Title: Predation by a ciliate community mediates temperature and nutrient effects on a peatland prey prokaryotic community.

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Running Title: Predation and temperature affect bacterial communities.

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Abstract:

Temperature significantly impacts microbial communities' composition and function, which subsequently plays a vital role in the global carbon cycle that ultimately fuels climate change. Interactions between different microorganisms might be critical in shaping how these communities react to both temperature changes. Additionally, rising temperatures are occurring in the context of increasingly nutrient-rich ecosystems due to human activity. Nonetheless, we lack a comprehensive understanding of how predation influences microbial communities in future climate scenarios and an increasingly nutrient-rich world. Here, we assess whether predation by key bacterial consumers—ciliates—influences a microbial community's freshwater temperature and nutrient response regarding biomass, diversity, structure, and function. In a three-week microcosm experiment, we exposed mostly prokaryotic microbial communities to a community of ciliate predators at two different temperature scenarios (ambient and +3°C, i.e., a conservative projection of climate change by 2050) and nutrient levels (low and elevated). Nutrients, temperature, and ciliate presence influenced microbial biomass and function separately, but their interaction had the largest explanatory power over the observed changes in microbial community biomass, structure, and function. Our study supports previous findings that temperature and nutrients are essential drivers of microbial community structure and function but also demonstrates that the presence of predators can mediate these effects, indicating that the biotic context is as important as the abiotic context to understand microbial responses to novel climates.

Importance:

While the importance of the abiotic environment in microbial communities has long been studied, how prevalent ecological interactions, like predation and the broader abiotic context, may influence these responses is largely unknown. Our study disentangles the complex interplay between temperature, nutrients, and predation and their joint effects on microbial community diversity and function. The findings suggest that while temperature and nutrients are fundamental drivers of microbial community dynamics, the presence of predators significantly mediates these responses. Our study underscores the profound impact of abiotic factors on microbial communities, but how to properly understand, let alone predict, these responses, we need to account for the biotic context in which these are occurring.

Introduction:

Rising global temperatures are currently affecting populations [1,2], communities [3], and ecosystems [4] through an increase in metabolic rates [5,6]. Increased metabolic rates then lead to increased energetic demands for organisms within populations [7–9], and this has cascading effects across ecological scales[5,10–12]. In addition to temperature-driven ecological change, ecosystems face increasingly large nutrient loads from anthropogenic sources (e.g., agricultural runoff) and increased mineralization rates[13], which have long been known to impact the structure and dynamics of communities and ecosystems [14–19]. While the independent effects of rising temperature and nutrients on ecological processes are relatively well understood [20–22], joint increases in temperature and nutrients have been shown to have interactive effects that can be hard to predict and are thus poorly understood [23–28]. Yet, this understanding is central to assessing whether and how ecological communities will respond to an increasingly warm, nutrient-rich, and human-dominated world.

Some of the most consequential biological communities worldwide are also some of the least well-understood –prokaryotic communities– which comprise upwards of 14% of all existing biomass on Earth [29]. These communities are present on all continents and ecosystems [30,31], and their central role in the global carbon and nutrient cycles –which ultimately determine the pace of global climate change– is increasingly recognized [32,33]. For example, prokaryotic decomposition recycles organic material into food webs [34,35], and prokaryotic respiration is one of the atmosphere's most significant sources of carbon dioxide (CO_2) [36,37]. These respiration rates can change dramatically across temperatures via shifts in individual metabolic rates [5–7,38–41], which could result in a "warming begets warming" scenario. Therefore, how prokaryotic

communities respond to joint changes in temperature and nutrients is central to understanding and better predicting the global effects of climate change.

In addition to rapid abiotic change, prokaryotic communities worldwide will likely also respond to a rapidly changing biotic environment as food webs rewire under climate change [42–44]. These shifting ecological interactions often determine community and ecosystem-level processes [45]. For example, predation is a crucial factor leading to decreased biomass in prokaryotic communities[46–49], and bacterivores are predicted to increase foraging rates to offset metabolic costs [50]. This increase in bacterivory –and predation, in general– may impact the composition and function of many prokaryotic communities in a rapidly changing world [49,51–53]. However, how these prokaryotic communities may respond to both temperature increases and nutrient additions in the presence of predators is an active area of research [54].

Ciliates are among the planet's most abundant predators of prokaryotes [29,30,55,56]. These microbial predators show strong, well-known temperature [41,57–59] and nutrient responses [28,60–62], as well as temperature-dependent population dynamics [41,59] and feeding interactions [6,58]. Ciliates have been shown to affect prokaryotic community structure, dynamics, and function strongly [63] and can affect species interactions and ecological dynamics within microbial food webs [28,58,64]. Under warmer conditions, predation by ciliates could, therefore, significantly impact prokaryotic respiration and overall function. Whether this would result in a "warming begets warming" scenario or a "warming dampens warming" scenario, however, depends on the specific temperature response of the predators and the prey and how this ecological interaction constrains the rising temperature and nutrient effects.

We thus examine whether and how predation of prokaryotes by a ciliate community mediates the combined effects of nutrient additions and temperature on community biomass,

diversity, composition, and function (measured as total respiration rates). We used experimental microcosms containing a synthetic community of ciliates preying on a natural peatland prokaryotic community to address the questions: 1) How do temperature, nutrients, and the presence of a ciliate community affect prokaryotic community biomass, diversity, and composition? 2) How do these factors affect total community respiration? 3) How does the ciliate community, in turn, respond to imposed changes in temperature and nutrients?

We hypothesize that prokaryotic biomass and respiration rates should increase under warming temperatures and high nutrient conditions [65,66]. We should also observe that ciliate predation decreases prokaryotic biomass [67,68], reducing total respiration rates [46], which also should result in a shift in the composition of the prokaryotic community due to differential consumption by ciliates on different species[67,68]. As predation by a single ciliate species has been observed to result in an interactive effect of predation, temperature, and nutrients on prokaryotic community respiration and biomass (Rocca et al. 2022), we hypothesize that an entire ciliate community should also have an interactive effect of temperature and nutrients on prokaryotic community respiration and biomass. Last, a recent study has shown that natural ciliate communities can respond interactively to temperature and nutrients[69,70], so we expect that the predator ciliate community should also respond differently to temperature depending on nutrient levels.

Results

Effects of temperature, nutrients, and ciliate presence on total prey community biomass and respiration

Preliminary data analysis suggested that ciliate presence, temperature, and nutrient levels, as well as their interactions, likely influenced OD600, while nutrient levels were the most important variable explaining respiration, followed by the presence of ciliates and temperature (Supplementary Figures S4, S5; Appendix 3).

Additional analyses showed that temperature, nutrients, and the presence of a ciliate community all had independent effects on total community biomass (OD600, Figure 1). Higher temperature (Figure 1A) and low nutrient levels (Figure 1B) resulted in less total biomass (i.e., smaller OD600, temp effect = -0.008 ± 0.003 SE, t-value = -2.788, df = 118, p-value = 0.006; nutrient effect = 0.007 ± 0.003 SE, t-value = 2.524, df = 118, p-value = 0.012), while the presence of ciliates resulted in more overall biomass (Figure 1C, effect = 0.009 ± 0.003 SE, t-value = 3.025, df = 118, p-value = 0.003). Temperature, nutrients, and the presence of ciliates had a significant interactive effect on total community biomass (Figure 1D, 1E). Under high nutrient levels, the presence of ciliates increased OD600 (effect = 0.016 ± 0.007 SE, t-value = 2.041, df = 112, p-value = 0.043, Figure 1D, 1E) compared to the treatments that do not have ciliates. Moreover, how nutrients affected total biomass in the presence of ciliates varied across temperatures (i.e., three-way interaction, effect = -0.035 ± 0.011, t-value = -3.215, p-value = 0.001, Figure 1D, E). Specifically, under low nutrient conditions, the microbial biomass increased in response to warmer temperatures when ciliates were added compared to a decrease in the absence of ciliates. Under high nutrient conditions, there was a decrease in the response to warming of the microbial OD600 in the presence of ciliates but no change in the absence of ciliates.

Respiration rates were not independently influenced by temperature (Figure 2A) or ciliate presence (Figure 2C) but were affected significantly by nutrient level (Figure 2B, effect = 2.213

$\times 10^{-4} \pm 4.84$ SE, t-value = 4.564, df = 118, p-value = 1.24×10^{-5}). However, respiration rates increased with temperature only under high nutrient levels for treatments that did not have ciliates, suggestive an interactive effect of nutrient levels and temperature (Figure 2D, 2E, effect = $3.313 \times 10^{-4} \pm 1.654 \times 10^{-4}$, t-value = 2.004, df = 112, p-value = 0.047).

Effects of temperature, nutrients, and ciliate presence on the prey community structure

Across all treatments, Proteobacteria were the most abundant group in the prey prokaryotic community, followed by Firmicutes and Cyanobacteria (Figure 3A). Temperature, nutrients, and ciliate presence all had a significant interactive effect on Shannon diversity (ANOVA, F = 13.449, p-value = 3.76×10^{-4}, Figure 3B), while temperature and ciliate presence also had a significant interactive effect (ANOVA, F = 7.3073, p-value = 0.007). Shannon diversity increased in the presence of ciliates under high nutrient levels and low temperatures (22°C, Figure 3B). In the high temperature (25°C), however, Shannon diversity increased in the absence of the ciliates and high nutrients but decreased in the presence of the ciliates and high nutrients (Figure 3B). This three-way interaction is likely the result of an increase in the dominance of Firmicutes relative to Proteobacteria in conditions of low nutrients, low temperature, and ciliate presence (Figure 3A).

Beta diversity was significantly affected by temperature, nutrient levels, and the presence of ciliates (Temp: PERMANOVA, F = 4.919, p-value = 0.001. Nutrients: PERMANOVA, F = 4.625, p-value = 0.001. Pro: PERMANOVA, F = 15.913, p-value = 0.001. Figure 3C). In addition, all two-way interactions and the triple interaction between temperature, nutrients, and ciliates were significant (Temp:Nutrient, PERMANOVA, F = 2.551, p-value = 0.002, Temp:Ciliate, PERMANOVA, F = 2.218, p-value = 0.008, Nutrient:Ciliate, PERMANOVA, F =

1.939, p-value = 0.011, Temp:Nutrient:Ciliate, PERMANOVA, F = 2.253, p-value = 0.003, Figure 3C). These results suggest that while prey alpha diversity remained constant across treatments, the composition responded strongly to all variables and their interaction, indicating substantial species turnover among treatments.

Effects of temperature and nutrients on the predatory ciliate community

Across all treatments, *Blepharisma sp.* was the most abundant ciliate species, followed by *Colpidium sp.* and *Paramecium bursaria* (Figure 4A); two species, *Tetrahymena pyriformis* and *Cyclidium glaucoma,* were not present in any samples. This is most likely due to being outcompeted and eaten by other ciliates within the system. Nutrient levels significantly impacted predator ciliate community alpha diversity, while temperature or temperature and nutrient interactions did not. Shannon diversity was higher in low nutrient conditions (ANOVA, F = 6.370, df = 1, p-value = 0.013 Figure 4B). All population counts were similar across experimental treatments except for *Blepharisma sp*. counts, which were significantly affected by temperature, nutrients, and their interaction (temperature effect = -205.533 ± 68.833 SE, t-value = 2.986, p-value = 0.003; nutrient effect = -277.450 ± 97.902 SE, t-value = -2.834, p-value = 0.005; nutrient:temp effect = 10.650 ± 4.161 SE, t-value = 2.559, p-value = 0.012).

Interestingly, the imposed treatments seem to have shaped the composition of the ciliate communities in such a way that three distinct community configurations were possible, each corresponding to a principal component: a *Colpidium sp*., *Paramecium bursaria, Paramecium aurelia,* and *Glaucoma sp*. dominated community (PC1, Figure 4D), a *Blepharisma sp.* and *Colpoda steinii* dominated community (PC2, Figure 4D), and a *Halteria grandinella*, and *Strombidium sp*. dominated community (PC3, Figure 4D). The overall PCA was significantly

affected by nutrient level and its interaction with temperature (Nutrient: PERMANOVA, F = 2.144, df = 1, p-value = 0.029; Nutrient:Temp: PERMANOVA, F = 2.022, df = 1, p-value = 0.034). This suggests that a combination of direct environmental effects and indirect responses of the prey community to the abiotic manipulations likely influence the structure and composition of the predator community.

Joint direct and indirect effects of temperature and nutrients on the prokaryotic and ciliate communities

The SEM model fitted the data well (Fisher.C = 5.017, p-value = 0.286) and confirmed that temperature alone influences prey biomass (0.72, p-value = 0.024) and the Shannon diversity of the prey community (1.328, p-value = 0.0001). However, the SEM suggested that neither temperature, nutrients, nor their interaction influence the predator's community structure (Fig 5), which does not align with our earlier findings (Fig 4). Nutrients alone affected total microbial biomass (5.31, p-value = 0.0009), diversity of the prey community (5.839, p-value = 0.0006), and the biomass of the ciliate predators (4.13, p-value = 0.0133), but not the diversity of ciliates or the overall function (Fig 5). The interaction between temperature and nutrients directly affected ciliate biomass (standardized effect size (ses) = -3.7601, p-value = 0.0240), the biomass of the prey (ses = -5.36, p-value = 0.0011), and prey community diversity (ses = -5.930, p-value = 0.0007). Total functioning (respiration) was directly affected by the biomass of the predators (ses = 0.44, p-value = 0.003) and prey (ses = -0.28, p-value = 0.028) and ciliate diversity (ses = 0.32, p-value = 0.006), and was indirectly affected by nutrients through the biomass of ciliates (Fig 5).

Additionally, the SEM uncovered critical indirect effects of temperature and nutrients, notably, an indirect negative effect of temperature on total respiration through prey microbial biomass (Fig 5). Nutrients, in turn, had two distinct indirect pathways: one positive indirect effect through ciliate biomass and one negative indirect effect through ciliate biomass's effect on prey microbial biomass (Fig 5). Last, temperature and nutrient interaction also indirectly affected respiration through their effect on ciliate biomass, and the effects of ciliate biomass directly on respiration and indirectly on respiration through total prey microbial biomass (Fig 5).

Discussion

Shedding light on how changes in environmental temperature and nutrient levels may impact prokaryotic biomass and function (e.g., total respiration) is crucial to our ability to recognize and predict future warming trends [33]. First, our study shows how temperature and nutrient levels determine a prokaryotic community's biomass, composition, and respiration rates (Figures 1, 2, 3). Second, results reveal that a ciliate predator community influences these responses (Figures 1A, 1B, and 1C). Last, we observe that the predatory community's biomass, diversity, and composition respond to temperature and nutrient levels directly, to shifts in the prey prokaryotic communities they prey upon, or a combination of both (Figure 4, Figure 5). These results indicate that accounting for ecological interactions between prokaryotic prey and eukaryotic predators is important to understanding prokaryotic communities' responses to compound environmental changes.

Increased temperatures and low nutrient levels reduced microbial biomass (Figures 1A and B), consistent with results in non-prokaryotic taxa across ecosystems[46,87]. Interestingly, joint increases in temperature and nutrient levels have been predicted to increase microbial biomass

by intensifying eutrophication [27,88,89]. We, however, do not see an increase in biomass in the high nutrient-high temperature treatment, perhaps due to the resilience of prokaryotic biomass to changes in nutrient loads through compositional shifts [90]. While under elevated temperatures, prokaryotic communities are known to shift to be mesophilic and perform anaerobic respiration; thus, the prevalence of Firmicutes tends to be higher [91,92]. Proteobacteria are found in higher relative abundances in warmer temperatures; perhaps this is most likely due to the presence of ciliates (also observed in Rocca *et al.* 2022) and could represent prey switching by the ciliates. The composition of the prokaryotic community, however, responded strongly to all treatments and their interaction, as beta diversity was significantly affected by temperature, nutrient levels, and the presence of ciliates (Figure 3C).

Total community respiration rates increased with temperature, but only under high nutrient levels (Figures 2D and 2E), regardless of ciliate presence. These results are partly consistent with theoretical expectations of increased metabolic demands with temperature [5,7,47] and, hence, respiration rates [40,93–95]. However, they also suggest that changes in metabolic demands with temperature depend on nutrient availability: metabolic rates cannot increase unless enough nutrients fuel that increases. Further, this result implies that under low nutrients, the function of these microbial communities is less likely to change with warming unless there is a concomitant increase in nutrient levels to allow for increased function [96]. Alternatively, increased respiration rates due to nutrient enrichment could indicate increased metabolic activity in organisms due to increased turnover [97,98].

Moreover, while temperature, nutrient levels, and ciliate presence interactively influenced prokaryotic biomass (Figure 1), only nutrient levels significantly impacted total respiration (Figure 2). In other words, despite the effects of ciliate presence on prey prokaryotic community

structure and biomass, there seems to be no effect on its function. Yet, recent studies have argued that direct and indirect effects of ecological interactions can influence the function of prokaryotic communities in shifting abiotic conditions [49,56,99–101]. Indeed, previous work has shown that individual ciliate species can influence the biomass and the temperature response of respiration of a prokaryotic community [53], while one study even found that predation could reverse the effects of warming in a tundra community [102], which seems at odds with our results. On the other hand, the SEM did reveal that temperature, nutrients, and their interaction did indirectly affect – albeit weakly– total microbial respiration (Fig 5) through ciliate biomass, suggesting that direct effects of temperature and nutrients on the predatory ciliate community mediate their effect on total respiration rates and ecosystem function of these microbial communities. This seeming inconsistency in our results likely means that any effects on total microbial function may only be observed in simpler communities, but perhaps not speciose, more complex ones. Additionally, all indirect pathways are accounted for, highlighting the need for a deeper understanding of how predation by ciliates and other Eukaryotes impacts the global carbon cycle through its influence on the function of prokaryotic communities.

The predatory ciliate communities strongly responded to nutrient levels and their interaction with temperature (Figure 4). We showed that the alpha diversity of the predator ciliate community was higher under low nutrient levels (Figure 4B) and that nutrient levels affected community composition significantly differently across temperatures (Figure 4C). However, temperature can magnify or dampen this nutrient effect. Ciliate community composition (and function) can change across temperatures in both tightly controlled laboratory conditions [41] and whole-ecosystem warming experiments in boreal peatlands [70]. These recent

results are consistent with ours and suggest that neither nutrients nor temperature alone can explain observed complex shifts in these ciliate predator communities.

Caveats. We did not track the responses or dynamics of the prey prokaryotic and ciliate communities over time. Consequently, our results only represent a snapshot in time, and some of the effects of predation reported here may be transient. Also, our temperature manipulations only represent the effects of mean temperature in relatively stable conditions. So, our results should not be used to infer the broader consequences of seasonal temperature shifts, as we do not consider diurnal or seasonal temperature variation. As global temperatures rise, seasonality, in particular, is undoubtedly also changing [103,104]; it remains an open question whether the effects of temperature variability are distinct from those of changes in mean temperatures shown here. While the results presented are strong and clear enough to suggest that the processes at play may also be acting in nature, the short-term nature of our experimental manipulations and our experimental setup in tightly controlled experimental conditions inevitably limit the possible reach of our inference.

Conclusions. Our results shed some light on how warming may affect carbon sequestration in peatlands. Indeed, peatlands are exceptionally susceptible to future climate change impacts [105–107]. Despite covering less than 3% of the Earth's surface, these ecosystems store approximately 25% -30 % of the world's soil carbon as recalcitrant peat [108]. However, peat moss growth in nutrient-poor peatlands depends on symbiotic interactions with prokaryotic associates [109,110]. Shifts in prokaryotic community composition due to warming [111] may impact the activity of moss symbionts and other important microbes, potentially leading to reductions in carbon sequestration in these peatlands, through changes in moss growth [112]. Our results highlight that to fully understand the breadth and consequences of these changes, we need to

consider the interactive effects of rising temperatures and increasing nutrient loads in these rapidly changing ecosystems. Moreover, our results suggest that essential but largely overlooked predatory interactions between these organisms and their predators may be important to understanding and predicting how climate change may affect carbon dynamics in peatlands[13].

We highlighted critical temperature and nutrient effects on prey prokaryotic community structure and function. We also show that these effects are often interactive, suggesting that we cannot predict these microbial communities' response to climate change without considering possible eutrophication or other anthropogenic nutrient additions. Last, we show that ciliate predation mediates the effects of temperature, nutrients, and their interactions on prokaryotic communities. The predators themselves respond to these abiotic conditions, possibly through a combination of direct and indirect effects. Our study suggests interesting mechanisms for predation to influence prokaryotic function in a changing world.

Materials and Methods:

Prey Prokaryotic Community

We isolated a prey –mostly prokaryotic– community from a peatland bog in the Marcell experimental forest (Minnesota, USA) right next to the SPRUCE (Spruce and Peatland Responses Under Changing Environments) whole-ecosystem warming experiment [71]. These communities were isolated from 5 cm core samples containing a top layer of living *Sphagnum* moss tissue and a lower layer of peat. To isolate the prokaryotes, we filtered out larger fungi and ciliates using autoclaved filters (11 μm pore size, Whatman), then removed smaller flagellates and fungal spores [49] using sterile GF/A filters (1.6 μm pore size, Whatman). While the community is not guaranteed only to have prokaryotes (e.g., eukaryotic nanoflagellates cannot be

filtered out using this approach), it should be composed in its majority by Prokaryotes and Archaea [53] and will henceforth be called the prokaryote community.

Experimental Treatments

The resultant community was homogenized and incubated in 120, 200 mL acid-washed, and autoclaved borosilicate jars filled in 150 mL of Carolina Biological Ciliate culture medium [72]. Jars were assigned to one of two possible nutrient treatments: low nutrients (75 mL of media and 75 mL of distilled water, 60 jars) and high nutrients (150mL of media, 60 jars), and one of two possible temperature treatments (60 jars at 22°C, representing ambient temperature, and 60 jars at 25°C, representing an increase in the average temperature of 3ºC). All microcosms were incubated under controlled environmental conditions in Percival AL-22 L2 growth chambers (Percival Scientific, Perry, Iowa) at 10% light intensity (1700 lux), 75% humidity, and a 16:8 hr. day-night cycle for three days before further manipulation.

We added the synthetic ciliate communities after the three-day pre-incubation period to acclimate the prokaryotic communities. To this end, we assigned each experimental microcosm to one of two possible ciliate treatments (40 jars had no ciliates, and 80 jars were inoculated with ciliates). The asymmetrical design for the ciliate treatment was to ensure that even small or nuanced effects of predation could be detected. These "ciliate" treatment microcosms were inoculated with the ciliates *Paramecium aurelia, Paramecium bursaria, Halteria grandinella, Tillina magna, Tetrahymena pyriformis, Cyclidium glaucoma, Colpoda steinii, Strombidium sp., Blepharisma sp., Glaucoma sp.,* and *Colpidium sp.,* all of which naturally occur in *Sphagnum* peatlands [30]. The ciliates came from long-term (> five years) lab cultures [41], sourced from Carolina Biological (Burlington, NC, US) or Sciento (Manchester, UK), except for *P. bursaria,*

which was cultured from SPRUCE samples. Ciliates were introduced by pipetting well-mixed stock cultures at carrying capacity into experimental microcosms. Each species was introduced at a density of at least 17 individuals per jar (see Supplementary Table 1, ST1; Appendix 1 for initial densities). To control for possible effects of other microbes already occurring in the ciliate cultures –notably, prokaryotes– we added the same volume of filtered (11 μm pore size, Whatman), homogenized, ciliate stock media from the original cultures into all "non-ciliate" treatment microcosms in the identical amounts these were introduced in the ciliate treatments, as done elsewhere [53,63,73]. This inoculation of filtered media through filters allows the prokaryotes to pass through but filters out the ciliates to ensure that all experimental jars start with almost identical prey prokaryotic communities. Following standard ciliate culturing procedures [72], we also added one wheat seed (~35 mg ea.) to high-nutrient treatments and half of a seed (~17.5 mg ea.) to the low-nutrient treatments to serve as a slow-release carbon source and prevent resource limitation issues [72]. The wheat seeds were pre-autoclaved and sourced from the same batch to control additional wheat seed-associated microorganisms.

Microcosms were assigned to one of eight possible treatments: (1) 22°C, no ciliates, low nutrient; (2) 22°C, no ciliates, high nutrient; (3) 25°C, no ciliates, low nutrient; (4) 25°C, no ciliates, high nutrient; (5) 22°C, ciliates, low nutrient; (6) 22°C, ciliates, high nutrient; (7) 25°C, ciliates, low nutrient; and (8) 25°C, ciliates, high nutrient.

Measurement of Prey Community Biomass, Diversity, and Respiration Rate

After 21 days, we quantified the prey community's biomass, diversity, and total community respiration rate to assess the individual and joint impacts of temperature, nutrient levels, and ciliate predation. We measured the optical density at 600 nm wavelength (or OD600) [74] as a

proxy for the total biomass of each community microcosm using a BioTEK Epoch-2 microplate spectrophotometer (Winooski, VT, USA). Greater OD600 measurements (indicating increased absorbance and scattering) signify elevated overall biomass [74]. Protists scatter light to a lesser extent due to their larger size and reduced densities [64]. Community respiration was determined using an optode-based real-time OXY-4 SMA respirometer (PreSens, Regensburg, Germany) [72,75]. The respiration rate was measured on 150 ml samples for at least 30 minutes, with a collection rate of one measurement every three seconds, after a 30-min acclimation period (n = 120) at their original experimental temperature and in the dark. Respiration rates were estimated as the rate of change (slope) of the estimated oxygen concentration over time (in μmol O_2/min; Supplementary Figures S1, S2, and S3; Appendix 2) following standard optode-based closed-system respirometry procedures [76].

Analysis of Prey Community Biomass, Diversity, and Respiration Rate Data

To understand the joint effects of temperature, time, and ciliate presence on biomass (OD600) and respiration, we did exploratory data analysis using the R package "MuMIn." In doing so, we fitted all possible models containing OD600 and respiration as the response variable and all combinations of temperature, nutrients, ciliate presence, and their interactions as explanatory variables. To disentangle the joint effect of nutrients, temperature, and ciliates on the response variables, we used multi-model inference to measure the relative importance of each model term using the package MuMIn version 1.47.1 [77]. This approach fits all possible models –i.e., models considering all possible combinations of explanatory variables and their interactions–then ranks them all by AIC to calculate the relative importance of each model term across all possible models [77]. To assess whether total community biomass, diversity, and respiration rate changed

with experimental treatment, we used linear models with ciliate presence, nutrient levels (high or low), and temperature (22 or 25°C) (and their possible interactions) as explanatory variables, and either biomass, diversity, or respiration rates as response variables. Response variables were log-transformed, while all explanatory variables were treated as discrete. All analyses were done in R version 4.2.2 [78]. One of the 80 ciliate jars had no ciliates at the end of the experiment and was discarded.

Prey Community Structure: Amplicon Sequencing and Processing

We used 16S rRNA amplicon sequencing to examine the impacts of temperature, nutrient level, and ciliates on the community structure of the prey prokaryotic community. After 21 days of incubation, we collected the prokaryotic communities by pelleting 1 ml from each microcosm into 1.5 mL Eppendorf tubes and storing them at -20°C until DNA extraction. Genomic microbiome DNA was isolated with an Omega Mag-Bind Environmental kit. Amplification and preparation followed the Illumina 16S rRNA amplicon sequencing protocol with a custom mixture of 515F and 806R primers [79] for archaea/bacteria and 18SV4F and 18SV4R primers [80] targeting the 18S region. Prokaryotic sequences were processed with the QIIME 2 v 2021.2 platform [81]. Paired sequences were demultiplexed with the plugin demux and quality filtered (denoised, dereplicated, chimera filtered, and pair-end merged) and processed in Amplicon Sequence Variants (ASVs) with the DADA2 plugin [82]. Taxonomy was assigned using a pre-trained Naive Bayes classifier based on the Silva database (version 138) trimmed to the 515F/806R primer pair (16S rRNA). Sequence variant-based alpha diversity (Shannon) and beta diversity (Bray Curtis distance) were calculated with the phyloseq package [83]. Alpha diversity was calculated from the analysis of variance tests of the relationship between alpha diversity and

moss species. Beta diversity was calculated from a linear model test of the relationship between Bray Curtis distance and site.

Ciliate Responses

The ciliate community may itself respond to the imposed nutrients and temperature treatments and understanding that response is crucial to shedding light on the direct and indirect effects of temperature and nutrients on the prokaryotic community. To understand how the predator-ciliate communities may have been affected by the treatments, we estimated ciliate population sizes at the end of the experiment through fluid imaging of 1 mL subsamples of all microcosms using a FlowCam (Fluid Imaging Technologies, Scarborough, ME, United States). We used linear models to assess whether ciliate population counts changed with either temperature or nutrient level. Shannon diversity was calculated using the diversity() function in the R package "vegan" (v2.6-4;[84]). Using principal components analysis on abundance data, we assessed whether the overall ciliate community structure responded to temperature and nutrient levels. We tested for individual and interactive treatment effects with a perMANOVA with the adonis2() function in the R package "vegan" (v2.6-4;[84]).

Direct and indirect influences on microbial communities using Structural Equation Modeling

Last, we assessed the possibility of joint direct effects of temperature and nutrients on the prokaryote community and indirect effects of temperature and nutrients through their effects on the ciliates, using Structural Equation Models we fitted piecewiseSEM version 2.3.0 [85]. Compared with the traditional variance covariance-based SEM, the piecewise SEM (1) pieces

together multiple separate (generalized) linear models into a single causal network, (2) uses Shipley's test of d-separation to test whether any paths are missing from the model, and (3) uses Akaike information criterion (AIC) to compare nested models. We then automatically calculated direct, indirect, and total effects for piecewise structural equation models using the package semEff version 0.6.1 [86] in R. We checked the model fit using Fisher's C test at the 0.05 level where the p-value needs to be larger than 0.05 for the model to describe the data appropriately.

Data Availability

The sequence data has been deposited in GenBank SRA under accession PRJNA1095004; https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1095004. All other data and code have been deposited in GitHub: https://github.com/kmd304/Predation-Mediates-TempXNut.git.

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Figures

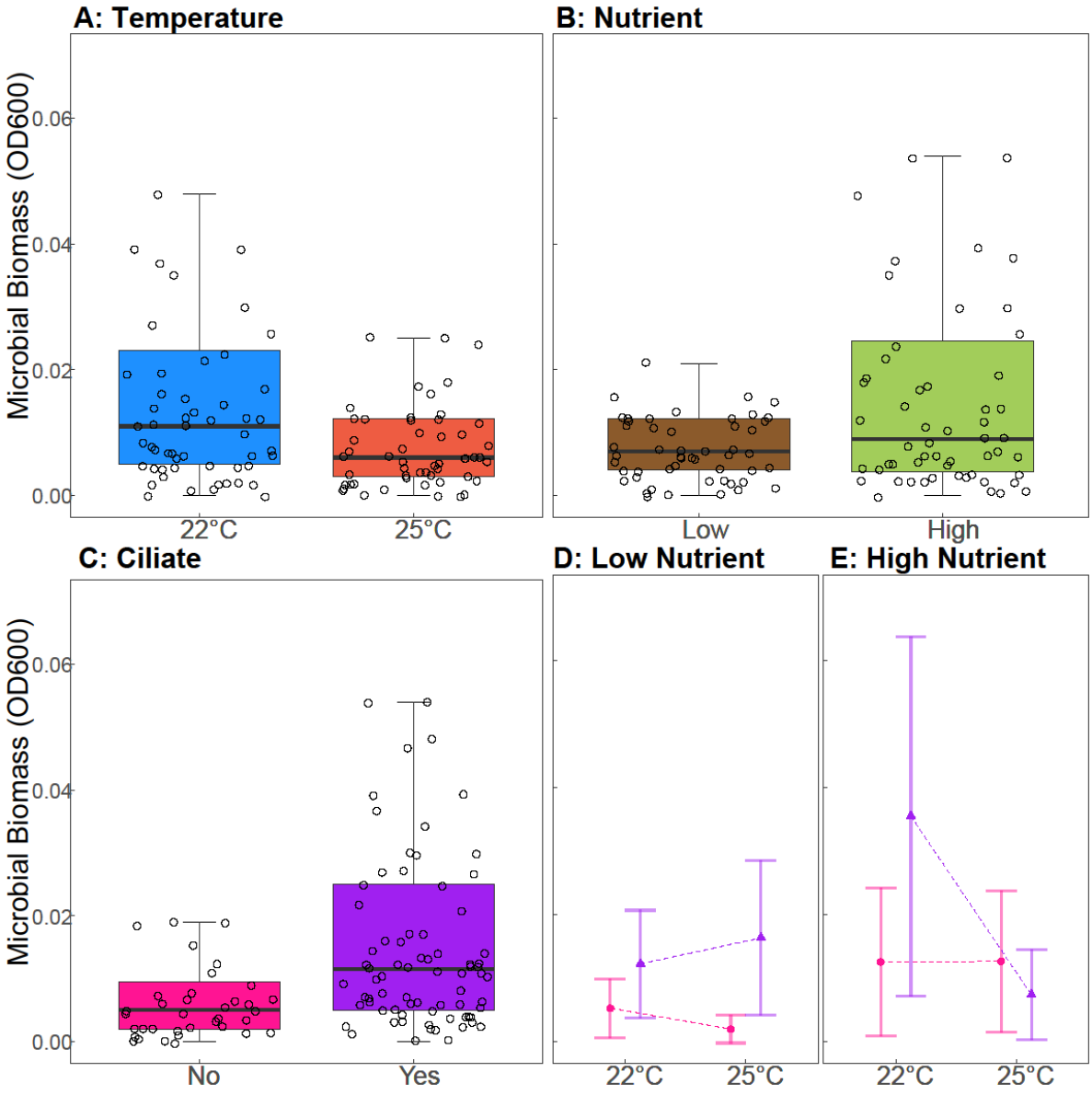

Figure 1. The independent and interactive effects of temperature, nutrients, and ciliate presence on prokaryotic biomass. (A) Boxplots of the temperature, (B) the nutrient level, (C) presence of ciliate community effects on prokaryotic biomass, (D) interaction between temperature, low nutrient level, and ciliate presence on prokaryotic biomass, and (E) interaction between temperature, high nutrient level, and ciliate presence on prokaryotic biomass. Lines within the

boxplots represent median, 25th, and 75th percentile values, while whiskers are defined by the largest value not greater than 1.5× the interquartile range (IQR). Individual open circles represent raw data points. Pink colors represent treatments with no ciliates, while purple represents those that have the addition of ciliates.

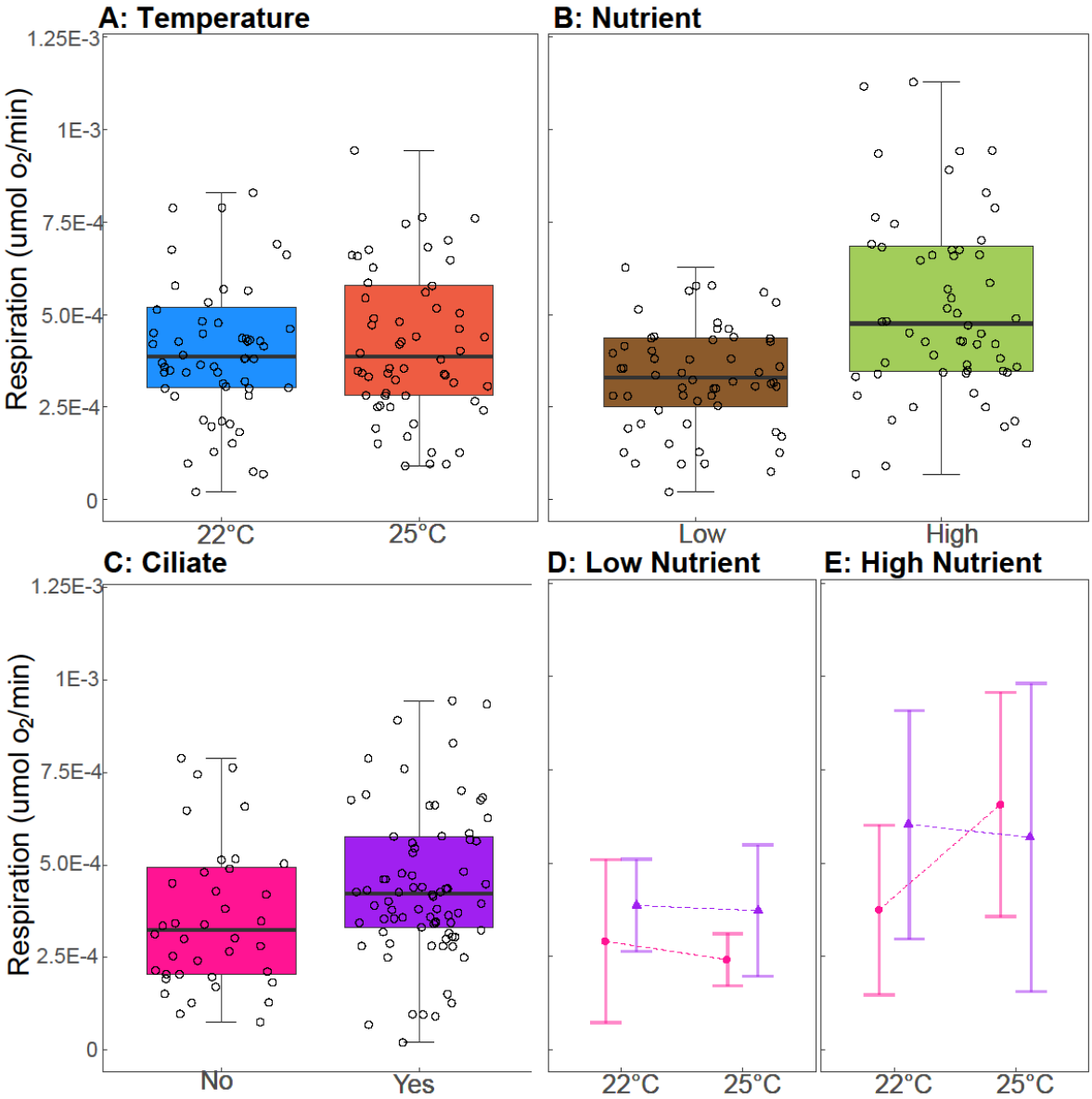

Figure 2. The independent and interactive effects of temperature, nutrients, and ciliate presence on prokaryotic respiration. (A) Boxplots of the temperature, (B) the nutrient level, (C) presence of ciliate community effects on prokaryotic respiration, (D) interaction between temperature, low nutrient level, and ciliate presence on prokaryotic respiration, and (E) interaction between temperature, high nutrient level, and ciliate presence on prokaryotic respiration. Lines within the boxplots represent median, 25th, and 75th percentile values, while whiskers are defined by the

largest value not greater than 1.5× the interquartile range (IQR). Individual open circles represent raw data points. Pink colors represent treatments with no ciliates, while purple represents those that have the addition of ciliates.

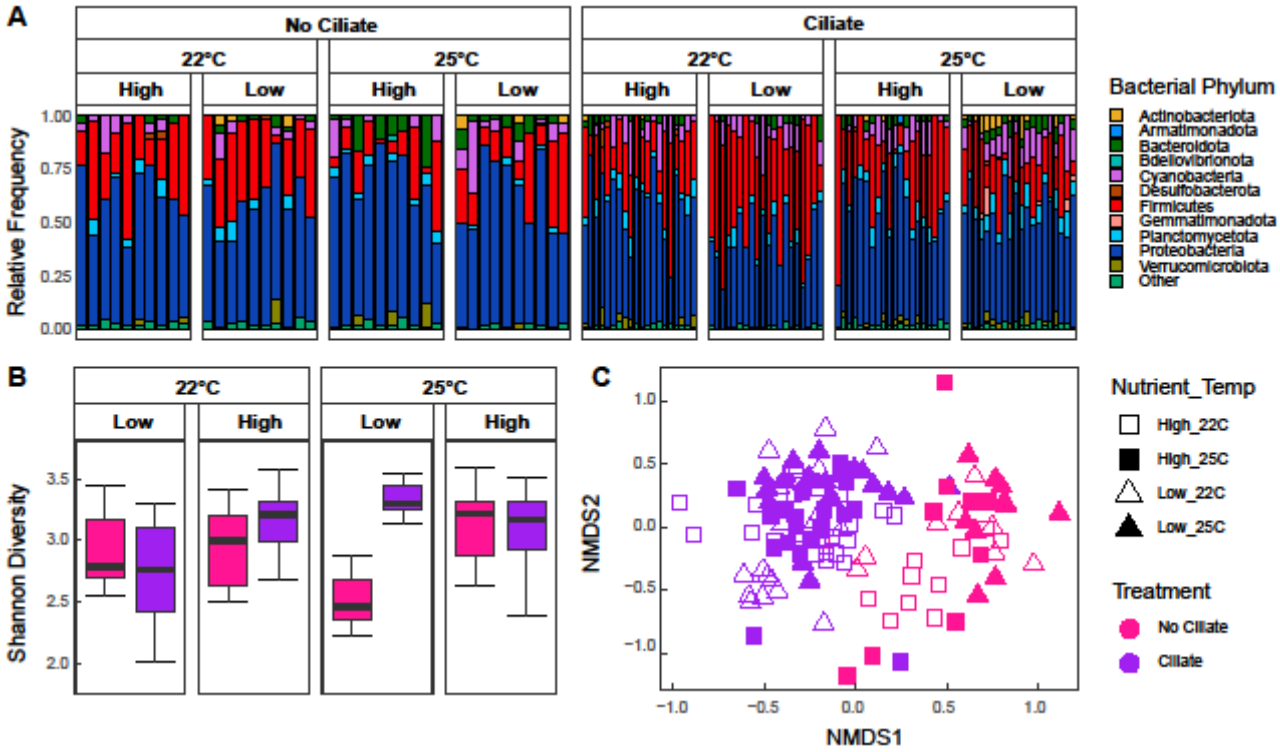

Figure 3. (A) Phyla relative abundances of prokaryotic (16S rRNA) prokaryotic communities across treatments. (B) Boxplot of alpha diversity of prokaryotic communities across temperature, nutrient level, and presence of ciliate community. Lines within the boxplots represent median, 25th, and 75th percentile values, while whiskers are defined by the largest value not greater than 1.5× the interquartile range (IQR). (C) NMDS ordination plot of Bray-Curtis dissimilarity of prokaryotic community composition (16S rRNA). Pink colors represent treatments with no ciliates, while purple represents those that have the addition of ciliates. Squares represent high

nutrient conditions, while triangles represent low nutrient conditions. If the shape is filled in with color, it is experiencing warming (25°C), whereas those that are not filled in are at base conditions (22°C).

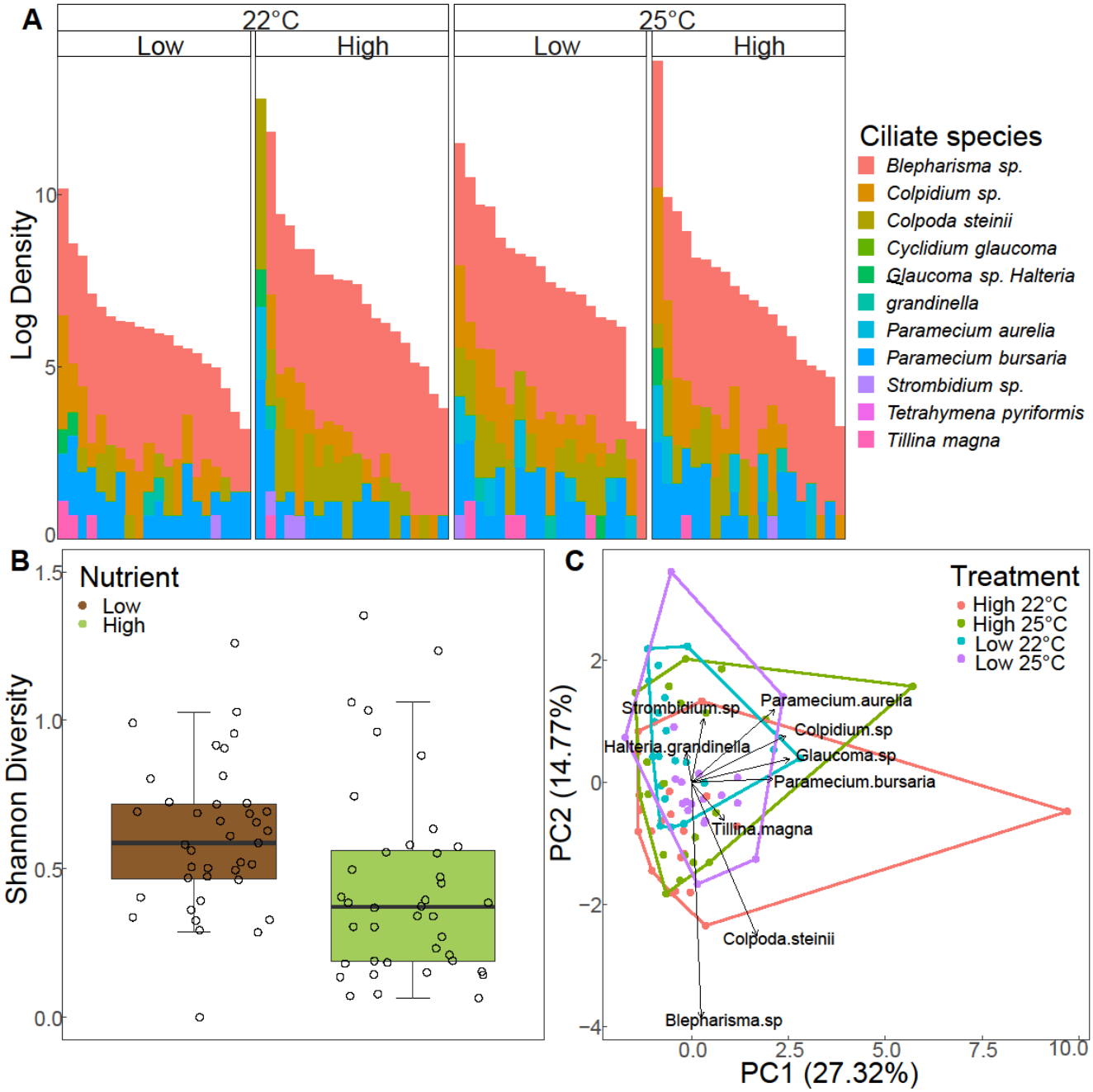

Figure 4. (A) Final density data of ciliate communities for each sample across treatments. (B)

Boxplot of alpha diversity of ciliate communities across temperature and nutrient level. Lines within the boxplots represent the median. (C) The ordination plot is based on a principal components analysis of ciliate community composition. Arrows show dominant communities' variable loadings within the functional trait space, while individual points show individual samples and are colored by treatment. Individual open circles represent raw data points. Lines within the boxplots represent median, 25th, and 75th percentile values, while whiskers are defined by the largest value not greater than 1.5× the interquartile range (IQR).

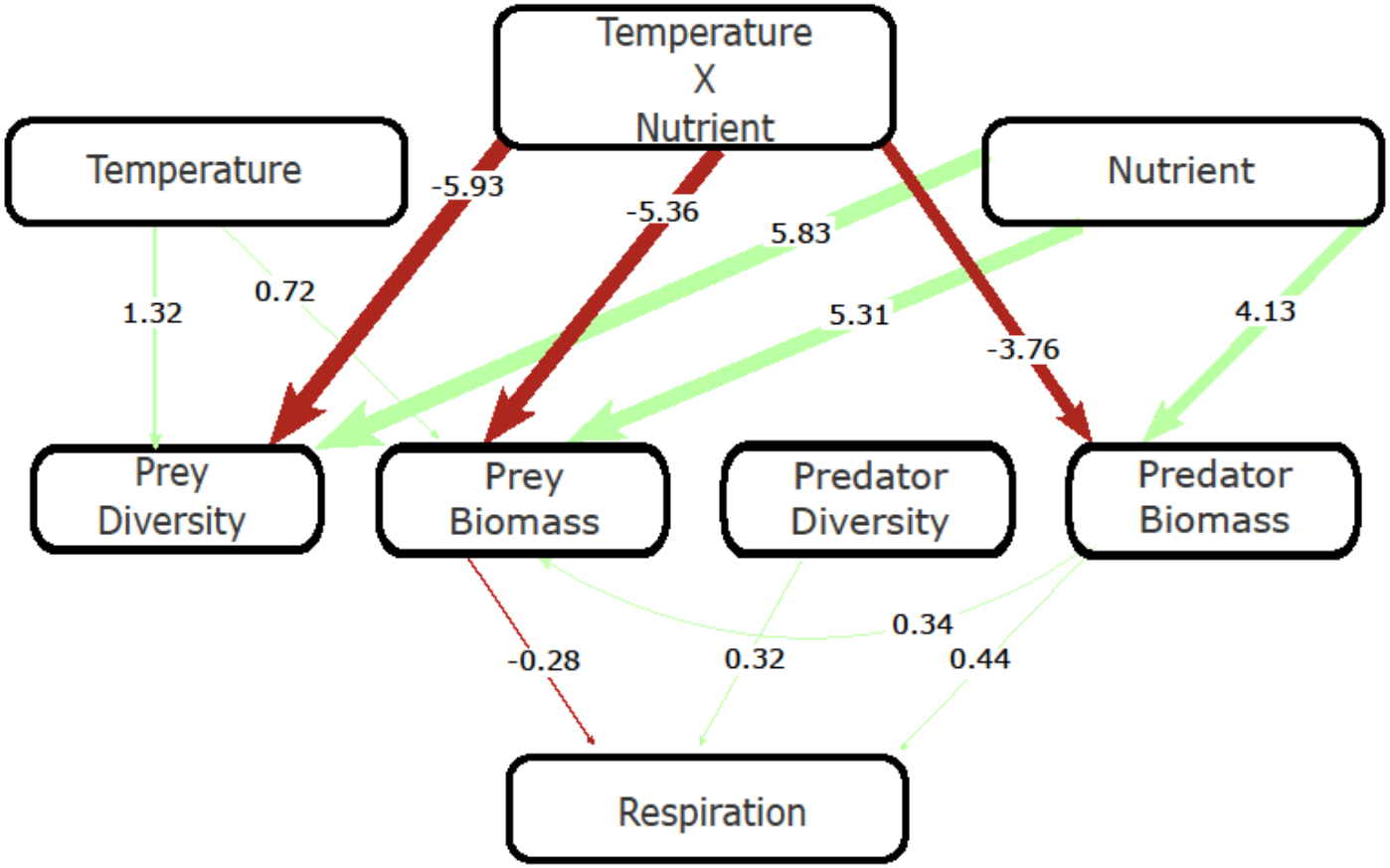

Figure 5. Structural equation model showing proposed relationships between latent variables temperature, nutrients, and their interaction on diversity and biomass of microbes and their function. All numbers correspond to the standardized path coefficients. Solid arrows shown are significant direct effects ($p < 0.05$). Red arrows represent a negative relationship, while green shows positive.