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**Effects of temperature on microbial parameters associated with decaying plant litter
in a stream microcosm experiment**

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Coastal Marine and Wetland Studies in the
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Abstract

Increases in mean surface air temperature under climate-change predictions are expected to affect microbial activity, as well as carbon dynamics in aquatic ecosystems. I explored responses of litter-associated decomposers along temperature ranges typical of temperate streams in laboratory microcosms simulating stream conditions. The objectives of this study were to determine (1) whether the effects of temperature on stream leaf-associated microorganisms can be explained by the Metabolic Theory of Ecology (MTE); (2) if the effects of temperature are the same across temperatures commonly found in temperate streams; and (3) if there are differences in the magnitude of responses to temperature among various microbial parameters. The experiment measured physiological responses to temperature (5 levels, 4-20°C) of natural microbial assemblages colonizing *Liriodendron tulipifera* leaf litter in a stream at the Coweeta Hydrological Laboratory, NC during peak litter fall. In microcosm experiments, leaf litter decomposition rates, fungal biomass (ergosterol), fungal and bacterial production (radiolabeled tracers), spore production by aquatic fungi, and microbial respiration rates were followed. I found that responses of aquatic litter-associated microorganisms to increases in temperature were more complex than predicted by the MTE, with more pronounced responses (higher apparent activation energy, E) at lower temperatures. For some parameters, estimates of E at lower temperatures were higher than values often reported for respiration (~ 0.65 eV), suggesting that microbial carbon processing in streams could be especially sensitive to temperature increases during the key winter period of high activity. These trends may have important implications for stream ecosystems under climate-change scenarios, since bulk leaf litter inputs and peak microbial activity coincide with the coldest season (autumn-winter) in temperate streams.

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Introduction

Temperature is a critical physical property of freshwater ecosystems. The predicted mean surface air temperature increases of up to 4°C by the end of this century (IPCC 2014) could potentially alter how organic carbon is processed in these systems (Schroter et al. 2005, Caissie 2006). Forested headwater stream networks play an essential role in the global C cycle (Cole et al. 2007), as they are capable of significant terrestrial organic carbon sequestration, processing and transport (Fisher & Likens 1973, Vannote et al. 1980, Wallace et al. 1999), making it imperative to determine how they may respond to warming (Yvon-Durocher et al. 2010, Graça & Poquet 2014, Martinez et al. 2014). These highly heterotrophic, cold-water systems are usually light-limited, causing them to rely heavily on the allochthonous inputs of leaf litter and wood (Danger et al. 2013, Kuehn 2016). Microbial decomposition of this organic matter is driven by fungi and bacteria (Kuehn 2016). In addition, microbial conditioning makes leaf litter more palatable to macroinvertebrates (Canhoto & Graça 2008, Tant et al. 2013), while also enhancing the nutritional value of detritus and further promoting litter fragmentation (Cummins et al. 1973, Bärlocher & Sridhar 2014). Thus, in streams and rivers, litter-associated microorganisms are predominantly responsible for the mediation of energy and nutrient transfer to higher trophic levels (Suberkropp 1992, Gessner et al. 2007).

Aquatic hyphomycetes (Ingold 1942) are the main colonizers and decomposers of leaf material that enters streams (Bärlocher 1992, Gessner et al. 2007). These fungi tend to dominate in terms of biomass on leaf litter, accounting for 95-99.9% of total microbial biomass (Gessner et al. 2007, Krauss et al. 2011), and may account for up to 23% of the total detrital mass (Suberkropp 1997, Gessner et al. 2007). Their production, per unit of

stream area can also surpass that of invertebrates and bacteria (Suberkropp et al. 2010). Aquatic hyphomycetes are good indicators of ecological functioning as they have been widely studied in low-order streams and can be potentially used in stream health assessment by following their diversity (via sporulation), biomass (via ergosterol), and function (via litter decomposition rate) (e.g. Gessner & Chauvet 2002, Ferreira et al. 2006, Gulis et al. 2006, Noel et al. 2016).

Several studies have explored temperature effects on aquatic microorganisms and microbially-driven processes (e.g. Bärlocher et al. 2008, Perkins et al. 2010, Woodward et al. 2010, Ferreira et al. 2015, Tiegs et al. 2019). Warming is known to speed up chemical reactions while increasing the rates of biological processes (Davidson & Janssens 2006, Davidson et al. 2006). Therefore, ecosystem processes such as primary production and decomposition may experience stimulatory effects (Knorr et al. 2005, Cornelissen et al. 2007). Fungal metabolism is also likely to be affected, as increases in temperature are predicted to increase fungal production (the amount of biomass produced per unit of time) (Chauvet & Suberkropp 1998, Dang et al. 2009), as well as to stimulate oxygen consumption (respiration) (Bergfur & Friberg, 2012). In terms of fungal exoenzyme-mediated decomposition of litter, it is recognized that decomposition of recalcitrant leaves and wood may accelerate with warming (Ferreira & Chauvet 2011, Canhoto et al. 2016), likely even more so than the decomposition of more labile detritus (Fernandes et al. 2012). In addition, Canhoto et al. (2016) suggested that temperature may affect fungal colonization of leaf litter by altering spore attachment rates and efficiencies.

To quantitatively predict how overall ecological functioning may change in the face of warming, Brown et al. (2004) devised the Metabolic Theory of Ecology (MTE). The MTE is a quantitative theory that predicts how “the most fundamental biological rate”, the metabolic rate, varies with body size and temperature within a biologically relevant range of temperatures (0-40°C). Although metabolism is an exclusively biological process, it still must obey the laws of mass and energy balance and thermodynamics (Brown et al. 2004). The biochemical reactions that comprise metabolism of litter-associated microorganisms are complex, but in general, they involve the heterotrophic incorporation of C into microbial biomass (secondary production) and loss of C via CO₂ during energy acquisition (respiration). According to MTE, metabolic activity (e.g. respiration) scales exponentially with temperature:

$$R \sim e^{-E/kT},$$

where R is respiration, k is Boltzman constant, T is absolute temperature, and *E* is activation energy of the process. The temperature dependence of the respiratory complex has previously been represented by an activation energy of ~0.65 eV (Gillooly et al. 2001, Yvon-Durocher et al. 2012). However, temperature sensitivity of different metabolic processes may vary, e.g. photosynthesis is less sensitive to temperature than respiration (lower activation energy), while methanogenesis responds to elevated temperature to a greater extent (Allen et al. 2005, Yvon-Durocher et al. 2010a).

The ‘metabolic balance’ of an ecosystem with significant primary production is the rate of carbon fixation by photosynthesis relative to remineralization by respiration (Yvon-Durocher et al. 2010b), determining whether an ecosystem acts as a source or a sink for atmospheric CO₂ (Woodward 2007). In a freshwater mesocosm experiment,

warming compromised the capacity of carbon sequestration, as a larger portion of carbon fixed by photosynthesis was released as CO₂, rather than being used for production (Yvon-Durocher et al. 2010b). Ecosystem respiration has been found to show a higher temperature dependence than primary production (Allen et al. 2005) and therefore ecosystems exposed to warming will become less productive while releasing more CO₂ into the atmosphere (López-Urrutia et al. 2006) resulting in a possible positive feedback to the greenhouse effect. However, the outcomes described above are only applicable to ecosystems with significant levels of primary production. Headwater streams generally have very low levels of primary production due to shading, and the responses of these mostly heterotrophic systems and aquatic microbial decomposers to warming are poorly understood.

To address the possible complexity of heterotrophic microbial responses to temperature, I performed an experiment in laboratory microcosms simulating stream conditions using plant litter naturally colonized by microbial assemblages in a stream at Coweeta Hydrologic Laboratory Long Term Ecological Research site (Macon County, North Carolina) during peak litter fall. The objectives of this study were to determine (1) whether the effects of temperature on stream leaf-associated microorganisms can be explained by the Metabolic Theory of Ecology; (2) if the effects of temperature are the same across temperatures commonly found in temperate streams (4-20°C); and (3) if there are differences in the magnitude of responses to temperature among various microbial parameters (litter decomposition rate, fungal growth rate and sporulation, bacterial production, and microbial respiration).

Materials and Methods

Experimental microcosm set up

This study was conducted in laboratory microcosms simulating stream conditions.

Microcosms were comprised of tissue culture flasks (250 mL, with membrane-filter caps) filled with 100 mL of sterile nutrient solution (see below) and leaf disks pre-colonized in a stream by natural microbial assemblages. Pre-weighed, dried tulip poplar

(*Liriodendron tulipifera*) leaf disks were placed in litter bags (1-mm mesh size) in groups of 45, sterilized by autoclaving (15-minute dry cycle) and submerged in a headwater stream draining watershed 5a at the Coweeta Hydrologic Laboratory (35°03'32.8"N 83°25'37.6"W) (Long Term Ecological Research site), Macon County, North Carolina during peak litter fall on November 8th, 2017. After being colonized by a natural microbial assemblage for four days, litter bags were transported back to the lab on ice.

Leaf disks were placed into corresponding flasks filled with nutrient solution.

Microcosms were incubated on shakers at 5 different temperatures for 28 days (see below). The nutrient solution contained inorganic nitrogen (N) and phosphorus (P) added as sterile stocks of NaNO₃ and KH₂PO₄ at final concentrations of 2.0 mg L⁻¹ of NO₃-N and 0.275 mg L⁻¹ of PO₄-P (N:P molar ratio 16:1), as well as 0.25 g L⁻¹ of 3-(N-morpholino) propanesulfonic acid (MOPS), pH adjusted to 6.5. Nutrient solutions were changed every two days via aseptic evacuation and replaced with fresh sterile nutrient solutions to ensure microbial activity was not limited by nutrient availability.

Sampling

Five environmental chambers were used throughout the experiment to maintain treatment temperatures of 4, 8, 12, 16, and 20°C and were monitored by temperature loggers. For

each treatment, flasks containing 45 leaf disks were harvested (4 replicates) on d. 0, 6, 12, 18, 24, and 28 after the start of laboratory incubations, and sets of 5-15 leaf disks were distributed into containers to measure litter dry mass (DM), fungal biomass, growth rate, production, and sporulation, bacterial growth rate and production, and microbial respiration. Aliquots of nutrient solutions were also preserved to quantify the released spores of aquatic hyphomycetes (see below).

Leaf mass loss and decomposition rates

The DM of leaf disks was obtained by drying the samples in an oven (60° C for at least 24 h) and weighing to 0.0001 g. Additional samples of leaf disks were also freeze-dried and then weighed. Mass loss of leaf litter, percentage of mass remaining, and decomposition rates were calculated by taking into account the number of disks weighed and the initial and final mass of disk sets. Leaf litter decomposition rates were determined by calculating exponential decay coefficient (k) based on fraction of initial DM remaining over time according to the model:

$$M_t = M_0 e^{-k},$$

where M_t is mass at time t , M_0 is mass at time 0, k is exponential decay coefficient and t is time in days (Webster & Benfield 1986).

Fungal biomass, growth rate, and production

Fungal biomass was estimated from ergosterol content of leaf litter whereas fungal growth rates were estimated from rates of [^{14}C]-acetate incorporation into ergosterol (Gulis & Bärlocher 2017). Five leaf disks from each sample were placed in plastic 20-mL scintillation vials with 3.95 mL sterile nutrient solution and acclimated at the appropriate treatment temperature. Sodium [$1\text{-}^{14}\text{C}$]-acetate (Vitrax, Placentia, CA)

solution (0.05 mL) was added (final acetate concentration 5 mM, activity 1 MBq per sample) and vials were incubated for 4 h at the respective treatment temperatures on a shaker. Corresponding sets of leaf disks with formalin added before the radioactive acetate served as killed controls. Acetate incorporation was stopped by preserving leaf disks in 5 mL of methanol. Samples were then stored in the freezer until being processed.

Fungal biomass was determined from ergosterol concentration of leaf disks by lipid extraction and quantification with high-performance liquid chromatography (HPLC). Ergosterol associated with leaf disks was extracted using the liquid-to-liquid extraction technique (Gulis & Bärlocher 2017). Lipids from leaf disks were extracted with alcoholic KOH and partitioned into pentane. Extracts were then evaporated to dryness, re-dissolved in 1 mL of methanol, and syringe-filtered (0.2- μ m pore size PTFE membrane) into HPLC vials. A Shimadzu Prominence (Columbia, MD) HPLC system equipped with a reverse phase C18 column (Whatman partisphere, 4.6 x 250 mm) was run isocratically at 1.2 mL min⁻¹ of methanol at 35°C; ergosterol in 250- μ L injections of lipid extracts was detected at 282 nm and eluted at ~8 min. HPLC areas were converted to ergosterol concentrations using external ergosterol standards (Acros Organics, Morris Plains, NJ) and a conversion factor of 5.5 mg ergosterol per g of fungal dry mass was used (Gulis & Bärlocher 2017). Ergosterol fractions were collected with a fraction collector (Shimadzu FRC-10A, Columbia, MD) and mixed with 10 mL of scintillation cocktail (Ecolume, MP Biomedicals, Solon, OH) in 20-mL scintillation vials. Radioactivity in the ergosterol fraction was measured in a scintillation counter (Triathler, LabLogic, Brandon, FL) and corrected for quenching. In cases of low activity, two 250-

μL injections of the same sample were pooled, and radioactivity measured. Instantaneous fungal growth rate and fungal production were calculated assuming the exponential growth model as described by Gessner and Chauvet (1997), and empirical conversion factor of 19.3 mg fungal biomass nmol⁻¹ acetate incorporated (Suberkropp & Weyers 1996).

Fungal sporulation rates, cumulative conidia production, and assemblage structure

Ten-mL aliquots of nutrient solutions collected from microcosms every two days during the experiment were preserved with 0.5 mL of formalin/Triton X-100 solution (final concentrations *ca.* 1.8% formalin and 0.025% Triton X-100) and stored at room temperature until analysis. To determine sporulation rates of aquatic hyphomycetes, 1-5 mL aliquots of spore suspensions were filtered through 8-μm membrane filters (25 mm diam., type SCWP, Millipore) and stained with 0.05% trypan blue in 85% lactic acid. Filters were then mounted on slides and examined under a light microscope at 100-400x magnification. Aquatic hyphomycete conidia were counted and identified to species by spore morphology using an illustrated key (Gulis et al. 2005). At least seven microscopic fields and at least 200 conidia were counted per sample. The number of conidia counted, number of fields scanned, total volume of solution in the microcosm, volume of conidia suspension preserved, and the aliquot filtered, time of incubation and DM of sample were used to calculate the sporulation rate following Gulis and Bärlocher (2017).

To determine the cumulative conidia production throughout the experiment, corresponding 1-mL aliquots (same temperature treatment and replicate) were pooled (14 sampling dates), and an aliquot of the resulting 14-mL spore suspension was filtered, stained, and examined as described above.

Bacterial production

Bacterial production was estimated from rates of [^3H]-leucine incorporation into bacterial protein (Buesing & Gessner 2006, Gillies et al. 2006, Suberkropp et al. 2010) with some modifications. Five leaf disks from each sample were incubated in 15-mL tubes containing 4 mL of sterile nutrient solution and 0.2 MBq of [4,5 ^3H]-leucine (1 μM final concentration) for 1 h. Radiolabel incorporation was stopped by adding trichloroacetic acid (TCA, 5% final concentration); bovine serum albumin (BSA) was also added to aid in co-precipitation of proteins. Corresponding sets of leaf disks with TCA added before the radioactive leucine served as killed controls. All samples were then heated at 95°C for 60 min to precipitate proteins and then stored at 4 °C in the dark. Samples were then filtered through membrane filters (0.22-mm pore size), leaf disks were washed 3× with ice-cold TCA, 2× with cold 80% ethanol and 1× with cold distilled water to remove unincorporated radiolabel. Leaf disks and filters were digested with 1 N NaOH for 1 h at 80°C and incubated for an additional 24 h at room temperature to solubilize proteins. An aliquot was then removed and decolorized with 30% H_2O_2 overnight, scintillation fluid (Ecolume, MP Biomedicals) was added, and then the radioactivity of each sample was determined with a scintillation counter (Triathler, LabLogic, Brandon, FL). Samples were then spiked with known amount of ^3H -leucine, radioactivity measured again, and raw counts corrected for quenching. Bacterial production was then calculated according to Suberkropp et al. (2010) and correcting for abiotic radiolabel incorporation in killed controls.

Microbial respiration

Respiration measurements were performed with an Orion 5-Star Plus multimeter (ThermoFisher, Beverly, MA) equipped with a BOD probe to measure dissolved oxygen concentration (Gulis & Suberkropp 2003, Harrington 2016). Fifteen leaf disks used for respiration were removed from the experimental flasks and placed in screw-cap vials (30 mL) filled with fresh sterile nutrient solution of the respective temperature. Blanks of nutrient solutions at the same temperatures were also used. Initial oxygen concentration ($\text{mg O}_2 \text{ L}^{-1}$) and temperature were measured, vials were sealed with no head space and incubated for 2-8 h at 4, 8, 12, 16, or 20° C. Final oxygen concentration and temperature were recorded after several hours of incubation. Microbial oxygen uptake rate ($\text{mg O}_2 \text{ g}^{-1} \text{ leaf DM h}^{-1}$) in sealed vials was estimated based on the change in O_2 concentration, vial volume, mass of leaf disks and incubation time; the values were blank corrected.

Statistical analyses

For statistical analyses, data were checked for normality and were ln-transformed when necessary. Analysis of variance (ANOVA) was performed for microbial variable datasets with sampling time as the blocking variable and temperature (5 levels) as the independent variable (i.e. main effects ANOVA). ANCOVA was performed to compare leaf litter decomposition rates among temperature treatments (time as covariate). Linear regression was used to estimate apparent activation energy of microbial processes by regressing ln-transformed activity against inverse temperature parameter ($1/kT$) from the MTE. To analyze the assemblage structure of aquatic hyphomycetes, traditional metrics such as

Shannon-Wiener diversity index and evenness were calculated based on relative abundances of the conidia of individual species (Magurran 1988, Gulis & Bärlocher 2017).

Results

Leaf mass loss and decomposition rates

Leaf litter decomposition rates (k) were accelerated by warmer temperatures (ANCOVA, $F_{4,114} = 12.53$, $p < 0.0001$, Figure 1, Table 1). The percentage of initial dry mass remaining at the end of the experiment (day 28) was 45.6% at 20°C compared to 63.4% at 4°C. Temperature sensitivity of leaf litter decomposition rates (k) was estimated by calculating the apparent activation energy of the process (E) for the 4-12°C interval, which is the slope from the linear regression of ln-transformed decomposition rate vs. $1/kT$ ($E_{4-12} = 0.42$ eV, $R^2 = 0.97$, $p = 0.112$, Figure 2). No further increases in decomposition rates were evident for higher temperatures (12-20°C interval, Figure 2).

Fungal biomass, growth rate, and production

Fungal biomass accrual differed among five temperature treatments throughout the experiment (ANOVA, $F_{4,110} = 10.28$, $p < 0.001$, Figure 3) with especially pronounced differences at early stages of decomposition (day 0-12). Early increases in fungal biomass were greater at warmer temperatures (16 and 20°C), however, respective biomass levels either leveled off or decreased past day 12. Although early increases in fungal biomass were more modest at lower temperatures (4 and 8°C), these treatments peaked later at comparable levels to warmer temperature treatments.

Fungal growth rates (ANOVA, $F_{4,110} = 5.42$, $p = 0.001$) and fungal production ($F_{4,91} = 2.49$, $p = 0.048$, excluding day 0 data due to identical fungal biomass) differed

among temperature treatments (Figure 4). Both fungal growth rate and production exhibited earlier and larger increases at warmer temperatures (12-20°C), peaking on day 6, followed by subsequent sharp declines. At lower temperatures, fungal production peaked later and at lower levels, with the 4°C treatment not reaching its peak until day 28 (Figure 4B). Temperature sensitivity of fungal growth rate early in the experiment (day 6, when both growth rate and production were the highest) was greater at lower temperature interval (4-12°C) ($E_{4-12} = 0.85$ eV, 95% CI 0.51-1.20 eV, $R^2 = 0.75$, $p = 0.0003$, Figure 5). At warmer temperatures, above 12°C, there was a decrease in growth rate with temperature.

Fungal sporulation rates and cumulative spore production

Sporulation rates of aquatic hyphomycetes expressed per g of leaf litter dry mass and per g of litter-associated fungal biomass differed among temperature treatments (ANOVA, $F_{4,91} = 8.08$, $p < 0.001$; $F_{4,91} = 3.94$, $p = 0.005$, respectively, Figure 6A,B). In general, sporulation rates peaked earlier and were highest at 12 and 16°C, while 4 and 8°C treatments had lower sporulation rates with delayed peaks (days 18 and 24, respectively). Temperature sensitivity of fungal sporulation on day 12 (around peak levels of sporulation for the warmest temperatures) was greater at lower temperatures (4-12°C interval) ($E_{4-12} = 2.17$ eV, 95% CI 1.71-2.64 eV, $R^2 = 0.92$, $p < 0.0001$, Figure 7A). Further increases in temperature from 12 to 20°C did not stimulate fungal reproductive activity.

Cumulative spore production of aquatic hyphomycetes throughout the experiment (day 0-28) was also greatest at lower temperatures (4-12°C) ($E_{4-12} = 1.57$ eV, 95% CI

1.11-2.04 eV, $R^2 = 0.85$, $p < 0.0001$, Figure 7B). There was no stimulating effect of further temperature increases from 12 to 20°C on cumulative spore production.

Bacterial production and growth rate

Bacterial production associated with leaf litter differed among temperature treatments (ANOVA, $F_{4,108} = 24.07$, $p < 0.0001$, Figure 8) and generally increased across all temperatures throughout the duration of the experiment. There were earlier increases and greater levels of bacterial production at warmer temperatures, with production peaking on day 28 across all temperatures. Bacterial growth rates were calculated based on production estimates and bacterial biomass data generated for early stages of decomposition by Robert Tracey (see Appendix I). Temperature sensitivity of bacterial growth rates was more apparent very early during the experiment (on day 0, i.e. litter pre-colonized in a stream and then incubated at different temperatures in the lab, rather than on day 6). On day 0, bacterial growth rate responded to temperature increases to the same extent across all temperature treatments ($E_{4-20} = 0.67$ eV, 95% CI 0.47-0.87 eV, $R^2 = 0.73$, $p < 0.0001$, Figure 9).

Microbial respiration

Microbial respiration rates per g of leaf litter dry mass differed among temperature treatments (ANOVA, $F_{4,109} = 13.62$, $p < 0.0001$, Figure 10A) and showed sharp increases at the early stages of decomposition (0-12 days), especially at warmer temperatures. In 12-20°C treatments, respiration peaked at day 12, with rates dropping steeply beyond that day. More gradual increases in respiration were found at lower temperatures, with a peak in respiration at 4°C only reached by day 28. Since contribution of bacterial biomass to total microbial biomass (fungi plus bacteria) was extremely low for days 0-12 (0.08 to

1.69%, typical for Coweeta streams), respiration data were standardized per unit of fungal biomass and not total microbial biomass due to the negligible relative contribution of bacteria, lack of complete bacterial biomass data and for the sake of comparison with other data. Microbial respiration rates standardized per unit of fungal biomass did not differ among treatments (ANOVA, $F_{4,109} = 1.33$, $p = 0.264$, Figure 10B). Temperature sensitivity of microbial respiration estimated for day 0 was similar across 4-16°C interval ($E_{4-16} = 0.65$ eV, 95% CI 0.38-0.93 eV, $R^2 = 0.65$, $p = 0.0002$, Figure 11), while respiration rates did not further increase from 16 to 20°C.

Fungal assemblage structure

A total of 11 species of aquatic hyphomycetes were identified from spores released from decomposing leaf litter in this experiment. Six species of aquatic hyphomycetes were found to be dominant (contributed greater than 5% to the total conidia pool) based on a pooled sample of spore suspensions throughout the duration of the experiment (Table 2, Figure 12, Appendices II-VI). Some differences in dominance patterns have been observed across temperature treatments, however, the temporal shifts in the assemblage structure complicated the picture. For example, *Flagellospora* sp. 1 dominated on day 6 at lower temperatures with 64.3% and 68.0% relative abundances at temperatures 4 and 8°C, respectively (Appendix II). *Anguillospora filiformis* dominated at warmer temperatures on day 6, with 31.6% at 16°C and 43.9% at 20°C. Conversely, *Flagellospora* sp. 1 dominated on day 28 at higher temperatures (Appendix VI), with relative abundances of 37.5% and 44.3% at 16 and 20°C, respectively. Additionally, some species that sporulated at one temperature may have failed to sporulate at other temperatures. Diversity (H) tended to be higher at warmer temperatures on day 6

(Appendix I) but it was greater at cooler temperatures on day 28 (Appendix V). Evenness (E) did not seem to be affected by temperature.

Discussion

Results from this experiment suggest that microbial responses to temperature may be more complex than predictions based on the Metabolic Theory of Ecology. Overall, the highest levels of microbial activity per unit of microbial biomass were observed early in the experiment (day 0-12), when microbial cells (e.g. fungal mycelium) were still physiologically young and actively metabolizing. During these early stages of leaf litter decomposition, greater temperature sensitivity of microbial processes was found at the lower temperature interval (4-12°C). In many cases, temperature levels above 12 or 16°C did not further stimulate microbial activity. Individual parameters also responded differently to temperature, with leaf litter decomposition being the least sensitive to temperature increases (estimates of apparent activation energy, $E_{4-12} = 0.42$ eV, 0-28 days). Bacterial growth rate ($E_{4-20} = 0.67$ eV, day 0) and microbial respiration ($E_{4-16} = 0.65$ eV, day 0) showed temperature sensitivity similar to MTE predictions for the respiratory complex (0.65 eV) (Brown et al. 2004). On the other hand, fungal growth rate ($E_{4-12} = 0.85$ eV, day 6) and reproduction (sporulation rates at peak levels on day 12, $E_{4-12} = 2.17$ eV, and cumulative spore production throughout the experiment, $E_{4-12} = 1.57$ eV) exhibited the greatest apparent temperature dependency at low temperatures, with no positive effects of temperature above 12°C.

Several studies reported that warmer temperatures often result in accelerated decomposition of submerged plant litter due to stimulation of associated microbial activity (e.g. Dang et al. 2009, Ferreira & Chauvet 2011b, Goncalves et al. 2013, Ferreira

& Canhoto 2014, Ferreira et al. 2014, Canhoto et al. 2016). As a result, the affected streams could experience faster substrate depletion, possibly affecting the resource availability to higher trophic levels (e.g. detritivorous invertebrates) and compromising ecosystem functioning (Fernandes et al. 2009). Similar substrate depletion driven by stimulation of microbial activity has been reported following whole-stream nutrient enrichment experiments (Rosemond et al. 2015). In this microcosm experiment, I found a significant positive effect of temperature on microbial leaf litter decomposition rates (Table 1, Figure 1). There was a trend for a greater temperature dependency of leaf litter decomposition rate at the 4-12°C range ($E_{4-12} = 0.42$ eV), with no further increase in decomposition for the 12-20°C interval. These results for the 4-12°C interval echo similar values reported from a recent study that included 1025 estimates of temperature dependency of leaf litter breakdown rates ($E = 0.34 \pm 0.04$ eV, Follstad Shah et al. 2017), with estimates in both cases being considerably lower than the predicted activation energy of the respiratory complex from the MTE (0.65 eV, Brown et al. 2004, Yvon-Durocher et al. 2012). However, recently, Tiegs et al. (2019) reported somewhat higher temperature sensitivity of decomposition for artificial standardized cellulosic substrates ($E = 0.68$ eV) in a worldwide survey of 514 streams and rivers. These differences in the estimates of E can likely be attributed to additional factors that can influence litter breakdown, e.g. substrate type and litter quality (Follstad Shah et al. 2017).

This experiment showed that fungal activity has a significant positive relationship with temperature. Higher fungal metabolic activity was more pronounced at early stages of decomposition, almost immediately following colonization by stream microorganisms. Fungal growth rate generally peaked on day 6, while reproduction (sporulation rates of

aquatic hyphomycetes) peaked around day 12 (Figures 4A, 6). This experiment suggested that fungal growth rate (and production) is more sensitive to warming at lower temperature interval ($E_{4-12} = 0.85$ eV), which is in line with the results from a study of soil fungi that used the same methodology and yielded comparable estimates of activation energy at lower temperatures ($E_{3-16} = 0.64$ eV, Bååth 2001), with decreases in temperature sensitivity of fungal growth rate with further warming. Suberkropp and Weyers (1996) reported exponential increases in fungal production rate on submerged leaf litter with temperature. At the ecosystem scale, enhanced fungal production and respiration (see below) can expedite organic matter turnover in response to climate change (Bärlocher et al. 2008). Therefore, it is imperative to explore possible consequences of warming during the crucial period of autumn bulk leaf litter input followed by the cold winter season in temperate streams (Gessner et al. 2007). Ecosystem-level implications may be especially critical if warming trends are found to be higher in the winter (Manning et al. 2018).

Fungal reproduction (sporulation rates of aquatic hyphomycetes during early stages of decomposition and cumulative spore production throughout the experiment) was greatest in the mid-range of our experimental temperatures (12°C, Figure 7). Similar findings were previously reported for fungal reproduction, with peaks estimated at 10°C (Fernandes et al. 2009, Fernandes et al. 2012), 12.5°C (Bärlocher et al. 2013) and 15°C (Ferreira & Chauvet 2011a). Responses of fungal sporulation to temperature are likely to be affected by the structure of fungal assemblages and individual responses of fungal species due to differences in their temperature optima (e.g. Chauvet & Suberkropp 1998, Dang et al. 2009, Ferreira et al. 2014). Both sporulation rate and cumulative spore

production exhibited tremendously high temperature sensitivity in the 4-12°C range (E_{4-12} = 2.17 eV (day 12) and 1.57 eV, respectively), which correspond to Q_{10} coefficients of around 16.1 and 7.9, respectively. Sporulation of aquatic hyphomycetes is known to be considerably more sensitive to changes in environmental variables (e.g. dissolved nutrient availability) than other parameters of fungal activity (e.g. Gulis & Suberkropp 2003, 2004).

Bacterial production (Figure 8) was the only parameter measured that increased throughout all temperatures and sampling dates without a plateau or decline. However, while bacterial biomass was the lowest on day 0, bacterial growth rate was high and showed temperature sensitivity very early in the experiment (Figure 9). Temperature sensitivity of bacterial growth rates on day 0 was similar across experimental temperatures with the estimate of apparent activation energy (E_{4-20} = 0.67 eV) comparable to the predicted value for respiration (0.65 eV). Psychrotolerant bacteria may have a relatively high temperature optimum, yet commonly reside in colder environments (Adams et al. 2010), which may explain why we saw increases in bacterial production throughout the entire experimental temperature range. Suberkropp and Weyers (1996) also reported exponential increases in bacterial production associated with leaf litter submerged in a stream across the 5-30°C range, which means no difference in temperature sensitivity between lower and higher temperatures.

An essential parameter for understanding microbial and stream metabolism is respiration. The standard range of activation energies reported for the respiratory complex according to the MTE is 0.60-0.70 eV (Brown et al. 2004). Estimates of apparent activation energy for microbial respiration at early stages of microbial

colonization from this experiment fell directly within that expected range for the 4-16°C interval ($E_{4-16} = 0.65$ eV, Figure 11). Increased metabolism (microbial respiration and ecosystem respiration) has been documented in several recent studies in streams or other freshwater ecosystems with elevated temperatures. Demars et al. (2011), Perkins et al. (2012) and Yvon-Durocher et al. (2012) estimated activation energy of ecosystem respiration in these aquatic systems to be around 0.67, 0.60-0.70 and 0.65 eV, respectively. Manning et al. (2018), working in the same area where our short stream incubations occurred (Coweeta LTER), reported slightly lower activation energies of microbial respiration associated with leaf litter, wood and fine benthic organic matter that averaged 0.30-0.43 eV. These lower values could be explained by their use of randomly collected submerged leaf litter at different stages of microbial colonization and activity, with some samples having relatively low levels of metabolism associated with senescing fungal mycelium at advanced stages of litter decomposition.

The ability to accurately predict changes in metabolic rates with temperature are important to our overall understanding of aquatic ecosystem responses to climate change. Aquatic ecosystems are reported to have considerably greater temperature sensitivity of ecosystem respiration (0.65 eV) compared to their terrestrial counterparts (0.32 eV) (Yvon-Durocher et al. 2012). Heterotrophic metabolism typically exceeds autochthonous primary production in aquatic ecosystems, further highlighting the importance of allochthonous leaf litter input (Yvon-Durocher et al. 2012). If ecosystem respiration increases with temperature at a faster pace than primary production, then a stream's ability to sequester and store organic carbon may be hindered, and thereby it would

intensify the positive climate feedback via increased CO₂ evolution (Yvon-Durocher et al. 2010).

Several studies have observed temperature-driven shifts in aquatic hyphomycete assemblages (Bärlocher et al. 2008, Dang et al. 2009, Ferreira & Chauvet 2011a, Canhoto et al. 2016), for example, when the dominant fungal species shift towards an assemblage better adapted to higher or lower temperatures (Fernandes et al. 2012). Gonçalves et al. (2013) suggests that it is reasonable to assume that the fungal taxa that initially colonized leaf disks in our experiment were acclimated to the stream's mean ambient temperature (~10°C). Changes that we observed in assemblage structure (Table 2, Appendices II-VII) may be due to the prevalence of cold-adapted species in the temperate stream in which we inoculated our leaf disks. This could explain why we saw a smaller number of species and lower diversity on day 6 compared to day 28, as cold-adapted species tend to have delayed increases in activity and production. Therefore, in this study, metabolism and activity of individual fungal species likely increased with temperature until reaching a species-specific temperature optimum (Manning et al. 2018), followed by a subsequent decrease in activity at even warmer temperatures (Chauvet & Suberkropp 1998). Gonçalves et al. (2013) also suggested that substrate impacts microbial assemblages even more so than temperature, as aquatic hyphomycete substrate preference typically differs among species. Future studies addressing this question should include the use of multiple leaf species.

Any temperature-induced changes in microbial activity and stream metabolism need to be better understood before we can predict the extent of climate-induced changes at the ecosystem or global scales. However, it is also important to consider if observed

changes are a result of short-term fluctuations, or rather long-term trends. The ability of microorganisms, including fungi, to downregulate their metabolism to return to previous levels of activity within several days following a temperature increase is a mechanism that has been recently documented (Crowther & Bradford 2013). This phenomenon of thermal acclimation, although controversial, should be explored further. Additionally, predicted increases in temperature due to climate change are expected to transpire over a relatively long period of time (e.g. up to 4°C by the end of the century, IPCC 2014). Some studies have suggested that although abrupt experimental manipulations mimicking climate change scenarios may affect the structure and function of microbial communities, it was not the case when the increases were gradual over five years (Klironomos et al. 2005).

The use of Metabolic Theory of Ecology to help predict anticipated effects of climate change has been common in recent years. While MTE is useful in establishing general patterns of temperature sensitivity of metabolic rates within the typical temperature range of biological activity (0-40°C), my results suggest that future studies should especially explore effects at lower temperatures (0-16°C). I found that responses of aquatic litter-associated microorganisms to increases in temperature are more complex than predicted by the MTE, with more pronounced responses (higher apparent activation energy, E) at lower temperatures. For some parameters, estimates of E at lower temperatures were higher than values often reported for respiration (~0.65 eV), suggesting that microbial carbon processing in streams could be especially sensitive to temperature increases during the key winter period of high activity. These trends may have important implications for stream ecosystems under climate-change scenarios, since

bulk leaf litter inputs and peak microbial activity coincide with the coldest season (autumn-winter) in temperate streams.

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Table 1. Decomposition rates (k) of leaf litter at various experimental temperatures in laboratory microcosms. Asymptotic standard errors (ASE) are also shown.

Temperature (°C)	k (day ⁻¹)	ASE	R ²
4	0.0176	0.0029	0.62
8	0.0249	0.0025	0.82
12	0.0286	0.0016	0.93
16	0.0272	0.0027	0.82
20	0.0286	0.0015	0.94

Table 2. Mean relative abundances (%) of aquatic hyphomycetes associated with leaf litter incubated at different temperatures throughout the experiment.

Species	Temperature				
	4°C	8°C	12°C	16°C	20°C
<i>Alatospora acuminata</i>	3.4	11.7	13.8	19.2	18.7
<i>Anguillospora filiformis</i>	3.5	12.5	21.9	16.6	17.1
<i>Articulospora tetracladia</i>	59.3	35.8	21.8	23.0	32.6
<i>Culicidospora aquatica</i>	0.3	0.2	0.0	0.1	0.0
<i>Flagellospora curvula</i>	4.8	15.0	11.1	16.9	11.0
<i>Flagellospora sp. 1</i>	3.3	1.9	6.3	5.5	7.7
<i>Fontanospora alternibrachiata</i>	18.3	18.0	21.9	13.7	11.0
<i>Lemonniera pseudofloscula</i>	2.5	2.5	1.1	2.5	0.8
<i>Lunulospora curvula</i>	0.0	0.0	0.6	0.0	0.0
<i>Tetrachaetum elegans</i>	4.7	2.5	1.4	2.4	1.1
<i>Tricladium chaetocladium</i>	0.0	0.0	0.0	0.0	0.0
Total	100	100	100	100	100
Total no. of species	9	9	9	9	8
Evenness (E)	0.620	0.792	0.834	0.854	0.842
Diversity (H)	1.363	1.740	1.832	1.877	1.752

*See Appendices II - VI for data from the individual sampling dates.

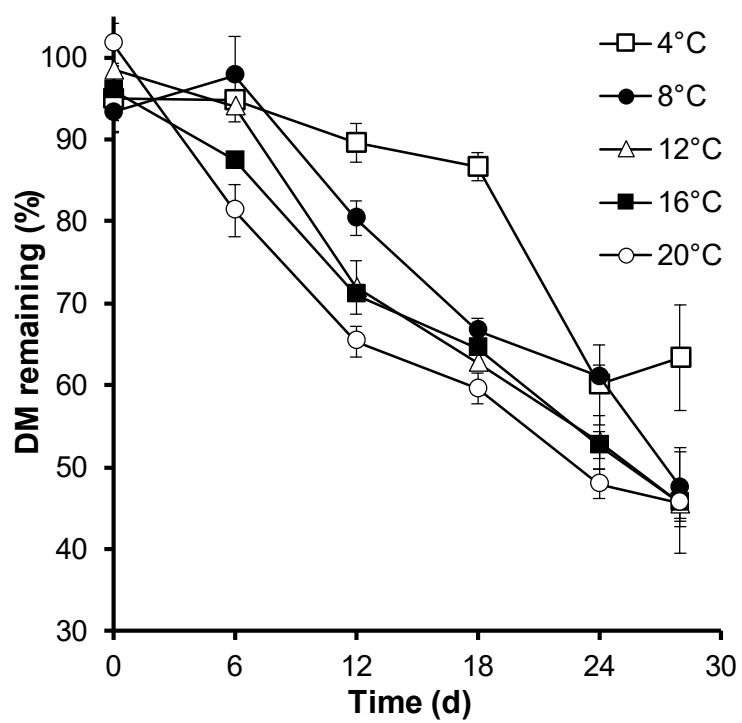


Figure 1. Leaf litter dry mass remaining (%) in laboratory microcosms at different temperatures on each sampling date. Error bars represent standard errors (SE).

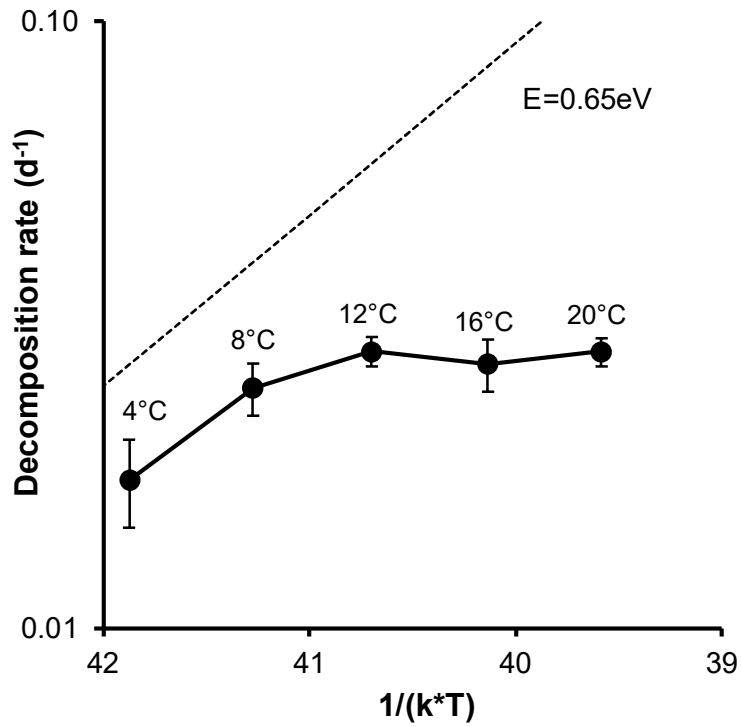


Figure 2. Temperature sensitivity of microbial leaf litter decomposition rates in laboratory microcosms within 4-20°C temperature range throughout the entire experiment (28 days). Dotted line indicates the slope corresponding to the activation energy of respiration (0.65 eV) according to the MTE and is shown for comparison. Error bars represent asymptotic standard errors (ASE). Activation energy of decomposition rate for 4-12°C interval was estimated as $E_{4-12} = 0.42$ eV, $R^2 = 0.97$, $p = 0.112$.

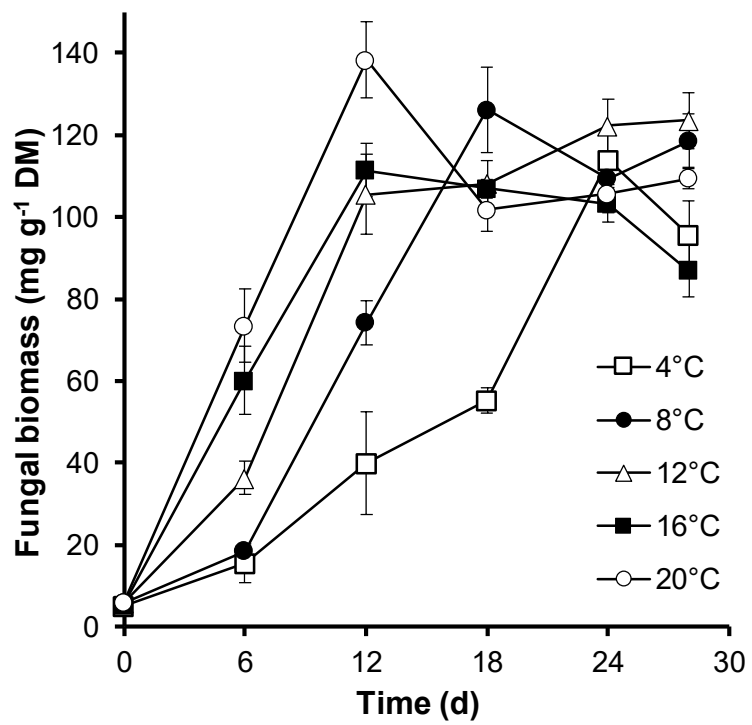


Figure 3. Fungal biomass associated with leaf litter in laboratory microcosms at different temperatures on each sampling date. Error bars represent standard errors (SE).

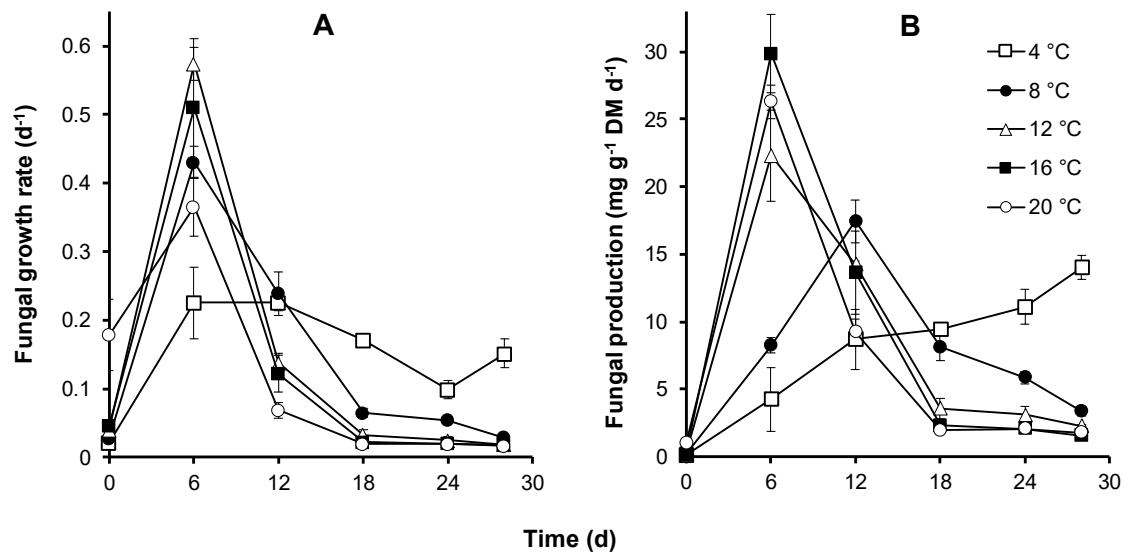


Figure 4. (A) Fungal growth rate and (B) fungal production associated with leaf disks in laboratory microcosms at different temperatures on each sampling day. Error bars represent standard errors (SE).

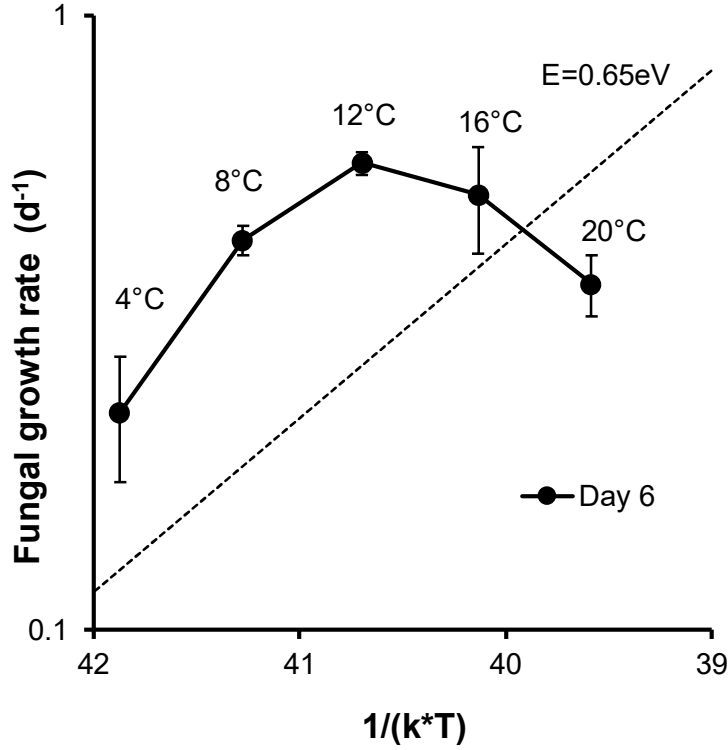


Figure 5. Temperature sensitivity of fungal growth rates associated with leaf litter in laboratory microcosms within 4-20°C temperate range on day 6 (highest growth rates, see Fig. 4A). Dotted line indicates the slope corresponding to the activation energy of respiration (0.65 eV) according to the MTE and is shown for comparison. Error bars represent standard errors (SE). Activation energy of fungal growth rate for 4-12°C interval was estimated as $E_{4-12} = 0.85$ eV, $R^2 = 0.75$, $p = 0.0003$.

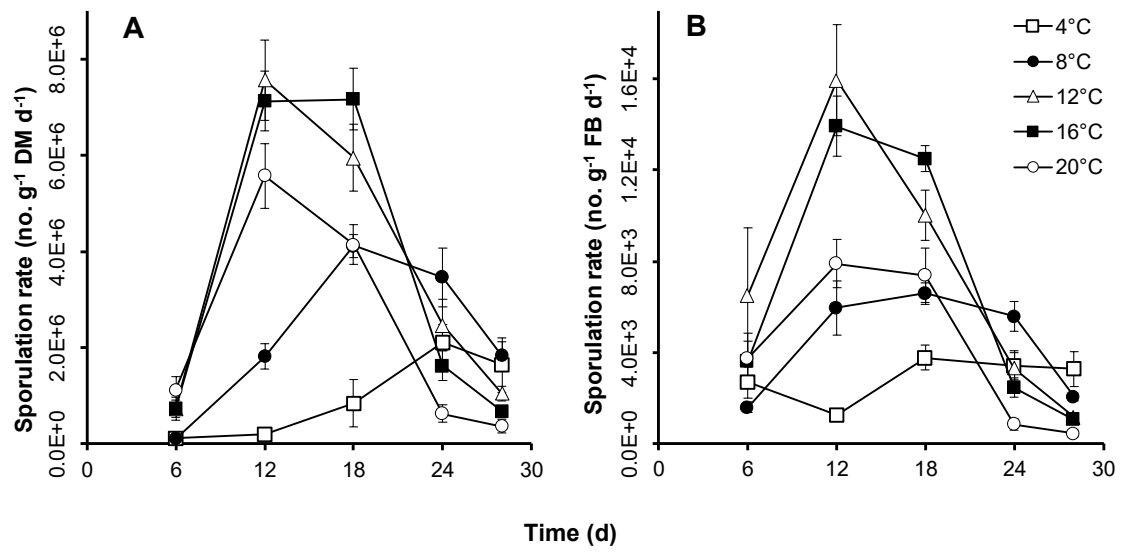


Figure 6. Sporulation rates of aquatic hyphomycetes associated with leaf litter in laboratory microcosms at different temperatures on each sampling day expressed (A) per g of litter dry mass and (B) per g of fungal biomass. Error bars represent standard errors (SE).

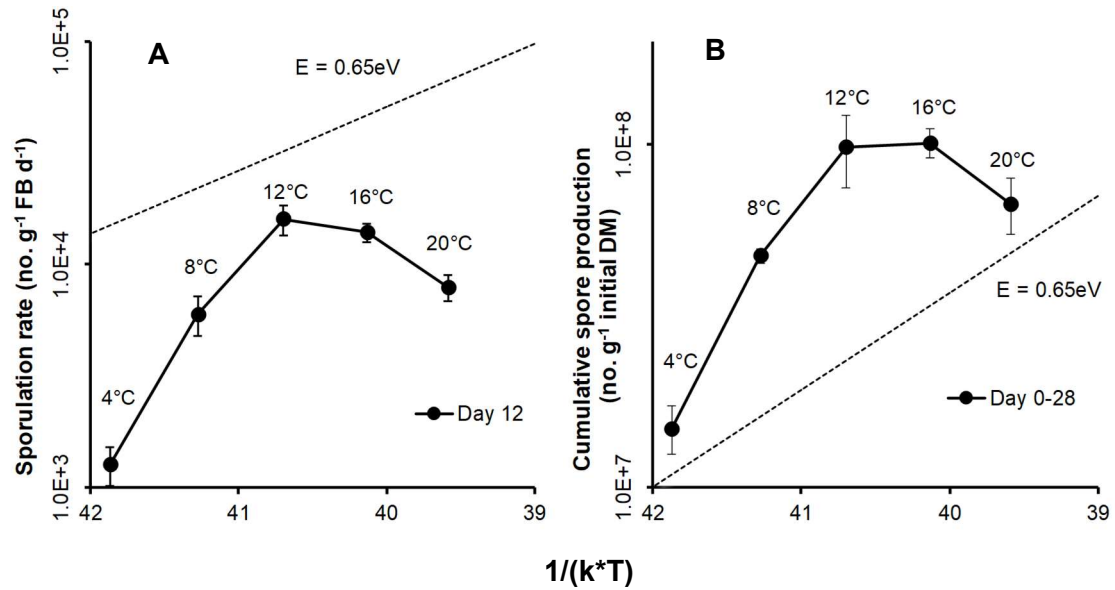


Figure 7. Temperature sensitivity of (A) sporulation rates of aquatic hyphomycetes on day 12 (highest sporulation, see Fig. 6B) and (B) cumulative spore production of aquatic hyphomycetes associated with leaf litter in laboratory microcosms throughout the experiment within 4-20°C temperate range. Dotted line indicates the slope corresponding to the activation energy of respiration (0.65 eV) according to the MTE and is shown for comparison. Error bars represent standard errors (SE). Activation energy of fungal sporulation rate for 4-12°C interval was estimated as $E_{4-12} = 2.17 \text{ eV}$, $R^2 = 0.92$, $p < 0.0001$, while activation energy for cumulative spore production was $E_{4-12} = 1.57 \text{ eV}$, $R^2 = 0.85$, $p < 0.0001$.

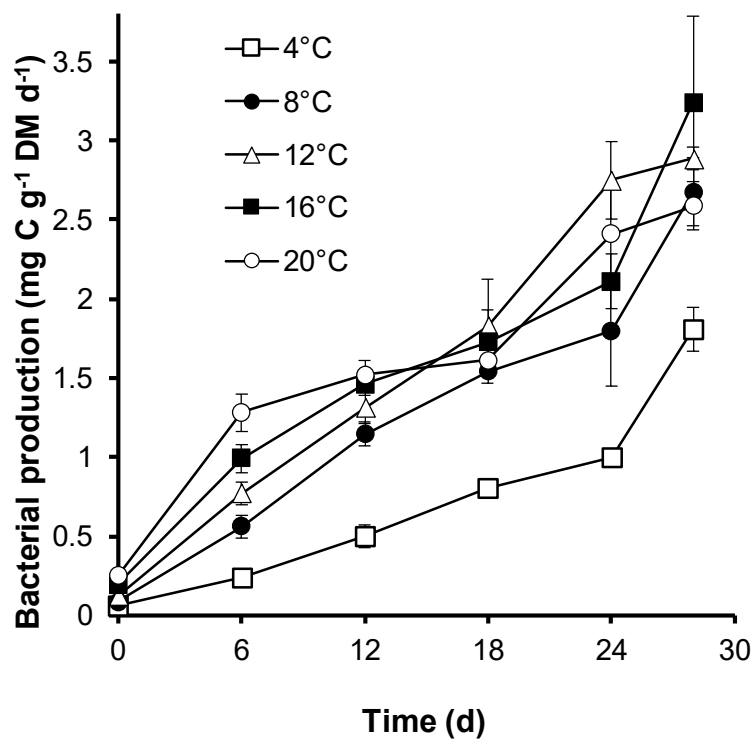


Figure 8. Bacterial production associated with leaf litter in laboratory microcosms at different temperatures on each sampling date. Error bars represent standard errors (SE).

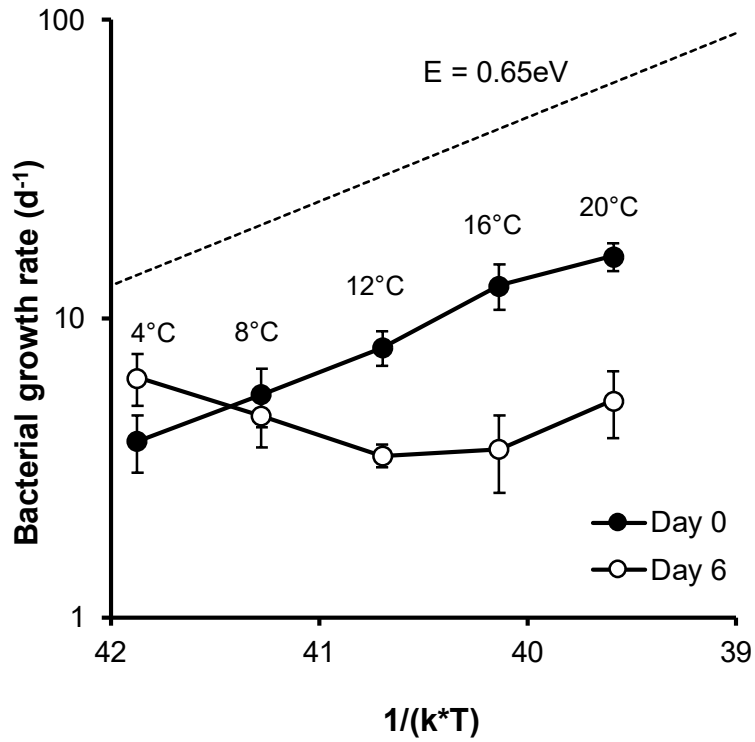


Figure 9. Temperature sensitivity of bacterial growth rate associated with leaf litter in laboratory microcosms within 4-20°C temperate range. Dotted line indicates the slope corresponding to the activation energy of respiration (0.65 eV) according to the MTE and is shown for comparison. Error bars represent standard errors (SE). Activation energy of bacterial growth rate on day 0 across all temperatures was estimated as $E_{4-20} = 0.67$ eV, $R^2 = 0.73$, $p < 0.0001$.

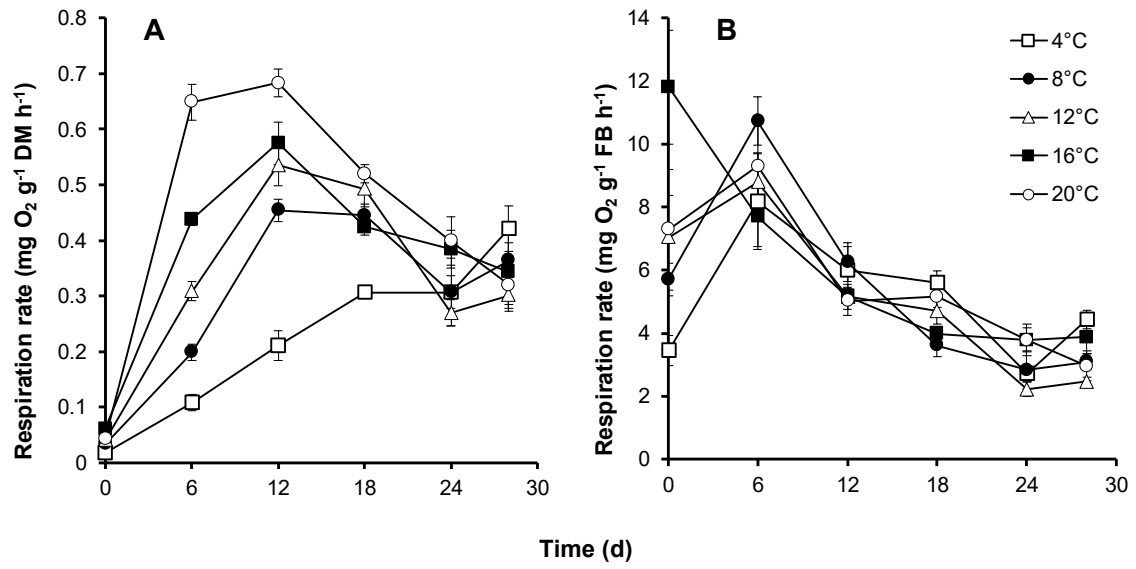


Figure 10. Microbial respiration associated with leaf litter in laboratory microcosms at different temperatures on each sampling date expressed (A) per g of litter dry mass and (B) per g of fungal biomass. Error bars represent standard errors (SE).

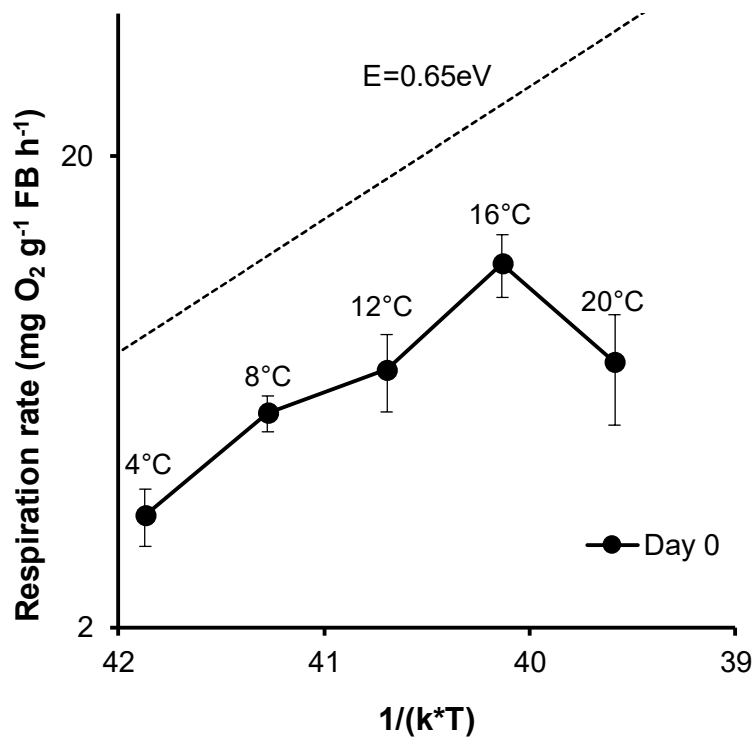


Figure 11. Temperature sensitivity of microbial respiration associated with leaf litter in laboratory microcosms within 4-20°C temperate range. Dotted line indicates the slope corresponding to the activation energy of respiration (0.65 eV) according to the MTE and is shown for comparison. Error bars represent standard errors (SE). Activation energy of microbial respiration rate on day 0 for 4-16°C interval was estimated as $E_{4-16} = 0.65$ eV, $R^2 = 0.65$, $p = 0.0002$.

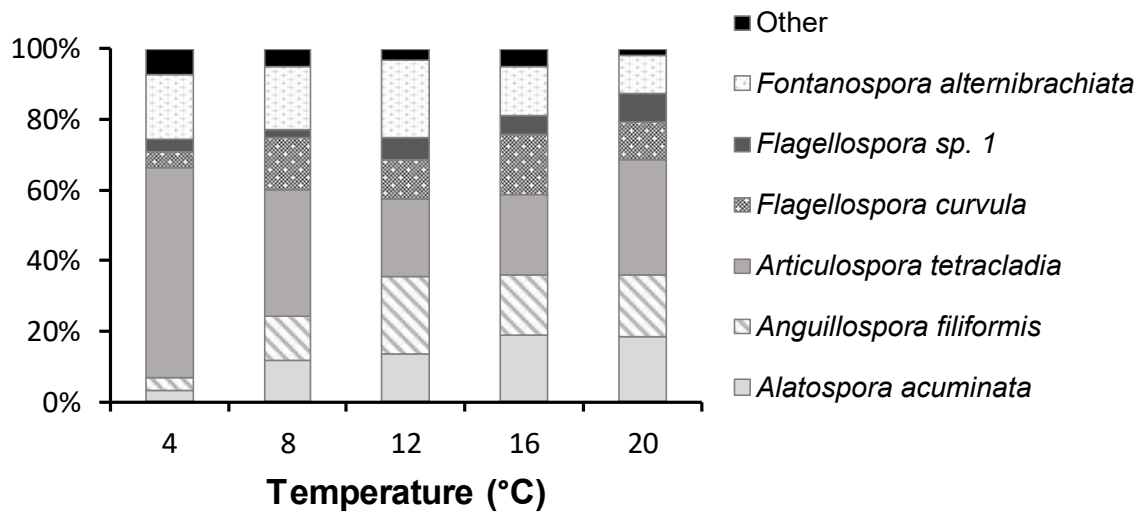
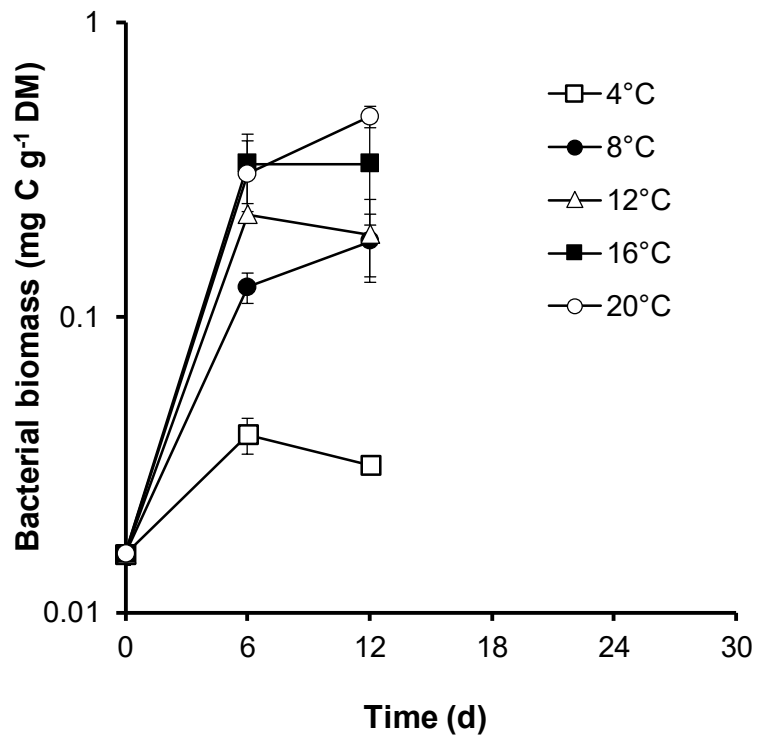


Figure 12. Relative contribution of dominant aquatic hyphomycetes to total conidia pool from each temperature treatment throughout the entire experiment. A dominant species contributed greater than 5% to the total conidia pool from at least one temperature treatment.

Appendix I. Bacterial biomass associated with leaf litter in laboratory microcosms at different temperatures on days 0-12 of the experiment. Error bars represent standard errors (SE).



Appendix II. Mean relative abundances (%) of aquatic hyphomycetes associated with leaf litter incubated at different temperatures on day 6.

Species	Temperature				
	4°C	8°C	12°C	16°C	20°C
<i>Alatospora acuminata</i>	0.0	0.0	0.0	0.1	10.5
<i>Anguillospora filiformis</i>	4.8	3.4	15.3	31.6	43.9
<i>Articulospora tetracladia</i>	0.0	0.2	9.7	22.6	8.0
<i>Culicidospora aquatica</i>	0.0	0.0	0.0	0.0	0.0
<i>Flagellospora curvula</i>	30.9	27.1	20.6	7.4	12.4
<i>Flagellospora sp. 1</i>	64.3	68.0	36.2	14.9	15.6
<i>Fontanospora alternibrachiata</i>	0.0	0.6	13.3	13.9	3.6
<i>Lemonniera pseudofloscula</i>	0.0	0.0	0.3	0.0	0.0
<i>Lunulospora curvula</i>	0.0	0.0	0.0	0.0	0.3
<i>Tetrachaetum elegans</i>	0.0	0.6	4.6	9.4	5.7
<i>Tricladium chaetocladium</i>	0.0	0.0	0.0	0.0	0.0
Total	100	100	100	100	100
Total no. of species	3	6	7	7	8
Evenness (E)	0.722	0.451	0.840	0.864	0.792
Diversity (H)	0.793	0.808	1.635	1.681	1.647

APPENDIX III. Mean relative abundances (%) of aquatic hyphomycetes associated with leaf litter incubated at different temperatures on day 12.

Species	Temperature				
	4°C	8°C	12°C	16°C	20°C
<i>Alatospora acuminata</i>	0.0	2.3	12.1	16.4	21.2
<i>Anguillospora filiformis</i>	0.6	12.6	14.0	10.8	5.9
<i>Articulospora tetracladia</i>	28.2	36.0	33.3	38.9	42.7
<i>Culicidospora aquatica</i>	0.0	0.0	0.0	0.0	0.0
<i>Flagellospora curvula</i>	12.2	10.2	13.2	12.7	13.6
<i>Flagellospora</i> sp. 1	29.3	6.8	5.7	6.4	11.4
<i>Fontanospora alternibrachiata</i>	23.5	19.1	10.6	8.7	3.7
<i>Heliscus lugdunensis</i>	0.0	0.0	0.0	0.0	0.0
<i>Lemonniera pseudofloscula</i>	1.0	1.6	4.8	2.0	0.2
<i>Lunulospora curvula</i>	0.0	0.0	0.5	0.5	0.0
<i>Tetrachaetum elegans</i>	5.3	11.5	5.7	3.4	1.3
<i>Tricladium chaetocladium</i>	0.0	0.0	0.0	0.0	0.0
Total	100	100	100	100	100
Total no. of species	7	8	9	9	8
Evenness (E)	0.794	0.847	0.866	0.744	0.754
Diversity (H)	1.546	1.761	1.904	1.783	1.568

APPENDIX IV. Mean relative abundances (%) of aquatic hyphomycetes associated with leaf litter incubated at different temperatures on day 18.

Species	Temperature				
	4°C	8°C	12°C	16°C	20°C
<i>Alatospora acuminata</i>	2.1	15.4	34.8	41.7	31.7
<i>Anguillospora filiformis</i>	1.3	6.7	8.8	2.1	5.9
<i>Articulospora tetracladia</i>	55.7	37.8	16.5	20.1	28.6
<i>Culicidospora aquatica</i>	0.4	0.1	0.1	0.0	0.0
<i>Flagellospora curvula</i>	3.6	15.9	13.5	12.2	13.5
<i>Flagellospora</i> sp. 1	1.7	4.4	10.6	12.1	12.5
<i>Fontanospora alternibrachiata</i>	25.2	12.5	8.9	8.4	7.1
<i>Heliscus lugdunensis</i>	0.0	0.0	0.0	0.0	0.0
<i>Lemonniera pseudofloscula</i>	4.0	4.4	2.6	2.0	0.2
<i>Lunulospora curvula</i>	0.0	0.0	1.6	0.8	0.2
<i>Tetrachaetum elegans</i>	6.0	2.8	2.6	0.6	0.1
<i>Tricladium chaetocladium</i>	0.0	0.0	0.0	0.0	0.0
Total	100	100	100	100	100
Total no. of species	9	9	10	9	9
Evenness (E)	0.599	0.806	0.810	0.744	0.748
Diversity (H)	1.317	1.771	1.864	1.636	1.644

APPENDIX V. Mean relative abundances (%) of aquatic hyphomycetes associated with leaf litter incubated at different temperatures on day 24.

Species	Temperature				
	4°C	8°C	12°C	16°C	20°C
<i>Alatospora acuminata</i>	5.2	30.1	48.1	36.6	30.7
<i>Anguillospora filiformis</i>	2.0	5.2	9.5	11.0	8.1
<i>Articulospora tetracladia</i>	42.4	20.8	8.1	16.6	21.2
<i>Culicidospora aquatica</i>	0.9	0.0	0.0	0.0	0.0
<i>Flagellospora curvula</i>	12.1	13.2	10.8	10.8	8.9
<i>Flagellospora</i> sp. 1	8.6	17.4	15.7	22.2	30.5
<i>Fontanospora alternibrachiata</i>	17.8	11.7	4.5	2.6	0.4
<i>Heliscus lugdunensis</i>	0.0	0.0	0.0	0.0	0.0
<i>Lemonniera pseudofloscula</i>	5.3	0.5	1.1	0.0	0.0
<i>Lunulospora curvula</i>	0.0	0.0	1.2	0.0	0.0
<i>Tetrachaetum elegans</i>	5.6	0.8	0.8	0.1	0.1
<i>Tricladium chaetocladium</i>	0.1	0.1	0.2	0.0	0.1
Total	100	100	100	100	100
Total no. of species	10	9	10	7	8
Evenness (E)	0.754	0.791	0.697	0.816	0.727
Diversity (H)	1.736	1.738	1.605	1.587	1.511

Appendix VI. Mean relative abundances (%) of aquatic hyphomycetes associated with leaf litter incubated at different temperatures on day 28.

Species	Temperature				
	4°C	8°C	12°C	16°C	20°C
<i>Alatospora acuminata</i>	8.1	32.7	49.0	26.6	25.1
<i>Anguillospora filiformis</i>	5.7	7.1	2.4	10.9	3.3
<i>Articulospora tetracladia</i>	23.0	12.8	8.9	13.6	23.0
<i>Culicidospora aquatica</i>	0.4	0.1	0.1	0.0	0.0
<i>Flagellospora curvula</i>	15.9	10.2	5.4	10.5	3.8
<i>Flagellospora sp. 1</i>	20.3	29.1	29.4	37.5	44.3
<i>Fontanospora alternibrachiata</i>	17.3	5.9	3.8	0.7	0.5
<i>Lemonniera pseudofloscula</i>	5.3	0.6	0.1	0.1	0.0
<i>Lunulospora curvula</i>	0.0	0.0	0.8	0.0	0.1
<i>Tetrachaetum elegans</i>	3.9	1.3	0.0	0.0	0.0
<i>Tricladium chaetocladium</i>	0.0	0.0	0.0	0.0	0.0
Total	100	100	100	100	100
Total no. of species	9	9	9	7	7
Evenness (E)	0.879	0.762	0.616	0.778	0.676
Diversity (H)	1.932	1.674	1.353	1.514	1.316