Climate Change Factors Interactively Shift Peatland Functional Microbial Composition in a Whole-Ecosystem Warming Experiment

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Microbes affect the global carbon cycle that influences climate change and are in turn influenced by environmental change. Here, we use data from a long-term whole-ecosystem warming experiment at a boreal peatland to answer how temperature and CO_2 jointly influence communities of abundant, diverse, yet poorly understood, non-fungi microbial Eukaryotes (protists). These microbes influence ecosystem function directly through photosynthesis and respiration, and indirectly, through predation on decomposers (bacteria, fungi). Using a combination of high-throughput fluid imaging and 18S amplicon sequencing, we report large climate-induced, community-wide shifts in the community functional composition of these microbes (size, shape, metabolism) that could alter overall function in peatlands. Importantly, we demonstrate a taxonomic convergence but a functional divergence in response to warming and elevated CO_2 with most environmental responses being contingent on organismal size: warming effects on functional composition are reversed by elevated CO_2 and amplified in larger microbes but not smaller ones. These findings show how the interactive effects of warming and rising CO_2 could alter the structure and function of peatland microbial food webs — a fragile ecosystem that stores 25% of terrestrial carbon and is increasingly threatened by human exploitation.

climate change | microbial diversity | community ecology | functional traits
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Introduction

Greenhouse gas emissions set the pace of climate change (1) and most ecosystems on Earth influence this process by serving as carbon (C) sources or sinks (2). Overall, terrestrial ecosystems mitigate ≈30 % of anthropogenic CO_2 emissions (3, 4). The predominant terrestrial carbon sink — peatlands — store 100% more carbon than all forests while covering less than 3% of the globe (5–8). *Sphagnum* mosses dominate peatlands (9) and are responsible for most C sequestration in the form of recalcitrant peat (10). They harbor diverse communities of single-celled (*i.e.,* bacteria, archaea, protists) and multi-celled (*i.e.,* fungi, metazoa) organisms (11) that influence C cycling directly (12, 13), through respiration of ≈100 $Pg \cdot C \cdot yr^{-1}$ in soils alone (14, 15). These organisms indirectly influence the C cycle by supplying nitrogen needed for *Sphagnum* moss growth (16–18), converting methane into the CO_2 necessary for photosynthesis (19–21), providing stress tolerance to the host mosses (22, 23), and assisting in host-pathogen defense (24).

How ecological interactions between members of the moss-associated community influence their effects on peatland C cycling (25–27), is poorly understood. Predation by metazoans, in particular, plays a prominent role in peatland C cycling (28), and is likely to also impact microbial communities (29, 30). Unicellular Eukaryotes collectively known as protists store two times more C globally than all animals combined (31, 32), contribute to C cycling through mixotrophic metabolism (33), and also serve as one of the principal biological controls on microbial respiration and photosynthesis through predation and competition (30, 34, 35). Protists also directly influence plant growth and health through their effects on the rhizosphere (34, 36, 37) and phyllosphere (38, 39) — thus indirectly influencing plant growth through their effects on beneficial microorganisms (29, 40). In turn, climate effects can induce changes in moss microhabitat that affect predation, resource acquisition, and life cycles of associated protists(33).

But, despite important advances (50–53), how the structure and function of protist communities may respond to climate change in peatlands remains poorly understood. Simple protist functional traits — *i.e.*, characteristics controlling how organisms respond to the environment or affect ecosystem properties (54) — including protist cell size, cell shape, and cellular contents (Table 1), can be used to predict warming effects on protist communities and ecosystem function (43). This is due to underlying links amongst these traits, metabolism, respiration rates, and energy acquisition mode (43, 44). By quantifying changes in functional traits of these protist communities — in addition to changes in abundances, biomass, and community composition — we can infer changes to their ecological function in these peatland ecosystems. For example, metabolic demand and total respiration rates increase with increasing temperature, favoring smaller organisms (47, 55) with a shape (long and slim) that maximizes energy intake and minimizes energetic costs (42, 56). Increased metabolic demands can impact resource acquisition strategies (e.g., increased heterotrophy over autotrophy) (57–60), resulting in measurable changes in cellular optical properties such as red/green color ratio or the heterogeneity of gray-scale values (*e.g.* sigma intensity),

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Table 1. Functional Justification of Selected Traits Related to Metabolism

Trait	Functional Linkage	References
Cell Size	Cell size determines the metabolic costs of single-celled organisms, and is well-known to decline with temperature through the temperature-size rule.	Brown et al. (41), Atkinson (42), Wieczynski et al. (43)
Cell Shape	Cell shape captures information on the surface area to volume ratio of unicellular organisms (i.e., rounder cells have a lower surface/volume ratio than oblong cells), thereby mediating diffusion of material across membranes and establishing metabolic costs of this transport.	Wieczynski et al. (43), Gibert et al. (44), Glazier (45), Hirst et al. (46)
Cell Contents	Measured as the optical heterogeneity of a cell, our measure of cellular contents (vacuoles, cytoskeleton, organelles), captures information on cellular storage and metabolism, e.g. *the metabolic machinery of the cell*.	Wieczynski et al. (43)
Resource Acquisition Mode	The ratio of the red-to-green reflectance signal of a cell — where higher redder cells are more likely heterotrophic while greener cells are more likely autotrophic — indicate the resource acquisition mode (heterotrophy, autotrophy, mixotrophy) of the cell, which in turn influences function and C cycling.	Atkinson et al. (47), Gibert et al. (48), Nakano et al. (49)

which have been linked to cellular contents and cell viability (61, 62). Consequently, changes in functional composition could alter net C fluxes and other elements within peatlands (63–66).

Here, we address how peatland protist communities — operationally defined as the community of all non-fungal Eukaryotes — change in novel climates. We quantify shifts in abundance, biomass, taxonomic composition, and functional trait composition in a peatland long-term whole-ecosystem experiment with temperature and CO_2 manipulations of living moss and superficial peat at the SPRUCE (*Spruce and Peatland Responses Under Changing Environments*) long-term (since 2014) whole-ecosystem warming experimental site (67) (Fig. 1A). We quantified protist abundance, biomass, and community functional traits through fluid imaging and used 18S rRNA gene sequencing to infer mechanisms of change in observed protist community functional trait composition (e.g., through taxonomic shifts or plasticity). We test the hypotheses that increasing temperatures lead to: (1) a decrease in protists' body size — i.e., the temperature-size rule (42, 47), (2) an increase in surface-to-volume ratio (aspect ratio), as protists optimize shape to increase energy uptake (surface) and reduce metabolic costs (volume) (68), (3) an increase in cellular optical heterogeneity (sigma intensity or cellular "contents"), as temperature-induced increased metabolic rates requires more cellular machinery (69), and (4) increased heterotrophy (increased red/green ratios) to keep up with metabolic demands. Additionally, we expect elevated CO_2 to result in an increase in total community biomass (70, 71) as more CO_2 fuels photosynthesis at the base of the food web.

This CO_2 driven increase in energy availability at the base of the microbial food web should also result in (Fig. 1C): 1) an increase in protist volume in response to more energy availability, 2) an increase in aspect-ratio as increased available energy releases cells from shape metabolic constraints, 3) an increase in sigma intensity, due to increased production of cellular machinery to process available energy, and 4) an increase in autotrophy (measured as decreases in the red/green ratio of cells) relative to heterotrophy (47–49). We show that warming and CO_2 have significant, negative interactive effects, on the taxonomic and functional composition of peatland protist communities that is in turn dependent on organismal size and could result in important consequences for C cycling in peatlands.

Materials and Methods

A. Study Site and Field Sampling. The study site is within the S1-Bog of the Marcell Experimental Forest (47° 30.4760′ N, 93° 27.1620′ W; 418 m above mean sea level), MN, USA. It sits on the southern edge of the temperate boreal forest range in North America, with a sub-humid continental climate. The S1-Bog — above a water table with marginal

groundwater influence — consists of an ombrotrophic peatland dominated by a forest canopy of black spruce (*Picea mariana*) and tamarack (*Larix laricinia*). Understory plants are mixture of ericaceous scrubs, herbaceous sedges and grasses, and a bryophyte layer dominated by *Sphagnum* spp. mosses. Mosses are distributed in hollows (*S. angustifolium* & *S. fallax*) and in drier hummocks (*S. divinum* previously called *S. magellanicum* and *S. fuscum*). Other bryophytes include feather mosses (*Pleurozium* spp.) and haircap mosses (*Polytrichum* spp.). For further description of the site and below-ground peat profile, see Hanson et al. (2016) and Tfaily et al. (2014).

The *P. mariana–Sphagnum* spp. raised bog experimental ecosystem consists of open-top chambers 12m in diameter in a factorial design of ambient 450 ppm and elevated 900 ppm CO_2 treatments across ambient (+0, +2.25, +4.5, +6.75, +9°C) soil (initiated June 2014) and air (initiated June 2015) warming treatments (Fig. 1; (72)). After 5 years of treatment — in September 2019 — we collected superficial samples (1-5 cm) from SPRUCE containing living plant tissue and superficial peat from two sub-plot locations within each enclosure. We limited sampling efforts to a minimally sufficient amount of material to avoid disturbing the overall peatland community and interfering with long-term experiments at SPRUCE. We placed the samples in individual sterile bags and overnight shipped them to Durham, NC for imaging of protist communities while additional samples were flash-frozen and over-night shipped to Oak Ridge, TN for DNA extraction and 18S amplicon sequencing. Upon reception of samples for imaging, we removed the material from sterile bags, placed them in cloth sieves within glass funnels, and washed them thoroughly with Carolina protist media (Liquid Protozoan Nutrient Concentrate; 13-2350). We collected the discharge from the funnels in 250 ml glass jars and kept the cultures at 22°C and 65% humidity for equilibration for 24 hours before fluid imaging. All samples were processed and imaged in a simultaneous, treatment-blind fashion, and within 24-36 hours of collection in the field. While samples could have been fixed *in situ* with lugol or formaldehyde, these fixative

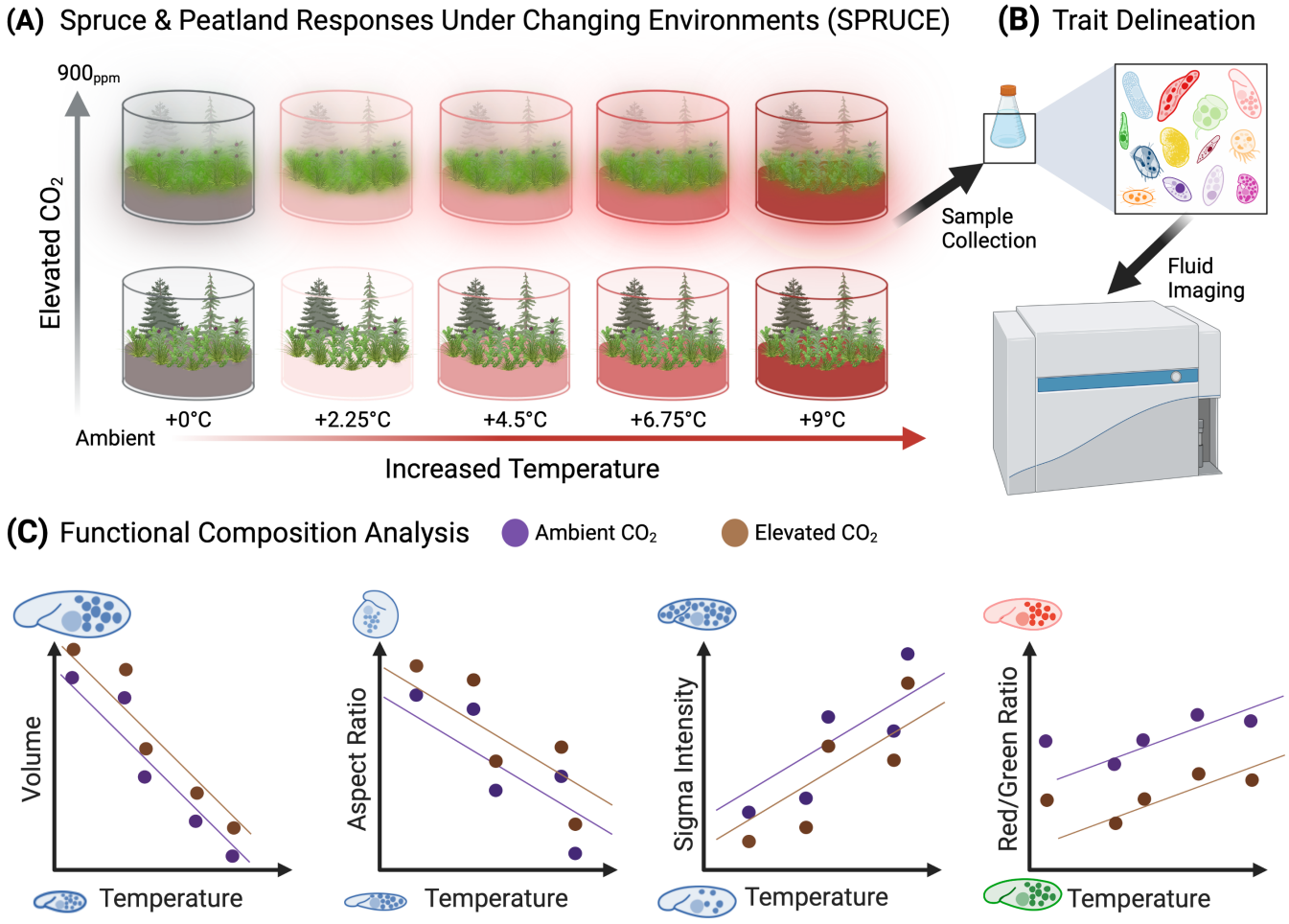

Fig. 1. (A) Samples were collected in a 2-factorial mesocosm design in boreal peatlands with variation in temperature (+0, +2.25, +4.5, +6.75, +9°C) and CO_2 (450 PPM, 900 PPM) concentrations. Protist samples were collected from the upper peat layer within each treatment and processed *ex situ*. (B) After equilibration (24 hours in climate-controlled growth chambers, Carolina protist liquid media, 22°C and 65% humidity), samples were analyzed via fluid imaging to quantify abundances across treatments and physical and optical functional traits. (C) Functional composition data hypothesized on first-principles of metabolic and ecological theory. We anticipate decreases in size (e.g., volume), shape (e.g., aspect ratio), and cellular contents (e.g., sigma intensity) under warming, with an increase in red/green ratio (e.g., photosynthesis). Under elevated CO_2 we expect larger cell volumes, more roundedness, increased cellular activity, and lower red/green ratios.

methods often alter the size and shape of cells and lead to an under-representation of larger size classes (74, 75), and would bias our quantification of functional trait shifts across size classes with temperature and CO_2.

B. Density and Biomass. Protists were operationally defined in this study as all non-fungal unicellular Eukaryotes. Their densities were estimated as simple counts ($\hat{E}$) of the number of imaged particles per volume of fluid analyzed through fluid imaging with a FlowCAM (Yokagawa Fluid Imaging, USA) at 10× magnification of 6 mL water subsamples of processed SPRUCE samples. The FlowCam screens roughly 75% of the sampled volume (called, the "efficiency" of the machine), which was used to efficiency-correct biomass and abundance estimates from total protist volume and densities (see supplementary materials; Fig. S2).

C. Trait Measurements. We quantified functional traits (Table S1) by manually curating the ensuing cell images in Visual Spreadsheet software program (Yokagawa Fluid Imaging, USA), discarding all non-protist images, cysts, and other organic materials and debris, and retaining only high-quality protist images for trait quantification. All protist images (n = 211,039) were used to quantify densities. To test our hypotheses about size, shape, cellular contents, and energy acquisition mode, we used four traits (volume, aspect ratio, sigma intensity, red/green ratio) representing these functional trait categories from a larger set of 37 physical and optical traits; these representative traits were chosen due to their linkages to the functions of interest (Table 1) and by eliminating highly correlated and co-linear traits (see supplementary materials; Table S1, Fig. S1A). Wieczynski et al. (2021) found these four traits to influence temperature performance curves (TPCs) of protists and their metabolic functioning (Table 1).

D. Regression Analyses. We tested the effects of temperature and CO_2 levels on the abundance and biomass of the protist communities with generalized additive models (GAMs) and used generalized linear models (GLMs) to test for effects on the traits. We pooled the data from each enclosure subplot, as the level of inference is that of the enclosure (Fig. 1). To test hypotheses 1-4, we first clustered individual cellular observations into size classes based upon the natural log of protists' geodesic length (e.g., longest axis) using Gaussian finite mixture modelling with the R package *mclust* (76). We chose length as it was less correlated with the traits of interest and would reduce co-linearity in our models between the size class cluster and the functional traits (Fig. S1). We selected an optimal set of five size classes with a Gaussian mixture model and Bayesian Information Criterion (BIC) (Fig. S3). We then ran GLMs for each functional trait against size class (factor), temperature (continuous), and carbon dioxide level (factor). Associated Pearson correlation coefficients and p-values were obtained via the *ggpubr* R package (77) and are reported for each size class (Fig. S4). Additionally, we examined changes across environments in the estimated abundance and biomass (grams) of the community per size class with GAMs (k = 5) using the R package *mgcv* (78), reporting associated Pearson correlations and p-values for each size class (Fig. S5).

E. Structural Equation Modeling (SEM) and Functional Diversity. To quantify possible direct and indirect effects of Temperature and CO_2 on protist communities, we used Structural Equation Modeling (SEM) with the R package *lavaan* (79), a robust Maximum Likelihood (MLM) estimator, and the 'csolnp' optimizer for enhanced convergence properties. The first SEM considered the effects of the environment (Temperature, CO_2, and their interaction) on compositional (Observed Richness, Shannon Diversity, and Biomass) and functional trait diversity (Aspect Ratio, Red/Green Ratio, Sigma Intensity, and Volume), and also accounted for possible effects of community composition and structure on functional trait diversity. A second SEM included latent variables representing compositional (Observed Richness, Shannon Diversity) and functional trait diversity (Aspect Ratio, Red/Green Ratio, Sigma Intensity, and Volume). A bootstrap procedure with 1000 replications was used to estimate robust standard errors and p-values for all model effects. We assessed model fit through conventional indices for goodness-of-fit (Comparative Fit Index (CFI), Tucker-Lewis Index (TLI), Standardized Root Mean Square Residual (SRMR), and Root Mean Square Error of Approximation (RMSEA)). To quantify the functional diversity of our protist communities, we used the *FD* and *fundiversity* packages in R (80, 81), leveraging the mean trait values for each size class within treatments.

F. Amplicon Sequencing. To understand the possible mechanisms behind observed changes in abundance, biomass and functional traits, we extracted genomic DNA from our samples with the DNeasy 96 Plant Kit (Qiagen) and amplified the V4 region of the 18S rRNA gene following (82). Samples were multiplexed, pooled and sequenced on an Illumina MiSeq instrument with paired end kit (2 x 250 bp). We processed protist sequences with the QIIME 2 v 2021.2 platform. Paired sequences were first demultiplexed with the plugin demux; then, we quality filtered (denoised, dereplicated, chimera filtered and pair-end merged) and processed in Sequence Variants (SVs) with the dada2 plugin (83). Taxonomy followed the Pr2 (18S) database (84) and sequences assigned as "bacteria" or Embryophyceae were removed. We used sequence variants to calculate alpha diversity as counts and the Shannon diversity (calculated beta diversity) and compositional dissimilarity, or Bray-Curtis distance, with the *phyloseq* package (85).

Results

Experimental manipulations of temperature and CO_2 levels induced an 80-fold change (+/- 4.45%) in total observed protist densities (F = 2.2554; p = 0.02244, Fig. S5A) and biomass (F = 2.2504; p = 0.02273, Fig. S5B) across treatments (Fig. 2A), as well as a shift in the relative dominance of size classes (Fig. 2B). Across all body size classes, estimated

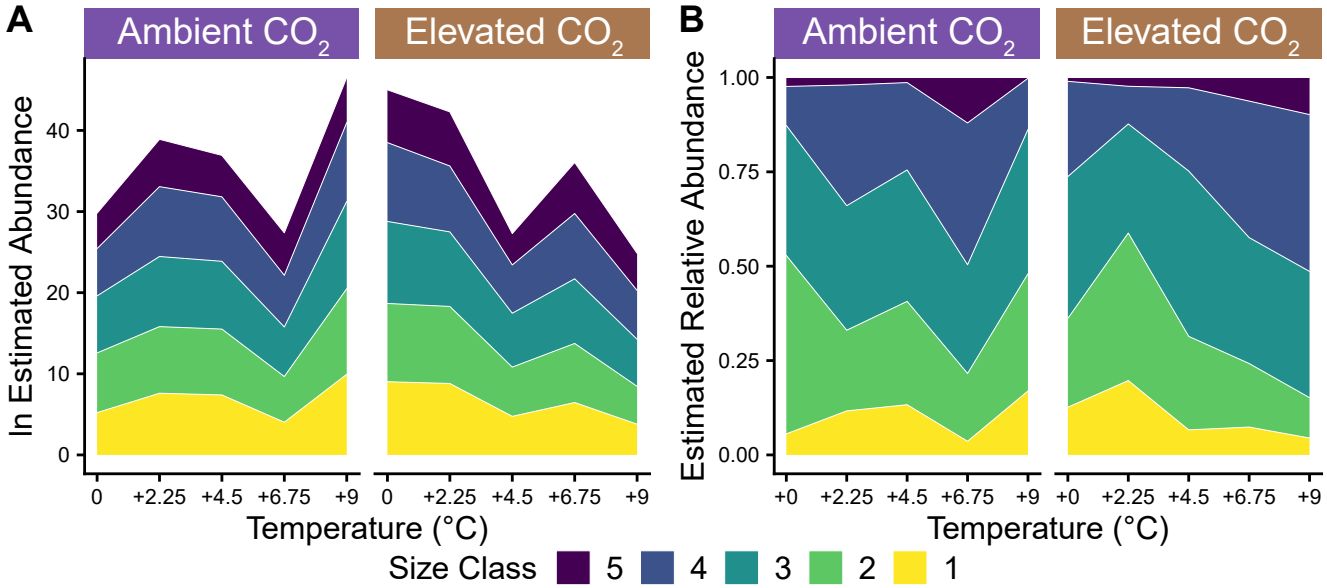

Fig. 2. Estimated protist abundances derived from FlowCam densities. (A) Estimated total abundance data on the natural log scale for size classes across temperatures under ambient and elevated CO_2 treatments. Error rates for all size classes are +/1 4.45%. Data indicates that abundances increase across size classes with temperature under ambient CO_2 levels but decreases under elevated CO2 levels. (B) Relative abundance of size classes by CO_2 treatment calculated from total estimated abundance (A) across temperatures. Results show that different size classes respond differentially to environmental treatments. Size Class 1: 12.43 μm - 17.07 μm; Size Class 2: 17.07 μm - 20.68 μm; Size Class 3: 20.68 μm - 28.24 μm; Size Class 4: 28.24 μm - 59.74μm; Size Class 5: 59.74 μm - 526.43 μm.

protist density and biomass increased with temperature under ambient CO_2 (Fig. 2A). This trend reversed under elevated CO_2, with major declines in biomass and density across size classes (Fig. 2A). The relative density within size classes remained generally consistent with increasing temperature under ambient CO_2 conditions; however, under elevated CO_2 conditions, the relative density of larger protists increased at the expense of the two smallest size classes (Fig. 2B).

A three-way interaction between temperature, CO_2 concentration, and size class drove significant changes in the functional trait composition of protist communities (Fig. 3): size (F = 4,007; $p \ll 2.2x10^{-16}$); shape (F = 13,204; $p \ll 2.2 \times 10^{-16}$); cellular contents (F = 789.05; p $\ll 2.2x10^{-16}$); and energy acquisition (F = 657.15; p $\ll 2.2x10^{-16}$). The magnitude of the change was driven by the CO_2 treatment, with elevated CO_2 rates of change outpacing that of ambient CO_2 by up to a factor of 25 (Fig. 3). Under ambient CO_2, protists were smaller (Figs. 3A, S6), less round (Figs. 3B, S7), showed more cellular contents (Figs. 3C, S8), and became redder (more heterotrophic) at lower levels of warming but greener (more autotrophic) at higher levels of warming (Fig. 3D, Fig. S9). Conversely, protists under elevated CO_2 were larger (Fig. 3A, S6), rounder (Fig. 3B, S7), had fewer cellular contents (Fig. 3C, S8), and got redder (Fig. 3D, S9). Thus, with the exception of the two smallest size classes for red/green ratio, protists displayed inverse patterns for all traits in response to increasing temperature under ambient and elevated CO_2 levels.

The observed changes in functional traits were protist-size dependent, as larger size classes responded more strongly to temperature than smaller ones (Fig. S10A, (0.014 $<$ p $<$ 0.066), c.f. (55)). While the rates of change in all traits displayed non-linear size-dependency, we found weak support for size allometries in shape, cellular contents, and energy acquisition (Fig. S10B-D).

Observed changes in community functional traits could be the result of plastic change across all or most protists in the community, or, instead, result from taxonomic compositional shifts. Amplicon sequencing results show that important taxonomic shifts (Fig. 4A) likely underlie the observed changes in functional traits (Fig. 3), not species-specific plastic change. Moreover, increasing temperature leads to a decrease in the diversity of microbial communities (Fig. 4B,C) — consistent with multiple studies across biomes that find climate change decreases diversity of species across scales (86–88). Importantly, temperature interacts with CO_2 such that the effects of CO_2 treatment decrease at warmer temperatures, leading to a near convergence in the taxonomic compositional similarity between ambient and elevated CO_2 (Fig. 4D), despite a dramatic divergence in functional traits (Fig. 3).

Our SEM model adequately described the data (CFI = 0.96, TLI = -0.771, SRMR = 0.025), and supported the existence of strong direct interactive effects of temperature and CO_2 on community structure and composition and community functional traits (Fig. 5A, Table S3). The model revealed stronger pressure on individual traits and community diversity from CO_2 than temperature — though their interactive effects weighed most heavily on both traits and community structure metrics (Fig. 5A). Moreover, we uncovered the existence of indirect effects of temperature, CO_2, and their interaction on community functional traits, through direct effects on species richness, which in turn influences cell size, shape, contents and energy acquisition mode (Figs. 5, S12).

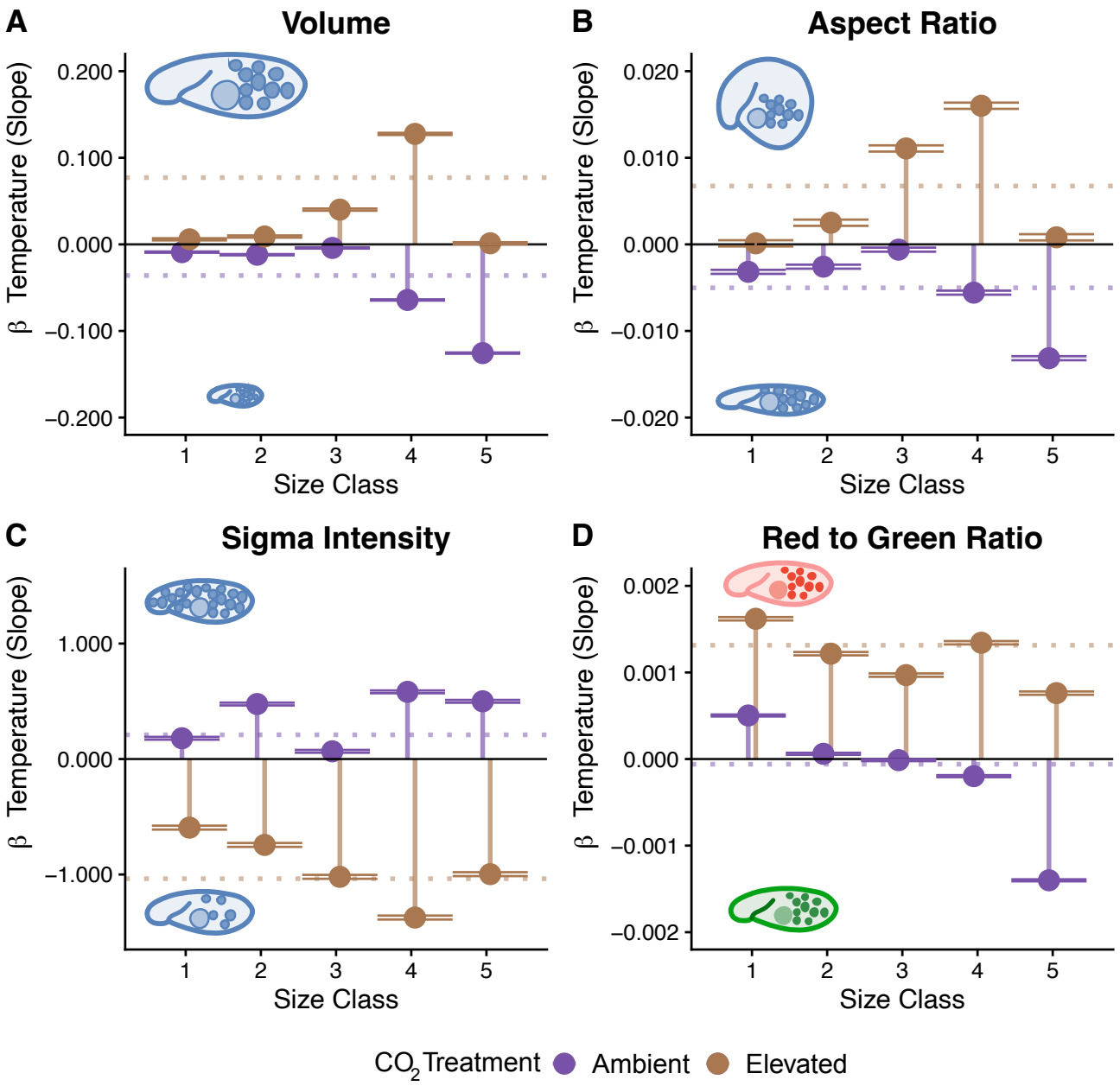

Fig. 3. Rate of change across temperatures (β or slope) for functional traits by protist size class and CO_2 treatments (lollipops). Slopes were calculated from GLMs. Mean slope across size classes displayed as dashed line. Positive slope values indicate: (A, Volume) increasing size; (B, Aspect Ratio) higher symmetry and roundedness (shape); (C, Sigma Intensity) higher optical heterogeneity; and (D, Red/Green ratio) increasing redness and/or decreasing greenness.

These indirect effects often had a reverse sign, suggesting small possible offsets through changes in total protist richness. For example, the interaction between temperature and CO_2 reduces protist size directly, but indirectly, it also can increase protist size through reducing richness, which itself negatively influences cell size (Fig. 5A). These results held while accounting for variations in the model structure of the SEM (i.e., including latent variables, Table S2, Fig. S12).

Lastly, functional diversity analysis supported the conclusion that temperature and CO_2 have complex joint effects on the structure of protist communities that diverge from the direct effects of each factor independently (Fig. S13). Surprisingly, we found no relationship between Functional Richness or Evenness and Temperature across CO_2 treatments (Fig S13A, B). We did, however, find a steep decline in functional dispersion with rising temperature under elevated CO_2, whereas the ambient CO_2 scenario showed a more moderate response (Figure. S13C).

Discussion

Understanding how moss-associated microbial communities change with temperature and CO_2 concentrations is vital for predicting how peatland ecosystems will respond to climate change (7, 89, 90) and alter the global C cycle (91–93).

Here we find strong evidence of changes in the abundance, biomass, functional traits, and composition of important but poorly understood members of peatland microbial communities. These changes are driven by an interaction between warming and CO_2 concentration (Figs. 2-5). We show that peatland protist communities are sensitive to climate change, and under ambient CO_2 match our expectations based upon metabolic theory — getting smaller, less round, more active, and more heterotrophic. However, these trends are reversed by elevated CO_2 concentrations (Fig. 3), and the magnitude and direction of these changes are driven by non-linear size-dependence (Fig. S10). While protist communities converge in taxonomic composition between ambient and elevated CO_2 levels as temperature rises, they diverge in functional trait composition (Figs. 3, 4, S13, S14), suggesting important but yet unknown effects on peatland function with environmental change.

Protist reductions in volume (Figs. 3A, S6) and roundedness (Fig. 3B, Fig. S7) observed under ambient CO_2 and elevated temperatures were predicted from metabolic theory, suggesting that we can anticipate complex temperature responses in natural and intricate microbial communities based on first principles. For example, temperature selects for smaller or-

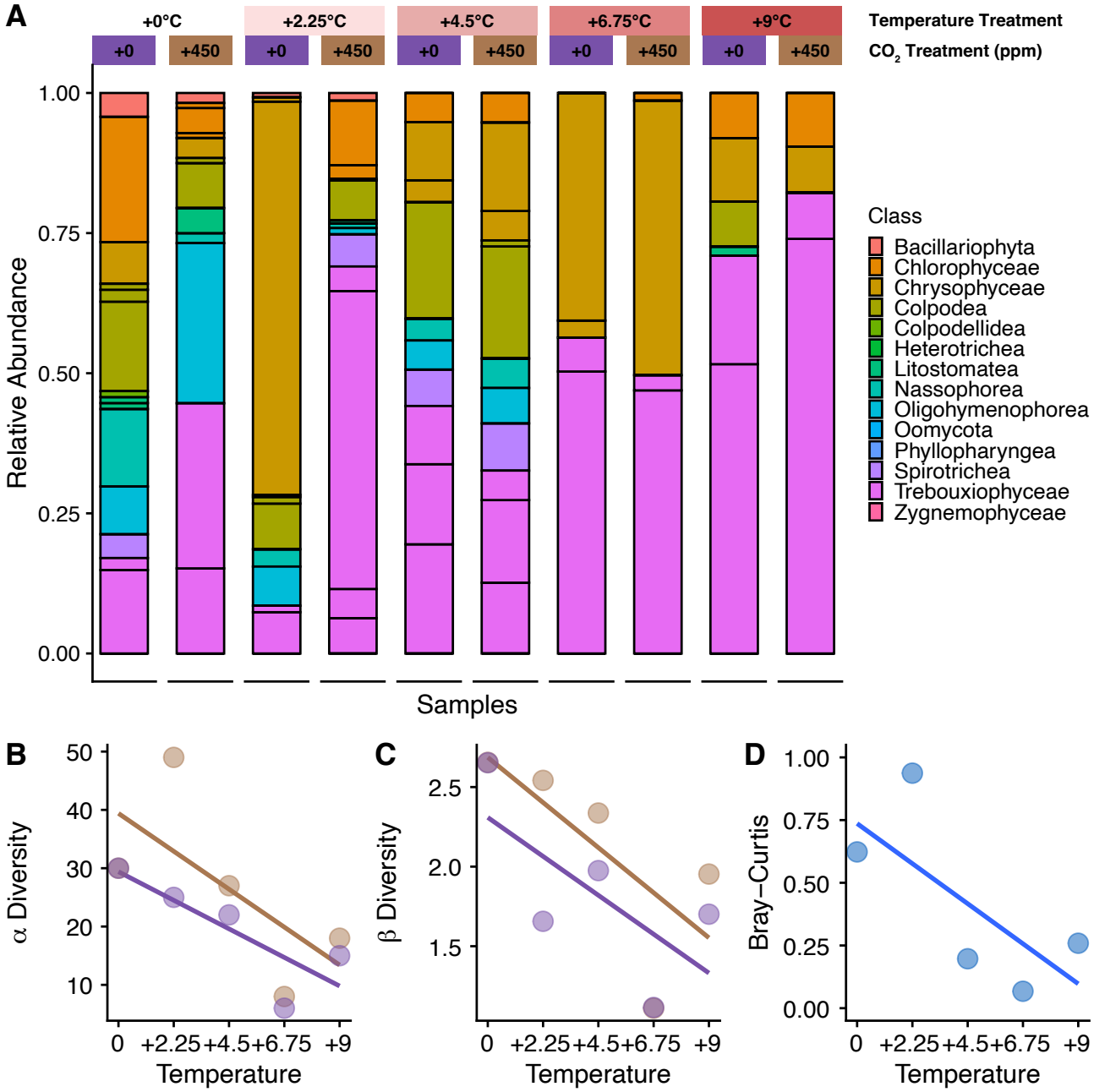

Fig. 4. Change in (A) protist Order relative composition (colored by Class) and (B, C) diversity of protist community across temperatures and CO_2 treatments. (A) Protists' Order composition (colored by Class) shifts both with temperature increase and within CO_2 treatment, with a decrease in (B) total species composition and (C) species diversity at higher temperatures. (D) Species composition becomes more homogeneous — lower Bray-Curtis distance — between CO_2 treatments at higher temperatures, indicating an interactive effect (pearson's correlation = -0.71).

ganisms and/or organisms with larger surface-to-volume ratios that can meet higher metabolic demands while conserving resources as metabolism costs increase at higher temperatures (94). This negative relationship between size and temperature — the "temperature-size rule" (42, 47) — is well documented for both single-celled (47) and multi-celled organisms (95–97), but was so far thought to be a plastic response.

We show that the temperature-size rule can result from community composition shifts that select for smaller organisms (Figs. 3-5). Similarly, shape shifts (e.g., a decrease in roundedness with temperature) likely results from selection to conserve energy by simultaneously reducing volume (which increases metabolic costs), and increasing surface (which increases resource acquisition) (98). Changes in protist shape and size have multiple ecological consequences, including population dynamics and ecological interactions within communities (30, 43, 44), as well as total respiration, which decreases with volume (99). Shifts in size and shape can occur quite rapidly and can be as dramatic as 50% or more (48, 100). Likely, species that cannot rapidly respond to temperatures are replaced by those that can, leading to high species turnover (Fig. 4A).

The increase in the red/green ratio of 70% of size classes in our study (Figs. 5B, S9) may be alarming. A decrease in the red/green ratio is associated with decreased autotrophy and therefore carbon uptake (101). However, when we normalize the β coefficents of our models, the overall increase in heterotrophy is outweighed by a decrease in biomass of heterotrophic organisms and an increase in biomass of photosynthetic organisms obtained through our 18S data (Fig. S14), likely indicating a net reduction in heterotrophy (Figs. 5B, S9, S12). The results of this increase in the green biomass may be linked to higher abundance (Fig. 4A) and shifts in the functional metabolic traits (Figs. 5A, S14, Table S4) of photosynthetic clades such as Trebouxiophyceae; this increase in phototrophic protists with warming — modulated by CO_2 — parallel to shifts in their traits suggests a shift in metabolic activity of the community, a fundamental result in light of the importance of phototrophic eukaryotes for both the peatland C cycle (102, 103) and the overall global carbon cycle (104). Coupled with decreases in the abundance of consumer microbes like Oligohymenophorans and Oomycota (Fig. 4A), our findings point to potential shifts in the functional metabolome and carbon cycling of peatlands under climate change (Fig. 5B, Fig. S14, Table S4).

Counter to our hypothesis, we report surprising reversals in functional trait change with temperature between CO_2 treatments (Figs. 3, 4, S6-S9) suggesting that joint temperature and CO_2 impacts in microbial food webs may be more complex than previously thought (Figs. 5, S12). Interactive effects of CO_2 and temperature have been reported in aquatic phytoplankton systems (70, 71, 105). However, these systems only displayed shifts in trait change magnitudes, not complete trend reversals, as reported here. Our results thus emphasize the importance of addressing temperature impacts on microbial organisms in the light of simultaneous CO_2 increases. These trend reversals may partially be due to CO_2-induced pH changes of the peat and/or the stoichiometry of diffusion under higher temperatures and elevated CO_2. Experimental work on ocean phytoplankton showed that rising

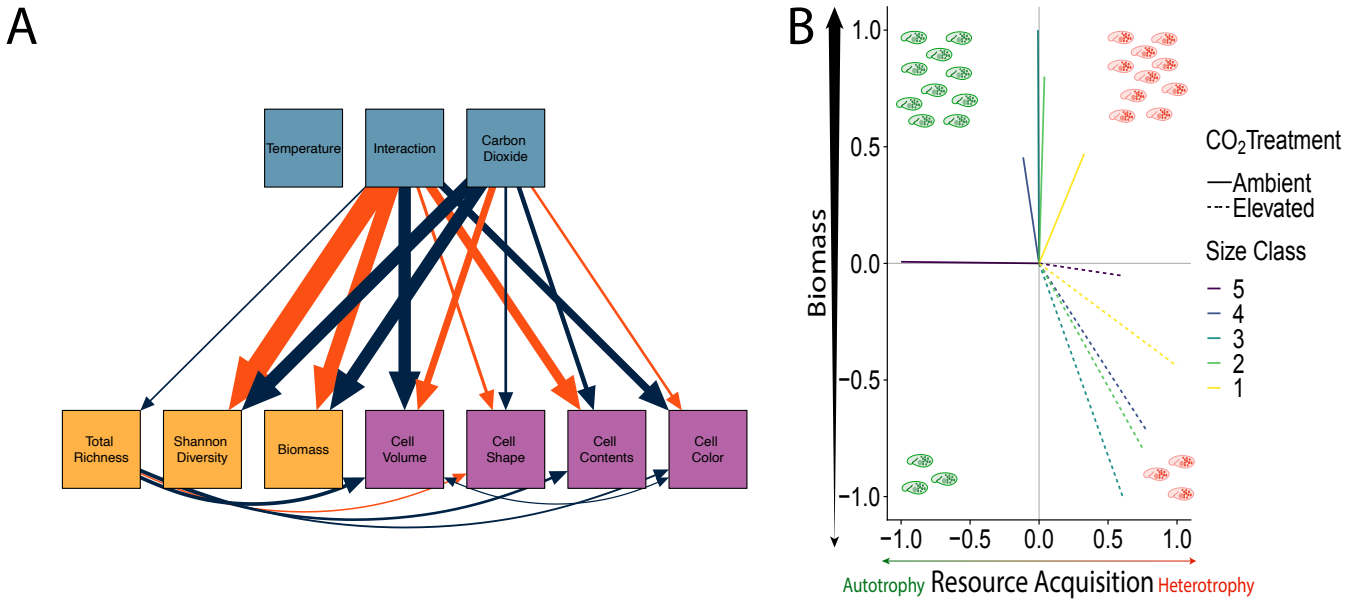

Fig. 5. (A) Structural Equation Model (SEM) illustrating the effects of environmental variables and their interaction (light blue) on protist community structure (light orange) and functional traits (purple). Solid lines — shown only the top 30% stronger effects — represent significant effects, with positive effects (blue) and negative effects (red). The thickness of the lines corresponds to the magnitude of the standardized estimates (Table S3). Traits are as follows: Shape = Aspect Ratio, Color = Red to Green Ratio, Cell Contents = Sigma Intensity. (B) Combined normalized change in biomass and resource acquisition across quadrant (I-IV) outcomes. Under elevated CO_2, the increase in respiration is offset by a loss in biomass of these organisms (IV). Given normalization of difference, slopes $\leq$ -1 indicate a net reduction in heterotrophy. Under ambient CO_2 conditions, an increase in net photosynthesis occurs for the two largest size classes (II), as both biomass and green reflectance increase. The net carbon dynamics in these plots may be offset by the normalized relative increase in biomass of the more heterotrophic smaller size classes (I).

temperatures and elevated CO_2 can induce a more rapid decrease in community biomass and chlorophyll-a concentration (106), and that elevated CO_2 may lessen the demand for a higher surface-to-volume ratio as diffusion becomes easier across the cellular membrane at higher concentrations of dissolved CO_2. These changes may also be due to the increased efficiency of heterotrophic resource acquisition compared to photosynthesis (107). The shift towards heterotrophic resource acquisition (Fig. 3D) under elevated CO_2 conditions may lessen the need for energy conservation.

These reported trend reversals under elevated CO_2 may also occur indirectly, through changes in nutrient cycling and mineralization (108) — especially of N (109). Elevated CO_2 often increases the exudation of soluble organic compounds by *Sphagnum* mosses (110) — especially sugars — that contribute to the structure of microbial communities, and can increase the abundance of bacteria and heterotrophic protists (111). With higher abundances of bacteria, protists may obtain more energy through predation. Petro et al. (2023) recently found similar interactive effects of warming and elevated CO_2 on N. Elevated temperature and ambient CO_2 conditions produced accumulation of nitrogen and a reduction in N_2 fixation, which was reversed under elevated CO_2 conditions. This in turn altered N immobilization by microorganisms, likely leading to a change in microbial food web dynamics. Elevated CO_2 may also indirectly increase nitrogen availability through changes in *Sphagnum* exudation of C and N organic compounds (26, 113) — indirectly influencing N-releasing enzymes (114, 115) — or through symbiosis with mycorrhizal fungi (116, 117).

We found that overall, the trend reversal reported here (biomass, abundance, traits) was size-dependent (Fig. S10). We find strong support for a super-linear scaling relationship between body size and volume that indicates protist populations might undergo density-dependent (population) and density-independent (individual phenotypic) responses to climate change (43, 48, 100, 118, 119). Our results indicate a significant relationship between proportional changes in traits and size. Larger protists' size (Fig. S10A) and shape (Fig. S10B) change more than smaller protists per unit volume (Figs. S10A, S10B). Cellular contents follow a sub-linear size-dependency (Fig. S10C). Because smaller protist phenotypes shift much less and these smaller individuals dominate the relative abundance of the system (Figs. 2B, S3B), there may be embedded resistance to phenotypic change in these communities overall. This trend portends potential eco-evolutionary responses of protists under environmental stressors, as phenotypic traits and plasticity may drive the success and survival of individuals responding to changing metabolic demands (120, 121).

Interestingly, our SEM model (Fig. 5A, Tables S2, S3) uncovers additional mechanisms through which these trend reversals are possible. For example, all SEM models found indirect pathways from community structure and composition to functional traits that reversed the direct effects of environmental conditions on functional trait changes (Figs. 5A, S11, S12). While these indirect effects are weaker in magnitude than the direct effects, they also somewhat offset the direct effects through trend reversals (Fig. 5A), perhaps shielding the community from even larger community functional trait shifts. Understanding these complex responses is pivotal for predicting how ecosystem services, such as nutrient cycling and primary productivity, might be reshaped in a future, warmer and CO_2-enriched world (122–124).

G. Caveats. Due to the limited temporal resolution of our sampling, it is possible that our results are only indicative of large shifts in the seasonal or transient dynamics of these moss-associated protist communities. However, given the magnitude of the observed changes, we suspect — but do not prove — that these reported shifts owe to broader non-transient community responses to temperature and CO_2 levels. It is also possible that our sample processing may have inserted bias in our protist counts across temperature treatments. This is because the equilibration period between extraction of the community and fluid imaging — roughly 24 hours at room temperature — could have deferentially affected our samples across treatments. However, we argue that such an effect should have been small, if at all present, as SPRUCE experiences major daily temperature fluctuations in September — when these data were collected — with daily temperatures that can range from below freezing to above 30°C (National Weather Service: https://www.weather.gov). If our samples had changed in composition so dramatically that our data could not be properly interpreted, it would also suggest that SPRUCE protist communities experience extremely large daily changes in composition. Such changes are likely on the longer timescales of months and seasons (125–127), but have, to our knowledge, not been shown to occur in the timescale of days. Last, because our density data was not controlled by total dry moss biomass — known to be positively correlated with microbial abundances (128) — it may show unintended levels of variability. However, we have designed our sampling regime so that living and dead moss biomass was consistent across samples and enclosures, so the amount of variation should be small. Also, the density data recovers patterns of temperature and CO_2 interactions that are consistent with those of trait and 18S data, both of which are unaffected by the actual amounts of dry moss biomass. Because of this, we acknowledge the possibility that these three sources of error may have affected our results, but remain confident that our inference is robust.

Conclusions

Our findings suggest important ways in which climate change may influence C cycling in peatland ecosystems by potentially altering important functional linkages within moss-associated microbial food webs (129). Further, while earth-system models may predict range shifts in *Sphagnum* spp. (130), our findings indicate that the combined effects of elevated carbon dioxide and temperature may alter the symbiotic moss-microbiome in unexpected ways — ways that could af-

fect the distribution of *Sphagnum* and peatlands in the future (23, 26, 27, 112). Given the diverse energy acquisition strategies of protists, changes in the balance of autotrophy versus heterotrophy can have cascading effects on the biological carbon cycle (131–134). Due to their predominant distributions in mid- and high-latitudes of the Northern hemisphere, peatlands are especially sensitive to climate change (135–138). While tantalizing, the data-driven hypotheses presented here need to be tested further, but are suggestive of important ways in which environmental change both influences and is influenced by rapid microbial responses in functional trait composition in globally important — and highly threatened — ecosystems such as peatlands.

AUTHOR CONTRIBUTIONS

CLK and JPG conceptualized the study. AC, DAP and DJW collected SPRUCE samples. SV, AY and JPG prepared the samples and extracted protist data. AAC prepared and provided amplicon sequencing data. CLK performed all analyses with support from DJW, AC, and JPG. CLK wrote the first draft and all authors contributed substantially to subsequent versions.

FUNDING

This work was supported by a US Department of Energy, Office of Science, Office of Biological and Environmental Research, Genomic Science Program Grant to J.P.G, award number DE-SC0020362, and a Simons Foundation Early Career Fellowship in Aquatic Microbial Ecology and Evolution to J.P.G, award number LS-ECIAMEE-00001588.

ACKNOWLEDGEMENTS

We would like to thank Dawn M. Klingeman from Oak Ridge National Laboratory for running the 18S rRNA samples on the sequencer and two anonymous reviewers for their feedback.

COMPETING INTERESTS

The authors report no competing interests.

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