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Research Article

Temperature and interspecific interactions drive differences in carbon use efficiencies and biomass stoichiometry among aquatic fungi

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Abstract

Saprotrophic fungi play important roles in transformations of carbon (C), nitrogen (N), and phosphorus (P) in aquatic environments. However, it is unclear how warming will alter fungal cycling of C, N, and P. We conducted an experiment with four aquatic hyphomycetes (*Articulospora tetracladia, Hydrocina chaetocladia, Flagellospora* sp., and *Aquanectria penicillioides*), and an assemblage of the same taxa, to test how temperature alters C and nutrient use. Specifically, we evaluated biomass accrual, C:N, C:P, δ^{13}C, and C use efficiency (CUE) over a 35-d experiment with temperatures ranging from 4°C to 20°C. Changes in biomass accrual and CUE were predominantly quadratic with peaks between 7°C and 15°C. The C:P of *H. chaetocladia* biomass increased 9× over the temperature gradient, though the C:P of other taxa was unaffected by temperature. Changes in C:N were relatively small across temperatures. Biomass δ^{13}C of some taxa changed across temperatures, indicating differences in C isotope fractionation. Additionally, the 4-species assemblage differed from null expectations based on the monocultures in terms of biomass accrual, C:P, δ^{13}C, and CUE, suggesting that interactions among taxa altered C and nutrient use. These results highlight that temperature and interspecific interactions among fungi can alter traits affecting C and nutrient cycling.

Keywords: aquatic hyphomycetes, ecological stoichiometry, leaf litter, metabolic theory of ecology, streams

Introduction

Saprotrophic fungi are ubiquitous in soil and aquatic ecosystems, where they play important roles in global cycles of carbon (C), nitrogen (N), and phosphorus (P) (Sterner and Elser 2002, Waring, Averill and Hawkes 2013, Danger, Gessner and Bärlocher 2016). Climate warming is expected to increase rates of fungal growth and metabolism, which should increase their demand for C, N, and P (Allen and Gillooly 2009, Cross et al. 2015). Fungi associated with decomposition of leaf litter in freshwater environments largely rely on plant litter as a source of C and nutrients while also accessing dissolved inorganic nutrients (N and P) from the water column (Suberkropp 1998, Danger, Gessner and Bärlocher 2016). Although it is well understood that dissolved nutrients control the growth and metabolism of aquatic fungi (Suberkropp 1998, Gulis and Suberkropp 2003a), it is unclear if total N and P demand scales similarly to C demand with changes in temperature. Studies of algae, plants, and poikilothermic organisms have highlighted increases in biomass C:nutrient ratios, and/or decreases in C use efficiency (CUE) at higher temperatures (Woods et al. 2003, Reich and Oleksyn 2004, Crowther and Bradford 2013, Li et al. 2019), but it is unknown whether aquatic fungi demonstrate similar patterns.

A critical factor determining how organisms cycle nutrients is the ratio of nutrients to C in their biomass (Chapin 1980, Sterner and Elser 2002). In particular, high C:N and C:P may be indicative of conditions under which organisms are able to use nutrients more efficiently (Chapin 1980). Higher temperatures appear to lower the concentrations of both N and P relative to C in plants from warmer locations (Reich and Oleksyn 2004). A similar pattern has been observed in large surveys of marine algae, with biomass C:P increasing with sea-surface temperature (Yvon-Durocher et al. 2015). Increasing C:nutrient ratios with temperature also may be common among poikilothermic animals (Woods et al. 2003), perhaps caused by differential changes in the activity of cellular components, such as P-rich RNA or N-rich proteins (Woods et al. 2003, Yvon-Durocher et al. 2015). Little is known about the response of fungal biomass stoichiometry to temperature. One meta-analysis found that higher temperatures were associated with higher content of C and N and higher C:P and N:P ratios in the fruiting bodies of Agaricomycetes (Zhang and Elser 2017). However, fruiting bodies may not be representative of bulk fungal biomass in soils or plant litter. Furthermore, the importance of shifts in fungal community structure vs. intraspecific changes in stoichiometry across temperature gradients remains unclear.

Temperature may also alter the CUE of fungi, which is the efficiency with which organisms convert C substrates into biomass. Estimates of the CUE of heterotrophic microbes can vary considerably with the environment or substrate and the timescale over which estimates are made, along with factors such as nutrient availability (Geyer et al. 2016). For example, for leaf-associated stream fungi, CUE was reported to range from 31 to 60% (Gulis and Suberkropp 2003b), while CUE of bacteria in rivers can be highly

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variable, ranging from 3% to 46% (Del Giorgio and Cole 1998). Declines in CUE with rising temperature have been observed in both soil and aquatic ecosystems and are generally attributed to increases in maintenance metabolic costs associated with higher temperatures (Manzoni et al. 2012, Li et al. 2019). Maintenance metabolic costs include processes such as respiration, osmoregulation, turnover of biochemicals, and motility (Wang, Post and Mayes 2013). Alternatively, higher temperatures may increase CUE by stimulating production of new biomass to a greater extent than respiration (Pirt 1965).

Responses of multi-species assemblages to temperature may differ from those of individual species for two distinct reasons. First, temperature may act as an environmental filter of physiological traits across taxa, and thus may influence ecosystem processes through changes in microbial community structure across gradients in temperature (Padfield et al. 2017, Garcia, Warfield and Yvon-Durocher 2022). For example, a survey of marine algae found that changes in biomass stoichiometry across a 30°C gradient in ocean surface temperature were primarily driven by changes in community structure (Yvon-Durocher et al. 2015). Second, the physiology of individual taxa may change when interacting with other species in the community. For instance, Maynard, Crowther and Bradford (2017) found that communities of wood-decay fungi had lower CUE than monocultures in laboratory experiments. This reduction in CUE may occur because of the additional metabolic costs of interspecific competition (Maynard, Crowther and Bradford 2017), which is likely to be more pronounced on wood than on leaf litter. Thus, changes in community structure and interactions among species in a community may both drive changes in CUE or biomass stoichiometry, in addition to taxon-level physiological changes.

Here, we evaluate the responses of aquatic fungi to temperature in microcosms with liquid media, using four fungal monocultures and an assemblage of all four species. We ran trials at five temperatures ranging from 4°C to 20°C and quantified fungal biomass accrual, the C:P and C:N ratios of biomass, and CUE as biomass accrual divided by C consumed during the incubation period. In addition, we examined potential changes in the physiology of C use with temperature by examining the $\delta^{13}C$ of fungal biomass. Based on previous empirical findings for other groups of organisms (Woods et al. 2003, Yvon-Durocher et al. 2015), we predicted that fungal C:N and C:P would increase with temperature. We also predicted that the CUE of fungi would decline with increasing temperature. Finally, we explored whether interactions among species in the 4-species assemblage affected the measured fungal responses to temperature.

Materials and methods

Fungal inoculum

We used monocultures of four aquatic hyphomycetes belonging to the phylum Ascomycota: *Articulospora tetracladia* (isolate VG25-1), *Hydrocina chaetocladia* (VG27-1), *Flagellospora* sp. (VG31-4), and *Aquanectria penicillioides* (VG230-7), and a mixed assemblage that included all four taxa. Additional information on fungal cultures is available from https://ww2.coastal.edu/vgulis/culture-collection.htm. ITS rDNA sequences of these isolates were deposited to GenBank under accession numbers OP362579, OP362580, KC834051 and OP362578, respectively. Single-spore isolates of these fungi were obtained from submerged decomposing leaves in streams as described by Descals (1997). Cultures of *A. tetracladia*, *H. chaetocladia*, and *Flagellospora* sp. were isolated from a stream draining watershed 54 at the United States Department of Agriculture Forest Service Southern Research Station Coweeta Hydrologic Laboratory in the southern Appalachian Mountains, Macon County, North Carolina, USA. This stream has a seasonal temperature range from ~4°C to 18°C (Gulis and Suberkropp 2004, Gulis, Suberkropp and Rosemond 2008). *A. penicillioides* was isolated from the Firehole River, Wyoming, USA, from a site with a temperature of around 25°C at the time of collection (June 2015). Cultures were maintained in the laboratory on either 2% malt agar or in sterile water. We induced sporulation from cultures grown for 4 weeks on 1% malt agar plates by transferring agar slivers to tissue culture flasks with sterile deionized water, which were shaken on an orbital shaker (100 r/m) for 48 h (Gulis and Suberkropp 2003b). We then determined the concentration of conidia in suspension by filtering aliquots through membrane filters (8-μm pore size, SCWP, Millipore, Burlington, Massachusetts, USA) and counting spores on the filter with a compound microscope. We normalized spore suspensions with sterile deionized water to the same concentration (120 spores mL^{-1} total for all treatments, and 30 spores per mL^{-1} of each of four species for the mixed culture) to be used as inocula.

Growth medium

We grew fungi in a liquid medium following the methods in Gulis et al. (2017). The C source used in this experiment was carboxymethylcellulose (CMC), which has a recalcitrance more similar to natural substrates (i.e. plant litter polymers) than labile carbohydrates (e.g. glucose). CMC was added as a sodium salt (8.38 g L^{-1} in the final solution). The growth medium also contained 0.1 g $CaCl_2$, 0.1 g $MgSO_4 \times 7\ H_2O$, 0.004 g $MnSO_4 \times 7\ H_2O$, 0.01 g $FeCl_3 \times 6\ H_2O$, 0.01 mg thiamine, 0.7 g NO_3-N as $NaNO_3$, and 0.097 g PO_4-P as KH_2PO_4 (all per L of final solution). These concentrations correspond to an initial N:P = 16, C:N = 14, and C:P = 226. All stock solutions were either filter-sterilized or autoclaved before they were combined with autoclaved CMC solution (Gulis et al. 2017). We aseptically dispensed 90 ml of sterile growth medium into 500-mL sterile tissue culture flasks with vented (membrane) caps and inoculated them with 10 ml of spore suspensions (1200 conidia per flask) for a total volume of 100 ml. We placed the flasks slightly tilted on orbital shakers (100 r/m) and incubated them at 4, 8, 12, 16, or 20°C for 35 days. We used four replicate flasks for each combination of temperature and fungal treatment and incubated an additional four uninoculated blanks at 4°C (104 flasks total). Temperature was monitored throughout the experiment with Hobo MX2201 data loggers (Onset, Bourne, Massachusetts, USA), and mean temperatures were used in subsequent analyses.

At the conclusion of the experiment, we separated fungal mycelium from the liquid medium by filtering through two pre-combusted and pre-weighed glass fiber filters (Grade A, Sterilitech, Auburn, Washington, USA). The volume filtered depended on the amount of biomass present, but similar amounts were filtered through each pair of filters. We washed the filters with diluted saline solution to remove excess media and dried the filters at 60°C for at least 48 h before reweighing them. We also saved subsamples of the remaining media from each flask to estimate the amount of substrate consumed during the experiment.

Estimating fungal biomass accrual, CUE, and stoichiometry

From each flask, we analyzed one filter containing fungal biomass for C and N using a CHN analyzer. We processed the other filter containing fungal biomass for P by combusting the filter at 500°C

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for 4 h and dissolving the resulting ash in aqua regia. We then diluted the resulting solution and measured the concentration of P using an Alpkem Rapid Flow Analyzer 300 (Alpkem, College Station, Texas, USA). All analytical chemistry was conducted at the Center for Applied Isotope Studies, Stable Isotope Ecology Lab (Athens, Georgia, USA). We converted measured masses of C, N, and P to concentrations per mL of growth medium based on the volume of medium filtered, and then converted to the total mass per flask by multiplying by the total volume (mL) in the flask at the conclusion of the experiment. We quantified fungal biomass accrual based on the mass of mycelial C filtered from each flask, with the assumption that the initial mass of spores was negligible. We calculated an apparent fungal CUE (similar to the 'ecosystem CUE' defined by Geyer et al. 2016) as the ratio of biomass accrued (mg C) to C consumed from the growth medium over the study period (mg C, Equation 1).

$$CUE = \frac{Biomass\ C\ produced}{Medium\ C\ consumed} \quad (1)$$

To quantify the amount of C consumed from the liquid medium, we diluted an aliquot of the filtered liquid medium collected at the end of the experiment ~50× and analyzed the diluted sample for the concentration of dissolved organic C (DOC) using a TOC-5000A (Shimadzu, Kyoto, Kyoto, Japan). We then calculated the mass of C remaining in the medium, from the product of the dilution-corrected DOC concentration and the volume of medium in the flask. We estimated the mass of C in the medium at the beginning of the experiment as the product of the initial volume of the medium added to each flask and the concentration of DOC in aliquots of uninoculated medium and corrected these values for the amount of C lost in the uninoculated blanks. We quantified the C:P and C:N ratio of fungal biomass, calculated on a molar basis (Sterner and Elser 2002). In addition to estimating the bulk amount of C in fungal biomass and the growth media, we also measured biomass $\delta^{13}C$ to evaluate possible changes in physiology based on differences in ^{13}C discrimination during growth. Measurements of $\delta^{13}C$ were made at the Center for Applied Isotope Studies, Stable Isotope Ecology Lab (Athens, Georgia, USA)

Estimating fungal community composition

An additional small aliquot of the liquid medium from the 4-species assemblage was filtered through a third filter (8-μm pore size, SCWP, Millipore) that we used to estimate potential shifts in fungal community structure with temperature using denaturing gradient gel electrophoresis (DGGE) as described by Gulis and Bärlocher (2017). Briefly, we extracted DNA from the filters (DNeasy UltraClean Microbial Kit, Qiagen) and amplified ITS2 rDNA region by PCR with ITS3GC and ITS4 primers. We then performed DGGE to separate *ca.* 400 bp amplicons from the 4 fungal species according to their GC content using the C.B.S. Scientific DGGE-4001 system. A denaturing gradient of 30%–65% was used (Gulis and Bärlocher 2017). We included 'ladders' on each gel that contained similarly amplified DNA from pure cultures of each of the four fungal species to reference their positions in the gel. Polyacrylamide gels were stained with SYBR Gold and imaged using the Bio-Rad Gel Doc XR+ gel documentation system (Gulis and Bärlocher 2017). We then estimated the relative abundances of the four taxa in each microcosm based on band intensities quantified with Bio-Rad Image Lab software (see supplemental material for images).

Data analysis

Effects of temperature on fungal biomass accrual, CUE, biomass stoichiometry, and isotope discrimination

Biological rates may increase exponentially with temperature, which is often described by the Boltzmann-Arrhenius equation following the conventions of the metabolic theory of ecology (Brown et al. 2004). The Boltzmann-Arrhenius equation describes increases in rates of biological processes, r_i, as a function of the activation energy (E_a), the absolute temperature (T, in Kelvin), the Boltzmann constant, K_B ($= 8.617\times10^{-5}$ eV K^{-1}), and a temperature-independent constant, r_0 (equation 2).

$$r_i = r_0 * e^{\frac{E_a}{K_B * T}}, \quad (2)$$

We fit our data to the linearized version of the Boltzmann-Arrhenius equation, with temperature centered on a standard temperature (T_{12}, 285.15°K = 12°C), by regressing the $\log_e$-transformed parameters against the centered inverse Boltzmann temperature, and estimating the E_a based on the slope of this line, which has units of *eV* (equation 3):

$$\log_e(r_i) = \log_e(r_0) + E_a * \left(\frac{1}{K_B * T_{12}} - \frac{1}{K_B * T}\right), \quad (3)$$

Over a sufficiently large temperature range, many biological processes are described by a unimodal relationship with temperature (Schulte, Healy and Fangue 2011, Ruiz et al. 2020, Arroyo et al. 2022). To test whether a unimodal relationship with temperature better described our data, we extended the Boltzmann-Arrhenius equation by adding a quadratic effect of temperature (Q) to the model (Equation 4):

$$\log_e(r_i) = \log_e(r_0) + E_a * \left(\frac{1}{K_B * T_{12}} - \frac{1}{K_B * T}\right) + Q * \left(\frac{1}{K_B * T_{12}} - \frac{1}{K_B * T}\right)^2, \quad (4)$$

However, we note that in equation 4, the E_a term is no longer interpretable as an activation energy, and that this relationship may also be fit to data with a minimum at intermediate temperatures.

To evaluate the effect of temperature on biomass accrual, C:N, C:P, $\delta^{13}C$, and CUE, we fit equations 3 and 4 independently for each fungal treatment along with the null model (intercept only). We used the Akaike Information Criterion adjusted for small sample sizes (AICc) to evaluate which of these models best described the data for each species and the 4-species assemblage. We ranked the models in order of complexity as null < exponential < quadratic and considered the more complex model to be supported by the data if the AICc was lower than the simpler models by at least two (Burnham and Anderson 2002). For growth and carbon use efficiency, we rejected quadratic models with a positive quadratic term, as we are not aware of a biological mechanism for a U-shaped response to temperature. We report model coefficients for the model with the most support, along with the difference in AICc between the best model and the second-best model and the proportion of variation in the data explained by the best model (R^2). Only the treatments with 4-species assemblage and monocultures of *Hydrocina chaetocladia* and *Articulospora tetracladia* had enough biomass across most temperatures to measure biomass N and P, so we limited our analysis of C:N and C:P to these three fungal treatments.

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Analyzing processes in the 4-species assemblage

We evaluated the effect of temperature on community structure in the 4-species assemblage using a PERMANOVA with relative abundances of fungal taxa as the response variable, and the temperature treatments as the only predictor. The PERMANOVA was conducted using the *adonis* function in the *vegan* package in the R environment (Oksanen 2013, R Core Team 2018). Then, we evaluated whether interactions in the community altered the biomass accrual, C:N, C:P, $\delta^{13}C$, and CUE of the assembled community. Because the monocultures and the assembled community started with the same total number of spores, and assuming that the growth of each taxon is exponential, our null expectation (i.e. no interspecific interactions in the 4-species assemblage that changed functional parameters) was that the 4-species assemblage would resemble a proportional mixture of the monocultures, with each taxon accumulating 25% of the biomass that they attained in the monoculture. Thus, this approach did not assume that the relative abundances in the assembled community at the conclusion of the experiment were 25% for each species, but that their contribution to the assemblage reflects the relative performance of each taxon in monoculture. We refer to this null expectation as the theoretical assemblage (TA) and calculated expected values of the TA for biomass accrual, C:N, C:P, $\delta^{13}C$, and CUE. To calculate these values for the TA we first calculated the biomass C, N, and P, and the amount of C consumed from the media in the TA for each parameter (X) at each temperature as the sum of each taxon's mean parameter value at that temperature $X_n \times 0.25$ (equation 5).

$$X_{TA} = \sum_1^n X_n * 0.25\ , \tag{5}$$

Then, we calculated CUE and nutrient ratios of the TA based on the calculated TA values for C, N, and P. Deviations between the TA and measured values in the 4-species assemblage are indicative of shifts in the relative abundance or performance of individual species in the 4-species assemblage due to interactions among the species. We evaluated differences between the TA and the mean of the measured parameters in the 4-species assemblage at each temperature using paired *t*-tests. Finally, we estimated the proportional difference between the TA and 4-species assemblage at each temperature for each parameter by dividing the value in the 4-species assemblage by the value in the TA. We used a linear model to test whether temperature affected the proportional difference between the TA and the 4-species assemblage. Some monocultures had biomass N and P below the limit of detection. Instead of assuming these values were zero, we handled non-detection of N or P (which occurred in roughly half of monoculture chambers) conservatively when calculating the values for the TA by assigning those samples N or P values corresponding to one-quarter of the respective detection limit (see supplement SD1 for a sensitivity analysis of this assumption, which showed no effect on the results of the analysis).

Results

Temperature affected the biomass accrual of each species of aquatic fungi and of the 4-species assemblage, though the effects differed across fungal treatments (Fig. 1A, Table 1). The biomass accrual of *A. penicillioides* increased exponentially with temperature with an E_a of 0.69 eV, and in *Flagellospora* sp. it increased with an E_a of 0.86 eV (Fig. 1A, Table 1). The other fungal treatments were best described by a quadratic relationship with peak biomass at an intermediate temperature. Biomass accrual of *H. chaetocladia*

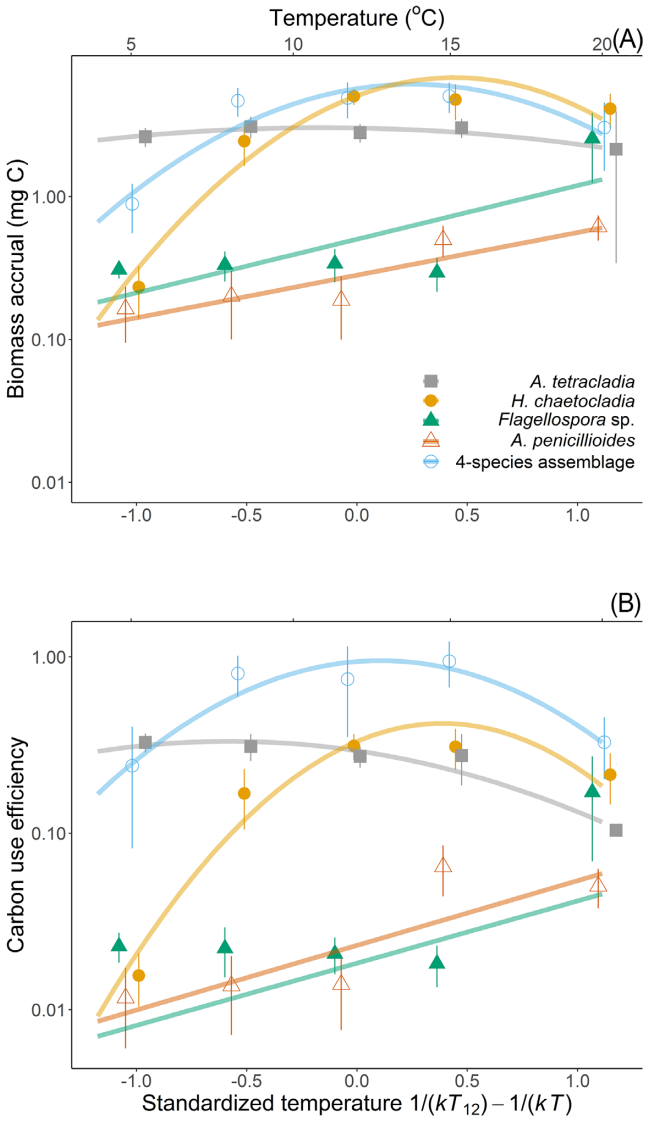

Figure 1. Biomass accrual **(A)** and carbon use efficiency **(B)** in different fungal treatments across temperatures. Temperature is transformed as the inverse Boltzmann temperature and is centered on 12°C (T_{12}). Treatments are jittered on the *x*-axis. Points represent the mean of four replicates and error bars represent 95% confidence intervals of the mean. Colors and shapes represent the different species treatments. Data for fungal treatments are fit to the quadratic model, except for *A. penicillioides* and *Flagellospora sp*, for which the exponential model provided a better fit. Model parameters are reported in Table 1.

was best described by a quadratic relationship and peaked around 15°C, *A. tetracladia* peaked at 11°C, while growth of the 4-species assemblage peaked around 14°C. Thus, for each fungal treatment, increases in temperature stimulated biomass accrual over at least some of the temperature range, but the quadratic models were generally better than exponential models over the full temperature range.

The CUE of fungi was also affected by temperature (Fig. 1B), with the relationship between CUE and temperature differing across fungal treatments. Similar to biomass accrual, we found that the CUE of *A. penicillioides* and *Flagellospora* sp. increased exponentially with temperature, while the responses of other fungal treatments were better described by quadratic relationships that were similar to those for biomass accrual (Fig. 1, Table 1). The CUE of *H. chaetocladia* peaked around 15°C, while the CUE of *A. tetracla-*

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Table 1. Temperature dependence of biomass accrual, carbon use efficiency, biomass C:N, biomass C:P, and biomass $\delta^{13}C$ for four monocultures of aquatic hyphomycetes and the 4-species assemblage. For each fungal treatment, we present the parameters of the best model based on model selection. Model selection included a null model with only an intercept, a model with an exponential effect of temperature (activation energy, or E_a), and a model with a quadratic effect (Q) of temperature. When the quadratic model was used the E_a does not represents an activation energy. We identified the best model as the model with the lowest AICc and required a more complex model to have a ΔAICc of at least 2 compared to the simpler models. When a simpler model was the best model, we report NA in the columns that represent terms in the more complex models. We report the ΔAICc relative to the next best model and the R^2 of the best model for each fungal treatment.

Fungal treatment	Intercept (SE)	E_a (SE)	Q (SE)	ΔAICc	R^2
Biomass accrual					
A. tetracladia	1.11 (0.04)	−0.06 (0.04)	−0.20 (0.05)	6.14	0.43
H. chaetocladia	1.63 (0.12)	1.32 (0.11)	−1.49 (0.16)	31.29	0.92
Flagellospora sp.	−0.69 (0.13)	0.86 (0.17)	NA	10.85	0.57
A. penicillioides	−1.27 (1.07)	0.69 (0.10)	NA	1.89	0.72
4-species assemblage	1.77 (0.09)	0.38 (0.07)	−0.96 (0.11)	30.63	0.85
Carbon use efficiency					
A. tetracladia	−1.22 (0.05)	−0.43 (0.06)	−0.37 (0.09)	10.83	0.78
H. chaetocladia	−1.10 (0.11)	1.21 (0.11)	−1.56 (0.15)	34.12	0.92
Flagellospora sp.	−3.41 (0.15)	0.81 (0.19)	NA	20.97	0.49
A. penicillioides	−3.76 (0.10)	0.84 (0.14)	NA	3.17	0.64
4-species assemblage	−0.06 (0.10)	0.23 (0.10)	−1.06 (0.14)	23.36	0.74
Biomass C:N					
A. tetracladia	2.12 (0.05)	0.18 (0.08)	NA	0.17	0.22
H. chaetocladia	1.92 (0.03)	−0.15 (0.06)	0.41 (0.08)	12.06	0.67
4-species assemblage	2.34 (0.03)	0.01 (0.03)	−0.16 (0.04)	8.15	0.45
Biomass C:P					
A. tetracladia	6.10 (0.06)	NA	NA	1.46	0.00
H. chaetocladia	8.08 (0.13)	0.87 (0.24)	0.85 (0.31)	3.68	0.84
4-species assemblage	6.18 (0.10)	NA	NA	1.55	0.00
Biomass $\delta^{13}C$					
A. tetracladia	−24.54 (0.06)	−0.62 (0.06)	−0.07 (0.09)	2.72	0.86
H. chaetocladia	−25.73 (0.47)	NA	NA	0.46	0.00
Flagellospora sp.	−26.13 (0.24)	1.31 (2.29)	NA	2.53	0.49
A. penicillioides	−26.46 (0.09)	NA	NA	0.88	0.00
4-species assemblage	−23.84 (0.08)	0.24 (0.07)	−1.05 (0.10)	33.76	0.85

dia peaked at 8°C, and the CUE of the 4-species assemblage peaked around 13°C.

Biomass C:N and C:P varied among the species treatments and with temperature (Fig. 2, Table 1). The biomass C:N of *A. tetracladia* increased exponentially with an E_a of 0.18 eV. The biomass C:N of *H. chaetocladia* was best described by a quadratic relationship with a minimum around 14°C while the 4-species assemblage had a quadratic relationship between temperature and C:N with a peak around 12°C. The biomass C:P of *A. tetracladia* and the 4-species assemblage showed no relationship with temperature. However, the biomass C:P of *H. chaetocladia* had large changes with temperature ranging from 150:1 at 12°C to >1000 :1 at 20°C, which was best described by a quadratic relationship with a minimum around 12°C.

Some fungal treatments had changes in biomass $\delta^{13}C$ across temperatures, but these relationships were variable. There was no relationship between temperature and the $\delta^{13}C$ of *A. penicillioides* or *H. chaetocladia*. The $\delta^{13}C$ of *Flagellospora* sp. increased with temperature, while the $\delta^{13}C$ of *A. tetracladia* declined (Fig. 3, Table 1), and the 4-species assemblage had a quadratic relationship between $\delta^{13}C$ and temperature that peaked around 13°C; these patterns were somewhat similar to those found for biomass accrual and CUE. The growth medium had a $\delta^{13}C$ of −27.3‰ (black dashed line in Fig. 3), which was lower than most of the biomass measurements, indicating that biomass was enriched in ^{13}C.

We found that there were significant differences in community structure of the 4-species assemblage across the temperature gradient (PERMANOVA, $F_{1,18} = 34$, $P = 0.001$, Fig. 4). Generally, we saw large increases in the relative proportion of *A. penicillioides* and declines in *A. tetracladia* with temperature. Furthermore, when we compared the parameters measured in the 4-species assemblage to the null expectation of the TA, we found many significant differences (Fig. 5). We found that biomass accrual was greater than expected (mean difference = 1.92 mg C, $t_4 = 3.06$, $P = 0.038$), as was CUE (mean difference 0.48, $t_4 = 3.88$, $P = 0.018$). In addition, biomass C:P was higher in the 4-species assemblage than in the TA (mean difference = 66, $t_4 = 3.21$, $P = 0.033$), but there was no difference in biomass C:N between the TA and 4-species assemblage ($t_4 = 1.50$, $P = 0.21$). Finally, the $\delta^{13}C$ of the 4-species assemblage was lower than in the TA ($t_4 = 3.47$, $P = 0.026$). While we found significant differences between the TA and actual 4-species assemblage, we did not detect linear relationships between temperature and the magnitude of the difference between our expectations (TA) and any of the measured microbial parameters in the 4-species assemblage (Fig. 5, Table S1).

Discussion

In evaluating the influence of a 4°C to 20°C temperature gradient on aquatic fungi, we predicted that warming would increase C:N and C:P ratios, while decreasing CUE, and lead to changes in $\delta^{13}C$. We found that temperature had effects on each of the parameters for at least one of the fungal treatments but many of these effects were quadratic rather than exponential over the temperature range we evaluated, and more often with peaks, but occasion-

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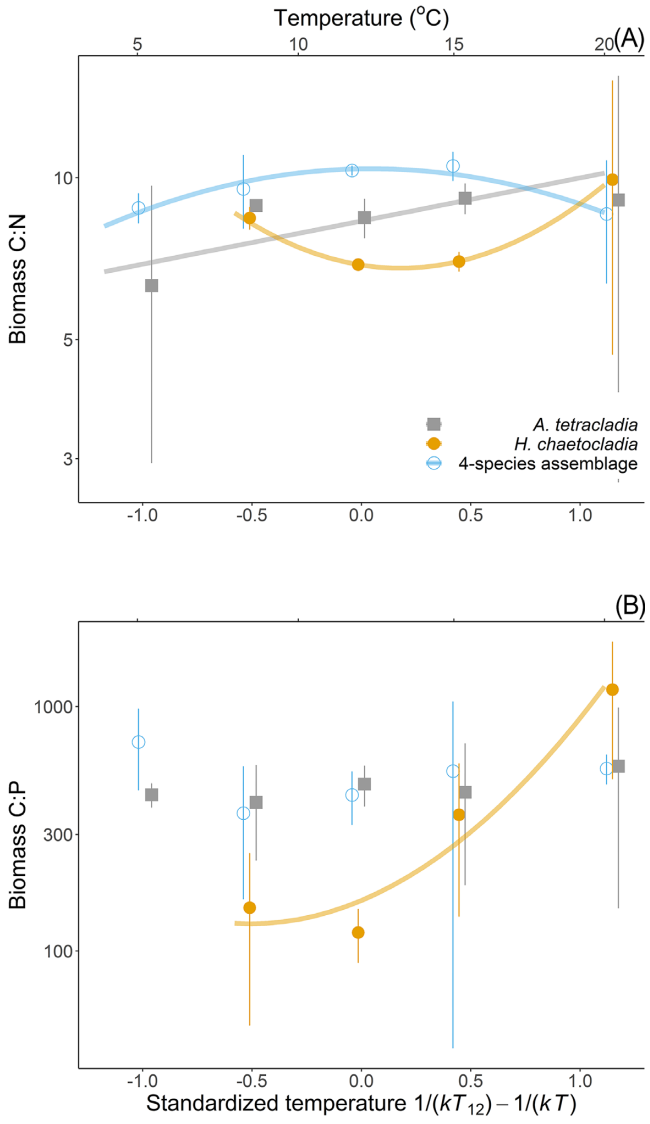

Figure 2. Biomass C:N **(A)** and C:P ratios **(B)** across temperatures. Temperature is transformed as the inverse Boltzmann temperature and is centered on 12°C (T_{12}). Treatments are jittered on the *x*-axis. Points represent the mean of four replicates and error bars represent 95% confidence intervals of the mean. Colors and shapes represent the different species treatments. Fitted lines are drawn for the best model (parameters reported in Table 1).

ally with minima, at intermediate temperatures. Thus, our results suggest that biomass accrual, C:N, C:P, CUE, and C isotope discrimination of aquatic fungi may increase or decrease with temperature, depending on the range of temperature considered and the taxon involved. Our results also highlight the importance of community processes in driving ecosystem-relevant parameters. We found that biomass accrual, C:P, and CUE were all higher (and $\delta^{13}C$ was lower) in the 4-species assemblage than in the theoretical assemblage, which suggests that interactions among taxa modified these functional parameters.

Microbial CUE is a critical parameter in the global C cycle as it defines the proportion of C that is incorporated into microbial biomass vs. what is lost to the atmosphere through respiration (Geyer et al. 2016). Prior studies have found declines in CUE with temperature (Lehmeier et al. 2016, Maynard, Crowther and Bradford 2017, Li et al. 2019), implying a greater response of respiration than biomass production to temperature. Here, we found considerable variation in CUE among taxa; however, most species treatments exhibited quadratic relationships between temperature and CUE, with declining CUE at higher temperatures. While low CUE of two species could be explained by species-specific traits or suboptimal conditions in our microcosms, some other CUE values were unusually high. For instance, the observed CUE of the 4-species assemblage approached 90% at some temperatures, which is unrealistically high (Pirt 1965, Roels 1980). Previous estimates of CUE for aquatic hyphomycetes have not exceeded 60% (2003a, Gulis and Suberkropp 2003c), suggesting there may have been some bias in our estimates of either C consumed or biomass accrual. This may have resulted from the large dilutions required to quantify DOC, or from variable abiotic losses of DOC across different temperatures, which we were unable to evaluate. Of our four focal species, three were isolated from a mountain stream that only reaches 15 to 18°C in summer, so these taxa are likely adapted to relatively low water temperatures. In contrast, *A. penicillioides* was isolated from a river with much higher temperatures. Thus, the thermal regimes of the source habitats for these taxa appear to play a role in how they responded to the gradient of temperatures we used (Padfield et al. 2017).

The stoichiometry of available nutrients and C relative to biomass is an important constraint on how ecosystems respond to warming (Buchkowski et al. 2019, Maaroufi and De Long 2020). While some previous studies of the effect of temperature on biomass stoichiometry found evidence for increases in C:nutrient ratios with temperature (Yvon-Durocher et al. 2015), the results of our experiment were more variable, with quadratic effects more common than simple exponential increases. Threshold elemental ratios are the ratios of elemental supply at which limitation of growth switches from one element to another, and biomass stoichiometry plays a role in determining these ratios. Recent analyses have found that threshold elemental ratios are U-shaped over a large temperature range, and lowest at intermediate temperatures (Ruiz et al. 2020, Laspoumaderes et al. 2022). When we calculated rough approximations of threshold elemental ratios with our data, we found the same U-shaped pattern in 5 out of 6 cases, with minimum threshold elemental ratios occurring between 8 and 14°C (supplemental data SD2). While we found effects of temperature on fungal biomass stoichiometry, it may be difficult to generalize how fungi respond to temperature, perhaps due to the limited elemental homeostasis, including flexibility of P content due to P storage, known to occur in aquatic fungi (Danger, Gessner and Bärlocher 2016, Gulis et al. 2017).

We found that fungi were generally enriched in ^{13}C relative to the growth medium and that isotopic fractionation varied across fungal and temperature treatments. Shifts in $\delta^{13}C$ are often used as evidence of changes in the sources of C being used (Phillips et al. 2014) and shifts in the $\delta^{13}C$ of respired CO_2 during field warming experiments have been taken as evidence of changes in microbial substrate utilization (Dorrepaal et al. 2009). Our experiment used a single source of C and found different degrees of isotopic fractionation between biomass and substrate at different temperatures. This highlights a potential challenge in using shifts in $\delta^{13}C$ as an indicator of shifts in substrate use with warming temperatures. Generally, isotope discrimination happens during the assimilation or uptake of elements, the synthesis of new molecules, or the excretion of waste products (Blair et al. 1985, Abraham, Hesse and Pelz 1998, Šantrůčková et al. 2000, Fry 2006). Prior studies have found that the difference between biomass and substrate $\delta^{13}C$ changes over the course of an incubation, with $\delta^{13}C$ values of biomass and substrate becoming more similar over time

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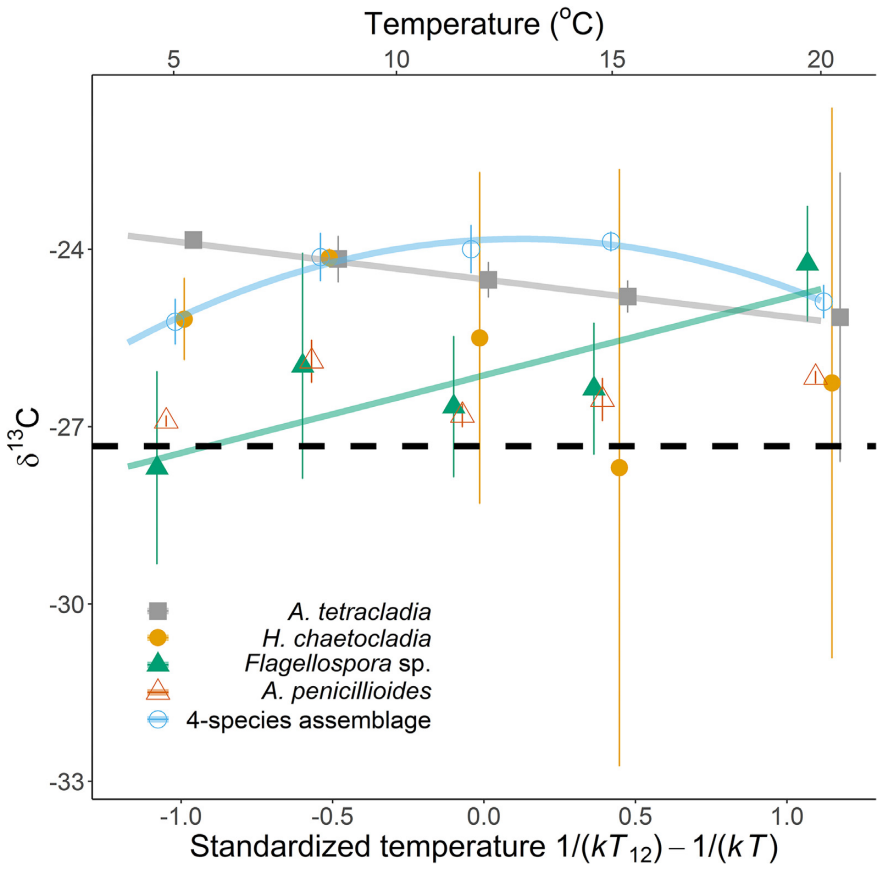

Figure 3. Values of $\delta^{13}C$ in fungal biomass across temperatures for the different species treatments. Temperature is transformed as the inverse Boltzmann temperature and is centered on 12ºC (T_{12}). Treatments are jittered on the *x*-axis. Points represent the mean of four replicates and error bars represent 95% confidence intervals of the mean. Colors and shapes represent the different species treatments. Data for fungal treatments are fit to the exponential or quadratic model (parameters reported in Table 1). For some taxa the null model best described the data, and no fit is shown for these taxa. The dashed line indicates the $\delta^{13}C$ of the growth medium.

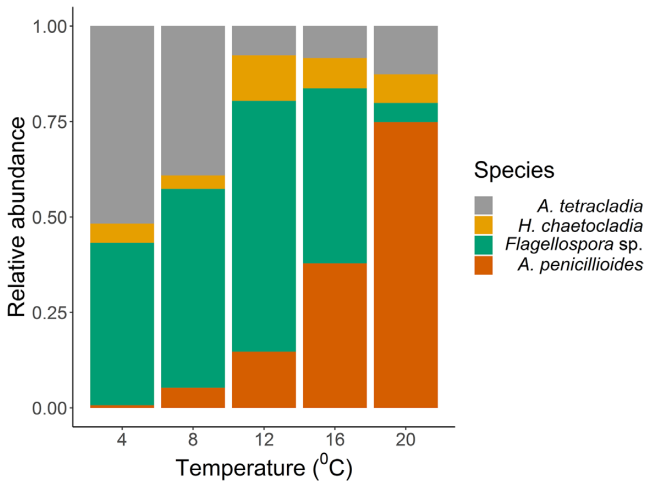

Figure 4. Relative abundance of fungal taxa in the 4-species assemblage at different temperatures based on analysis of amplified ITS rDNA by denaturing gradient gel electrophoresis (DGGE gels are shown as Figure S1 in Supplemental material). Bars represent the average across the replicate microcosms.

as substrate is depleted (Henn, Gleixner and Chapela 2002). If substrate depletion was driving patterns of isotopic discrimination in our experiment, we would expect smaller differences between the medium and the biomass in the chambers with more growth. However, we found chambers that accumulated more biomass tended to have $\delta^{13}C$ values that were more different from the medium than the chambers that accumulated less biomass (Fig. S2), suggesting that substrate depletion dynamics were not the primary driver of $\delta^{13}C$ patterns in our experiment. Steady-state cultures of *Pseudomonas fluorescens* have revealed that discrimination between ^{13}C in biomass and CO_2 increased with temperature (Lehmeier et al. 2016), suggesting that the physiological processes underlying isotopic discrimination may change with temperature. While we suspect that physiological changes are the primary cause of the differences in biomass $\delta^{13}C$ we observed, it is also possible that differences in the $\delta^{13}C$ of the carboxymethyl groups vs. glucose subunits in carboxymethylcellulose, and the relative enzymatic degradation and use of these two subcomponents, changed with temperature.

Interactions among fungal taxa may also play an important role in modifying functional traits, and these interactions may be sensitive to environmental conditions such as temperature (Lammel et al. 2023). A previous study with terrestrial wood-decomposing fungi found that monocultures had higher CUE than a mixed community, an effect that was attributed to the costs of interspecific competition (Maynard, Crowther and Bradford 2017). Maynard, Crowther and Bradford estimated CUE from measurements of respiration and production, which is distinct from our methods based on biomass produced relative to C consumed (Geyer et al. 2016). In addition, we used a more chemically complex substrate than easily digestible carbohydrates, which may

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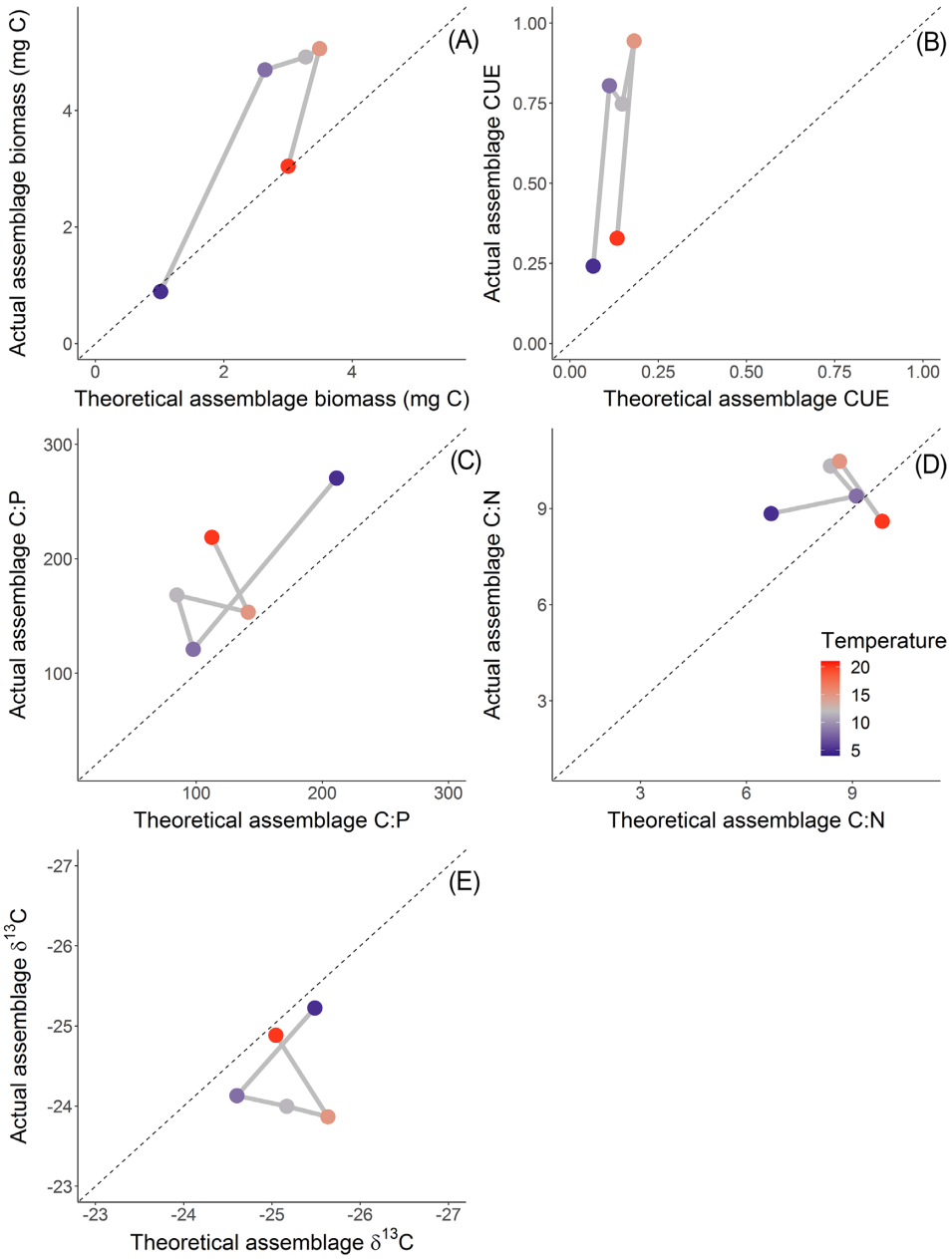

Figure 5. Comparison of biomass accrual **(A)**, carbon use efficiency (CUE) **(B)**, C:P **(C)**, C:N **(D)**, and δ^{13}C **(E)** between the theoretical assemblage (TA, proportional mixture of 4 species, no interactions or shifts in relative abundances) and the actual experimental 4-species assemblage. Deviations from the dashed 1:1 line indicate shifts in the relative abundance or performance of individual species in the 4-species assemblage due to interactions among the species. Gray lines connect adjacent temperature treatments. Results of the *t*-tests comparing the TA to the actual 4-species assemblage, and regressions evaluating the effect of temperature are reported in Table S1.

have also affected our CUE estimates. We found higher CUE in the 4-species assemblage than in our monocultures, suggesting mutualistic or synergistic effects rather than competition; however, potential bias in our estimates of CUE limits the strength of inference from this comparison. On a natural substrate such as plant litter, niche-partitioning could be a possible explanation, but the use of essentially single C, N and P sources in our experiment make this mechanism unlikely, though the CMC-based medium we used is relatively recalcitrant compared to easily digestible carbohydrates (Maynard, Crowther and Bradford 2017). The seemingly mutualistic interaction that we observed is somewhat surprising given that some species of aquatic hyphomycetes, and at least two of the species used in our experiment, are known to produce antimicrobial, sometimes antifungal, secondary metabolites (Gulis and Stephanovich 1999, Gulis and Suberkropp 2003c). However, leaf-decaying aquatic hyphomycetes as used in our experiment are generally less antagonistic than wood-decaying fungi (Shearer 1992). The identity of taxa involved in interspecific interactions is an important factor determining how interactions may play out and so influence ecosystem processes (Webster, Moran and Davey 1976, Frund et al. 2013, Ferreira et al. 2014).

In batch experiments such as ours, kinetic effects of substrate depletion can drive patterns across treatments, but we do not believe that this was an issue in our experiment. Initial masses of C, N, and P per microcosm were ~ 85, 7, and 0.97 mg, respectively. The maximum amount of N and P incorporated into biomass was 0.87

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and 0.19 mg, respectively, representing substrate depletion of only 12.4 and 19.5%, respectively. In the treatment with the most substrate C consumed, there was still ∼48 mg of substrate C remaining at the conclusion of the experiment, indicating that ∼43% of C was consumed. Thus, substrate depletion was unlikely to be a major factor driving changes in biomass stoichiometry, CUE, or isotope ratios across the treatments and variation among treatments was likely due to fungal responses to temperature.

The CUE and biomass stoichiometry of organisms are critically important traits as they have ecosystem-level consequences by altering the fluxes of elements within and among ecosystems (Ylla, Canhoto and Romaní 2014, Hood et al. 2018). Previous work with algae, plants, and animals indicates that C:nutrient ratios tend to increase with temperature (Woods et al. 2003, Reich and Oleksyn 2004). Our work highlights that, while the biomass stoichiometry of aquatic fungi does appear to be affected by temperature, the relationships are more variable, even over a relatively moderate gradient in temperature. These changes in biomass stoichiometry should influence whole-ecosystem rates of nutrient cycling (Webster, Newbold and Lin 2016), though the direction and magnitude of the change likely depends on the taxa present and the range of temperatures considered. Similarly, even though our temperature gradient barely extended beyond what most temperate stream fungi would experience naturally over the course of a year, we found that their growth and CUE generally had quadratic responses to temperature, with peak biomass accrual and CUE often at temperatures considerably lower than 20°C. Thus, in temperate forest streams the effects of warming on CUE may also have an important seasonal component, with fungal growth stimulated and CUE increasing in the winter when resources (i.e. leaf litter) are plentiful, while possibly being suppressed by higher temperatures during summer.

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Supplementary data

Supplementary data are available at *FEMSEC* online.

Conflict of interest. None declared.

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Data availability statement

The data and code associated with this manuscript are accessible at https://zenodo.org/record/7194880#.Y3ZdH3bMJaQ

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