:127-148.

Duarte S., Bärlocher F., Pascoal C. & Cássio F. (2016). Biogeography of aquatic hyphomycetes: Current knowledge and future perspectives. *Fungal Ecology,* 19:169-181.

Duarte S., Fernandes I., Nogueira M.J., Cássio F. & Pascoal C. (2013). Temperature alters interspecific relationships among aquatic fungi. *Fungal Ecology*, 6:187-191.

Easterling D.R., Meeh G.A., Parmesan C., Changnon A., Karl T.R. & Mearns L.O. (2000). Climate extremes: observations, modeling, and impacts. *Science*, 289:2068-2074.

Fernandes I., Uzun B., Pascoal C. & Cássio F. (2009). Responses of aquatic fungal communities on leaf litter to temperature-change events. *International Review of Hydrobiology*, 94:410-418.

Ferreira V. & Chauvet E. (2011). Future increases in temperature more than decrease in litter quality can affect microbial litter decomposition in streams. *Oecologia,* 167:279-291.

Ferreira V., Gulis V., Pascoal C. & Graça M.A.S. (2014). Stream pollution and fungi. *In:* Jones E.B.G., Hyde K.D., Pang K.L. (Eds.) *Freshwater fungi and fungal-like organisms*, pp. 389-412. De Gruyter, Berlin.

Gessner M.O., Gulis V., Kuehn K.A., Chauvet E. & Suberkropp K. (2007). Fungal decomposers of plant litter in aquatic ecosystem. *In*: Kubicek C.P. & Druzhinina I.S. (eds.). *The Mycota: Microbial and Environmental Relationships*, pp. 301-324. Springer, Berlin.

Geraldes P., Pascoal C. & Cássio F. (2012). Effects of increased temperature and aquatic fungal diversity on litter decomposition. *Fungal Ecology,* 5:734-740.

Godfrey B.E.S. (1983). Growth of two terrestrial microfungi on submerged alder leaves. *Transaction of the British Mycological Society,* 79:418-421.

Graça M.A.S. (1993). Patterns and processes in detritus-based stream systems. *Limnologica,* 23:107-114.

Gulis V. (2001). Are there any substrate preferences in aquatic hyphomycetes? *Mycological Research,* 105:1088-1093.

Gulis V., Su R. & Kuehn K.A. (2019). Fungal decomposers in freshwater environments. In: Hurst C.J. (Ed.) *Advances in environmental microbiology. The structure and function of aquatic microbial communities,* pp. 121-155. Springer Nature, Switzerland.

Gulis V. & Suberkropp K. (2003a). Effect of inorganic nutrients on relative contributions of fungi and bacteria to carbon flow from submerged decomposing leaf litter. *Microbial Ecology,* 45:11-19.

Gulis V. & Suberkropp K. (2003b). Interactions between stream fungi and bacteria associated with decomposing leaf litter at different levels of nutrient availability. *Aquatic Microbial Ecology,* 30:149-157.

Gulis V. & Suberkropp K. (2004). Effects of whole-stream nutrient enrichment on the concentrations and abundance of aquatic hyphomycete conidia in transport. *Mycologia*, 96:57-65.

Gulis V., Suberkropp K. & Rosemond A.D. (2008). Comparison of fungal activities on wood and leaf litter in unaltered and nutrient-enriched headwater streams. *Applied and Environmental Microbiology*, 74:1094-1101.

Hayer M., Wymore A.S., Hungate B.A., Schwartz E., Koch B.J. & Marks J.C. (2021). Microbes on decomposing litter in streams: entering on the leaf or colonizing in the water? *The ISME Journal,* 16:717-725.

IPCC (2022). Climate Change 2022: Impacts, Adaptation, and Vulnerability. Contribution of Working Group II to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change. [H.-O. Pörtner, D.C. Roberts, M. Tignor, E.S. Poloczanska, K. Mintenbeck, A. Alegría, M. Craig, S. Langsdorf, S. Löschke, V. Möller, A. Okem, B. Rama (eds.)]. Cambridge University Press. *In Press.*

Jentsch A., Kreyling J. & Beierkuhnlein C. (2007). A new generation of climate change experiments: events, not trends. *Frontiers in Ecology and the Environment,* 5:315-324.

Katoh K., Misawa K., Kuma K. & Myata T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research,* 30:3059-3066.

Krauss G.J., Solé M., Krauss G., Schlosser D., Wesenberg D. & Bärlocher F. (2011). Fungi in freshwater: ecology, physiology and biochemical potential. *FEMS Microbiology Reviews,* 35:620-651.

Kuehn K.A. (2016). Lentic and lotic habitats as templates for fungal communities: traits, adaptations, and their significance to litter decomposition within freshwater ecosystems. *Fungal Ecology,* 19:135-154.

Legendre P. & Legendre L. (2012). Numerical ecology, 3rd edition. Elsevier, Amsterdam.

Magurran A.E. (1988). Ecological diversity and its measurement. Princeton, Princeton University Press.

Mas-Martí E., Romaní A.M. & Muñoz I. (2015). Consequences of warming and resource quality on the stoichiometry and nutrient cycling of a stream shredder. *PLoS ONE*, 10:1-21.

Nikolcheva L.G. & Bärlocher F. (2005). Seasonal and substrate preferences of fungi colonizing leaves in streams: traditional versus molecular evidence. *Environmental Biology*, 7:270-280.

Nilsson R.H., Larsson K., Taylor A.F.S., Bengtsson-Palme J., Jeppesen T.S., Schigel D., Kennedy P., Picard K., Glöckner F.O., Tedersoo L., Saar I., Kõljalg U. & Abarenkov K. (2018). The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Research,* 47:D259-D264.

Oksanen J., Blanchet F.G., Friendly M., Kindt R., Legendre P., McGlinn D., Minchin P.R., O'Hara R.B., Simpson G.L., Solymos P., Stevens M.H.H., Szoecs E. & Wagner H. (2018). *vegan: Community Ecology Package,* version 2.5-3. https://CRAN.R-project.org/package=vegan

Price M.N., Dehal P.S. & Arkin A.P. (2010). FastTree 2-approximately maximum-likelihood trees for large alignments. *PloS ONE*, 5:e9490.

Panneerselvam P., Kumar U., Senapati A., Parameswaran C., Anandan A., Kumar A., Jahan A., Padhy S.R. & Nayak A.K. (2020). Influence of elevated $CO_2$ on arbuscular mycorrhizal fungal community elucidated using Illumina MiSeq platform in sub-humid tropical paddy soil. *Applied Soil Ecology,* 145:103344.

Pates H. (2019). Effects of temperature on microbial parameters associated with decaying plant litter in a stream microcosm experiment. MS thesis. Coastal Carolina University.

Pompanon F., Coissac E. & Taberlet P. (2011). Metabarcoding a new way to analyze biodiversity. *Biofutur,* 3:30-32.

Riaz T., Shehzad W., Viari A., Pompanon F., Taberlet P. & Coissac E. (2011). ecoPrimers: inference of new DNA barcode markers from whole genome sequence analysis. *Nucleic Acids Research,* 39:e145.

Schoch C.L., Seifert K.A., Huhndorf S., Robert V., Spouge J.L., Levesque C.A., Chen W. & Fungal Barcoding Consortium. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences,* 109:6241-6246.

Schmidt P., Bálint M., Bastian G., Cornelia B., Römbke J. & Schmitt I. (2013). Illumina metabarcoding of a soil fungal community. *Soil Biology & Biochemistry,* 65:128-132.

Seena S., Bärlocher F., Sobral O., et al. (2019). Biodiversity of leaf litter fungi in streams along a latitudinal gradient. *Science of the Total Environment,* 661:305-315.

Seena S., Pascoal C., Marvanová L. & Cássio F. (2010). DNA barcoding of fungi: a case study using ITS sequences for identifying aquatic hyphomycete species. *Fungal Diversity,* 44:77-87.

Siddique A.B. & Unterseher M. (2016). A cost-effective and efficient strategy for Illumina sequencing of fungal communities: a case study of beech endophytes

identified elevation as main explanatory factor for diversity and community composition. *Fungal Ecology,* 20:175-185.

de Souza L.M.S., Lirio J.M., Coria S.H., Lopes F.A.C, Convey P., Carvalho-Silva M., de Oliveira F.S., Rosa C.A., Câmara P.E.A.S. & Rosa L.H. (2022). Diversity, distribution and ecology of fungal communities present in Antarctic lake sediments uncovered by DNA metabarcoding. *Scientific Reports,* 12:8407.

Sridhar K.R. & Bärlocher F. (1993). Effect of temperature on growth and survival of five aquatic hyphomycetes. *Sydowia*, 45:377-387.

Suberkropp K. (1984). Effect of temperature on seasonal occurrence of aquatic hyphomycetes. *Transactions of the British Mycological Society,* 82:53-62.

Suberkropp K. (1992). Interactions with invertebrates. In: F. Bärlocher (Ed.) *The Ecology of Aquatic Hyphomycete*, pp. 118-134. Springer-Verlag, Berlin.

Suberkropp K. & Klug M.J. (1980). The maceration of deciduous leaf litter by aquatic hyphomycetes. *Canadian Journal of Botany*, 50:1025-1031.

Taberlet P., Bonin A., Coissac E. & Zinger L. (2018). Environmental DNA: for biodiversity research and monitoring. Oxford University Press, Oxford.

Walters W.A., Caporaso J.G., Lauber C.L., Berg-Lyons D., Fierer N. & Knight R. (2011). PrimerProspector: de novo design and taxonomic analysis of PCR primers. *Bioinformatics*, 27:1159-1161.

White T.J., Bruns T., Lee S. & Taylor J.W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: M.A. Innis, D.H. Gelfand, J.J. Sninsky & T.J. White (Eds). *PCR protocols: a guide to methods and applications*, pp. 315-322. Academic Press, New York.

Yang S., Xu W., Gao Y., Chen X. & Luo Z. (2020). Fungal diversity in deep-sea sediments from Magellan seamounts environment of the western Pacific revealed by high-throughput Illumina sequencing. *Journal of Microbiology*, 58:841-852.

Yuen T.K., Hyde K.D. & Hodgkiss I.J. (1998). Physiological growth parameters and enzyme production in tropical freshwater fungi. *Material und Organismen*, 32:1-16.

Zhang X., Wang G., Xu X., Nong X., Wang J., Amin M. & Qi S. (2016). Exploring fungal diversity in deep-sea sediments from Okinawa Trough using high-throughput Illumina sequencing. *Deep-Sea Research I,* 116:99-105.