

RESEARCH ARTICLE

Temperature and CO₂ interactively drive shifts in the compositional and functional structure of peatland protist communities

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Abstract

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 CO₂ 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 and 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, and metabolism) that could alter overall function in peatlands. Importantly, we demonstrate a taxonomic convergence but a functional divergence in response to warming and elevated CO₂ with most environmental responses being contingent on organismal size: warming effects on functional composition are reversed by elevated CO₂ and amplified in larger microbes but not smaller ones. These findings show how the interactive effects of warming and rising CO₂ levels could alter the structure and function of peatland microbial food webs—a fragile ecosystem that stores upwards of 25% of all terrestrial carbon and is increasingly threatened by human exploitation.

KEYWORDS

climate change, climate interactions, community ecology, functional traits, microbial diversity, peatlands, protists

1 | INTRODUCTION

Greenhouse gas emissions set the pace of climate change (Steffen et al., 2018) and most ecosystems on Earth influence this process by serving as carbon (C) sources or sinks (Stocker et al., 2013). Overall, terrestrial ecosystems mitigate ≈30% of anthropogenic CO₂ emissions (Canadell et al., 2007, 2021). The predominant terrestrial carbon sink—peatlands—store 100% more carbon than

all forests while covering less than 3% of the globe (Humpenöder et al., 2020; Loisel et al., 2012; Xu et al., 2018; Yu et al., 2010). *Sphagnum* mosses dominate peatlands (Turetsky et al., 2012) and are responsible for most C sequestration in the form of recalcitrant peat (Hooijer et al., 2010). These mosses harbor diverse communities of single-celled and multi-celled organisms (i.e., bacteria, archaea, protists, fungi, metazoa; Kostka et al., 2016), that influence C cycling directly (Gilbert et al., 1998; Gilbert & Mitchell, 2006) through

respiration of $\approx 100 \text{ Pg C year}^{-1}$ in soils alone (Gougoulas et al., 2014; Trumbore, 2006). Also, these microbial organisms indirectly influence the C cycle by supplying nitrogen needed for *Sphagnum* moss growth (Berg et al., 2013; Rousk et al., 2013; Vile et al., 2014), converting methane into the CO_2 necessary for photosynthesis (Kip et al., 2011, 2012; Reumer et al., 2018), providing stress tolerance to the host mosses (Carrell, Lawrence, et al., 2022; Shcherbakov et al., 2013), and assisting in host–pathogen defense (Opelt et al., 2007).

How ecological interactions between members of the moss-associated community influence their effects on peatland C cycling, however, is poorly understood (Carrell et al., 2019; Carrell, Velicković, et al., 2022; Salmon et al., 2021). Predation by metazoans, in particular, plays a prominent role in peatland C cycling (Wyatt et al., 2021), and is likely to also impact microbial communities (Geisen et al., 2020; Rocca et al., 2022). Unicellular Eukaryotes, collectively known as protists, store two times more C globally than all animals combined (Bar-On et al., 2018; Bond-Lamberty & Thomson, 2010), contributing to C cycling through mixotrophic metabolism (Jassey et al., 2015; Wieczynski, Moeller, & Gibert, 2023), and also serve as one of the principal biological controls on microbial respiration and photosynthesis through predation and competition (Gao et al., 2019; Rocca et al., 2022; Thakur & Geisen, 2019). Protists also directly influence plant growth and health through their effects on the rhizosphere (Ceja-Navarro et al., 2021; Gao et al., 2019; Xiong et al., 2020) and phyllosphere (Bashir et al., 2022; Gómez-Pérez et al., 2022)—thus indirectly influencing plant growth through their effects on beneficial microorganisms (Geisen et al., 2020; Guo et al., 2021). In turn, climate effects can induce changes in moss microhabitat that affect predation, resource acquisition, and life cycles of associated protists (Jassey et al., 2015).

But, despite important advances (Basińska et al., 2020; Jassey et al., 2016; Reczuga et al., 2020; Tsyganov et al., 2012), how the structure and function of protist communities may respond to climate

change in peatlands remains insufficiently understood. Simple protist functional traits—that is, characteristics controlling how organisms respond to the environment or affect ecosystem properties (Violle et al., 2007)—including protist cell size, cell shape, and cellular contents (Table 1), can be used to predict warming effects on protist communities and ecosystem function (Wieczynski et al., 2021). This is due to underlying links among these traits, metabolism, respiration rates, and energy acquisition mode (Gibert et al., 2017; Wieczynski et al., 2021). 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 (Atkinson et al., 2003; Montagnes et al., 2002) with a shape (long and slim) that maximizes energy intake and minimizes energetic costs (Atkinson, 1994; Clarke & Fraser, 2004). Increased metabolic demands can impact resource acquisition strategies (e.g., increased heterotrophy over autotrophy; Fussmann et al., 2017; Menden-Deuer et al., 2018; Wilken et al., 2013; Yvon-Durocher et al., 2010), 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), which have been linked to cellular contents and cell viability (Matsubayashi et al., 2010; Sediq et al., 2018). Consequently, changes in functional composition could alter net C fluxes and other elements within peatlands (Brose et al., 2012; Gibert, 2019; O'Connor et al., 2009; Wieczynski, Yoshimura, et al., 2023).

Here, we address how peatland protist communities—operationally defined as the community of all non-fungal microbial 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 and*

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	Atkinson (1994), Brown et al. (2004), Wieczynski et al. (2021)
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	Glazier (2010), Gibert et al. (2017); Hirst et al. (2014), Wieczynski et al. (2021)
Cell contents	Measured as the optical heterogeneity of a cell and our measure of cellular contents (vacuoles, cytoskeleton, organelles), it captures information on the metabolic machinery of the cell, i.e., cellular storage and metabolism.	Wieczynski et al. (2021)
Resource acquisition mode	The ratio of the red-to-green reflectance signal of a cell—where 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. (2003), Gibert et al. (2022), Nakano et al. (1996)

Peatland Responses Under Changing Environments (SPRUC) long-term (since 2014) whole-ecosystem warming experimental site (Hanson et al., 2017; Figure 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—that is, the temperature–size rule (Atkinson, 1994; Atkinson et al., 2003), (2) an increase in surface-to-volume ratio (aspect ratio), as protists optimize shape to increase energy uptake (surface) and reduce metabolic costs (volume; Atkinson et al., 2006), (3) an increase in cellular optical heterogeneity (sigma intensity or cellular “contents”), as temperature-induced increased metabolic rates requires more cellular machinery (Shin et al., 2016), and (4) increased heterotrophy (increased red/green ratios) to keep up with metabolic demands. Additionally, we expect elevated CO₂ to result in an increase in total community biomass (Sommer et al., 2015, 2017) as more CO₂ fuels photosynthesis at the base of the food web.

This CO₂-driven increase in energy availability at the base of the microbial food web should also result in (Figure 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 (i.e., cellular contents), 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 (Atkinson et al., 2003; Gibert et al., 2022; Nakano et al., 1996). We show that warming and CO₂ 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 have important consequences for C cycling in peatlands.

2 | MATERIALS AND METHODS

2.1 | 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; 418m 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 laricina*). 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* and *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 belowground 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 450ppm and elevated 900ppm CO₂ treatments across soil (initiated June 2014) and air (initiated June 2015) warming treatments (Figure 1; Hanson et al., 2016). Warming magnitudes within the enclosures fluctuate alongside outdoor ambient temperatures, but remain, on average, +0, +2.25, +4.5, +6.75, +9°C above ambient. After 5 years of treatment—in September 2019—we collected roughly equally sized superficial samples (3–5cm) 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 overnight 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 250mL glass jars and kept the cultures at 22°C and 65% humidity for equilibration for 24h before fluid imaging. All samples were processed and imaged in a simultaneous, treatment-blind fashion, and within 24–36h of collection in the field. While samples could have been fixed in situ with lugol or formaldehyde, these fixative methods often alter the size and shape of cells and lead to an under-representation of larger size classes (Modigh & Castaldo, 2005; Stoecker et al., 1994), and would bias our quantification of functional trait shifts across size classes with temperature and CO₂, so no fixative was used.

2.2 | 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 10X magnification of 6mL water sub-samples of processed SPRUCE samples. The FlowCam screens roughly 75% of the sampled volume (called the “efficiency” of the instrument), which was used to efficiency-correct biomass and abundance estimates from total protist volume and densities (see Supporting Information; Figure S1).

2.3 | Trait measurements

We quantified functional traits (Table S1) by manually curating the ensuing cell images in the Visual Spreadsheet software (Yokagawa Fluid Imaging), 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

(a) Spruce & Peatland Responses Under Changing Environments (SPRUCE) (b) Trait Characterization

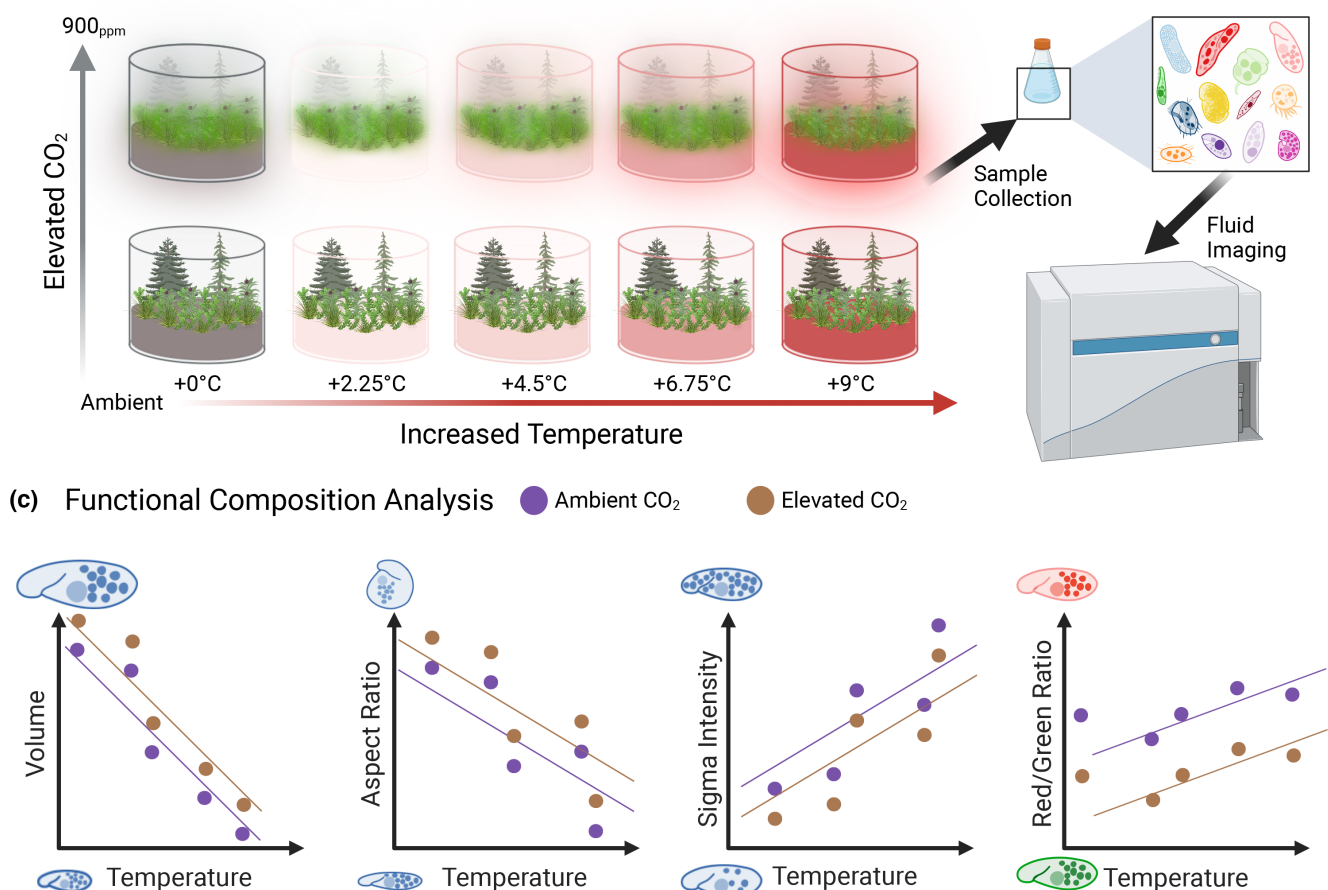


FIGURE 1 (a) Samples were collected in a 2-factorial mesocosm design in boreal peatlands with variation in temperature (+0, +2.25, +4.5, +6.75, +9°C) and CO₂ (450, 900 ppm) concentrations. Protist samples were collected from the upper peat layer within each treatment and processed ex situ. (b) After equilibration (24 h 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 CO₂, we expect larger cell volumes, more roundedness, increased cellular activity, and lower red/green ratios.

(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 Supporting Information; Table S1; Figure S2a). Wieczynski et al. (2021) found these four traits (Table 1) to influence the temperature performance curves of protists as well as their community and ecosystem-level responses to warming.

2.4 | Regression analyses

We tested the effects of temperature and CO₂ 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 sub-plot, as the level of inference is that of the enclosure (Figure 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 modeling with the R package *mclust* (Fraley & Raftery, 2002). 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 (Figure S2). We selected an optimal set of five size classes with a Gaussian mixture model and Bayesian Information Criterion (Figure 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 (Kassambara & Kassambara, 2020) and are reported for each size class (Figure 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 (Wood & Wood, 2015), reporting associated Pearson correlations and *p*-values for each size class (Figure S5). To ensure that variability within and across enclosures did not unduly influence our statistical results, we employed a bootstrap procedure with 1000 pseudo-replicates to estimate standard errors and *p*-values for all model effects, using each resample instance to refit all models (Figures S6–S9).

2.5 | Structural equation modeling and functional diversity

To quantify possible direct and indirect effects of temperature and CO₂ on protist communities, we used structural equation modeling (SEM) with the R package *lavaan* (Rosseel, 2012), a robust maximum likelihood estimator, and the “csoInp” optimizer for enhanced convergence properties. The first SEM considered the effects of the environment (temperature, CO₂, 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. We then pruned this a priori model by eliminating non-significant pathways and variables, which resulted in an overall better model fit. 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).

We assessed all model fits 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 (Grenié & Gruson, 2023; Laliberté et al., 2014), leveraging the mean trait values for each size class within treatments, to calculate functional richness, evenness, and dispersion (Laliberté & Legendre, 2010).

2.6 | 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, Hilden, Germany) and amplified the V4 region of the 18S rRNA gene following Hugerth et al. (2014). Samples were multiplexed, pooled, and sequenced on an Illumina MiSeq instrument with paired end kit (2×250 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 (Callahan et al., 2016). Taxonomy followed the PR2 (18S) database (Guillou et al., 2012) and sequences assigned as “bacteria” or Embryophyceae were removed.

We used SVs to calculate alpha diversity as counts and the Shannon diversity (calculated beta diversity) and compositional dissimilarity, or Bray–Curtis distance, with the *phyloseq* package (McMurdie & Holmes, 2013).

3 | RESULTS

Experimental manipulations of temperature and CO₂ levels induced an 80-fold change ($\pm 4.45\%$) in total observed protist densities ($F=2.2554$; $p=.02244$, Figure S5a) and biomass ($F=2.2504$; $p=.02273$, Figure S5b) across treatments (Figure 2a), as well as a shift in the relative dominance of size classes (Figure 2b). Across all body size classes, estimated protist density and biomass increased with temperature under ambient CO₂ (Figure 2a). This trend reversed under elevated CO₂, with major declines in biomass and density across size classes (Figure 2a). The relative density within size classes remained generally consistent with increasing temperature under ambient CO₂ conditions; however, under elevated CO₂ conditions, the relative density of larger protists increased at the expense of the two smallest size classes (Figure 2b).

A three-way interaction between temperature, CO₂ concentration, and size class drove significant changes in the functional trait composition of protist communities (Figure 3): size ($F=4007$; $p\ll 2.2\times 10^{-16}$); shape ($F=13,204$; $p\ll 2.2\times 10^{-16}$); cellular contents ($F=789.05$; $p\ll 2.2\times 10^{-16}$); and energy acquisition ($F=657.15$; $p\ll 2.2\times 10^{-16}$). The magnitude of the change was driven by the CO₂ treatment, with elevated CO₂ rates of change outpacing that of ambient CO₂ by up to a factor of 25 (Figure 3). Under ambient CO₂, protists were smaller (Figure 3a; Figure S10), less round (Figure 3b; Figure S11), showed more cellular contents (Figure 3c; Figure S12), and became redder (more heterotrophic) at lower levels of warming but greener (more autotrophic) at higher levels of warming (Figure 3d; Figure S13). Conversely, protists under elevated CO₂ were larger (Figure 3a; Figure S10), rounder (Figure 3b; Figure S11), had fewer cellular contents (Figure 3c; Figure S12), and got redder (Figure 3d; Figure S13). 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 CO₂ levels.

The observed changes in functional traits were size dependent, as larger size classes responded more strongly to temperature than smaller ones (Figure S14a, $[.014 < p < .066]$, c.f. Montagnes et al., 2002). 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 (Figure S14b–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 (Figure 4a) likely underlie the observed changes in functional traits (Figure 3), not species-specific plastic change. Moreover, increasing temperature leads to a decrease in the diversity of microbial communities

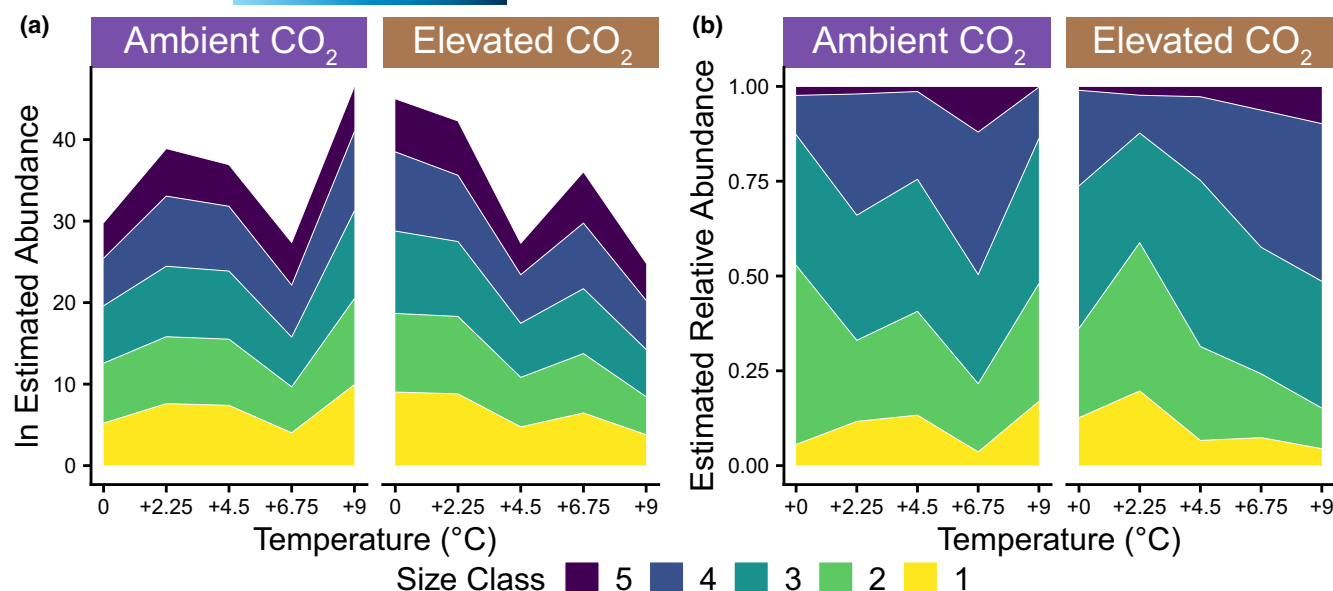


FIGURE 2 Estimated protist abundances derived from FlowCam densities. (a) Estimated total abundance data on the natural log scale for size classes across temperatures under ambient and elevated CO₂ treatments. Error rates for all size classes are $\pm 4.45\%$. Data indicate that abundances increase across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

(Figure 4b,c)—consistent with multiple studies across biomes that find climate change decreases the diversity of species across scales (Danovaro et al., 2004; Frishkoff et al., 2014; Zhou et al., 2020). Importantly, temperature interacts with CO₂ such that the effects of CO₂ treatment decrease at warmer temperatures, leading to a near convergence in the taxonomic compositional similarity between ambient and elevated CO₂ at warmer temperatures (Figure 4d), despite a dramatic divergence in functional traits—amplified by size (Figure 3; Figures S4 and S15).

Our a priori 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 CO₂ on community structure and composition as well as community functional traits (Figure 5a; Table S3). The model revealed stronger pressure on individual traits and community diversity from CO₂ than temperature—although their interactive effects weighed most heavily on both traits and community structure metrics (Figure 5a). Moreover, we uncovered the existence of indirect effects of temperature, CO₂, 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 (Figure 5; Figure S18). 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 CO₂ reduces protist size directly, but indirectly, it also can increase protist size through reducing richness, which itself negatively influences cell size (Figure 5a). These results held while accounting for variations in the model structure of the SEM (i.e., including latent variables, Table S2; Figure S16). While the individual regressions of our a priori model were both significant

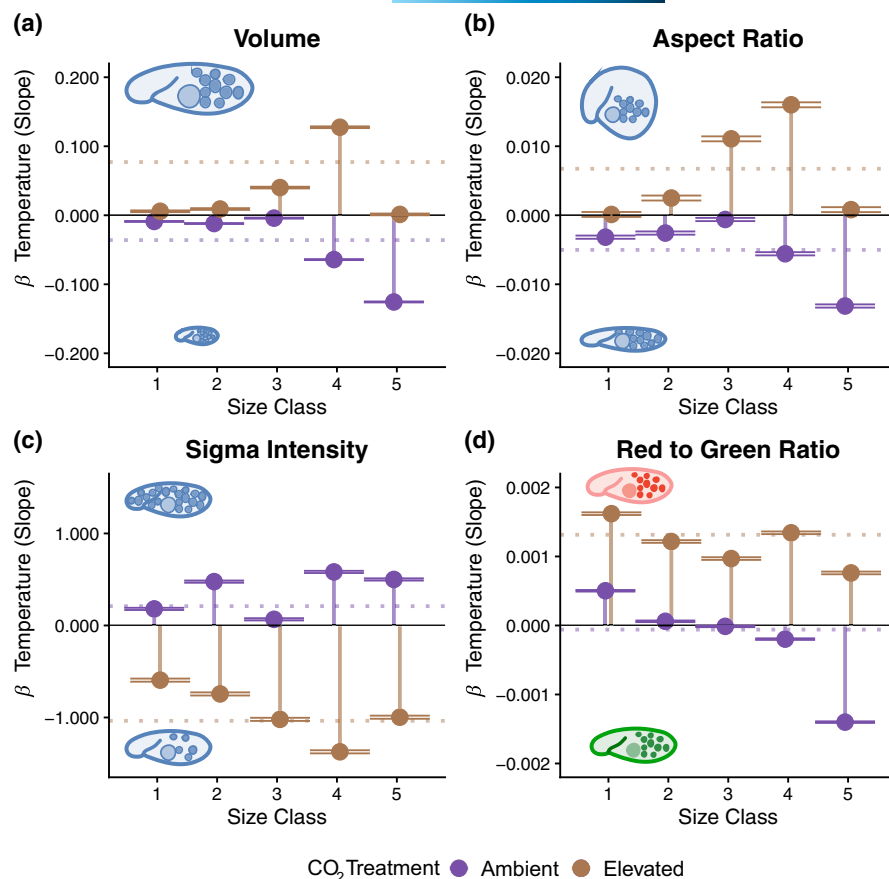
and supported findings from our other analyses, the overall model poorly fitted the totality of the effects in the system ($\chi^2 < 0.05$). By eliminating less significant pathways and variables (Tables S3 and S4), we arrived at a simpler model (Figure S18) that retained significant direct pathways and also adequately described all observed effects simultaneously ($\chi^2 = 0.06$). Interestingly, this new model does not retain some of the most salient features of the complex one and those shown in Figures 3 and 4, namely the interactive effect of temperature and CO₂.

Lastly, functional diversity analyses supported the conclusion that temperature and CO₂ have complex joint effects on the structure of protist communities that diverge from the direct effects of each factor independently (Figure S19). Surprisingly, we found no relationship between functional richness or evenness and temperature across CO₂ treatments (Figure S19a,b). We did, however, find a steep decline in functional dispersion with rising temperature under ambient CO₂, whereas the elevated CO₂ scenario showed a more moderate positive response (Figure S19c). Thus, the interaction of CO₂ with temperature drove functional divergence between the ambient and elevated CO₂ groups in the overall distribution of their traits (Figure S19).

4 | DISCUSSION

Understanding how moss-associated microbial communities change with temperature and CO₂ concentrations is vital for predicting how peatland ecosystems will respond to climate change (Loisel et al., 2012; Rydin et al., 2006; Waddington et al., 2010) and alter

FIGURE 3 Rate of change across temperatures (β or slope) for functional traits by protist-size class and CO₂ treatments (lollipops). Slopes were calculated from generalized linear models. Mean slope across size classes is displayed as a 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.



the global C cycle (Bardgett et al., 2008; Smith et al., 2019; Zhou et al., 2012). 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 CO₂ concentration (Figures 2–5). We show that peatland protist communities are sensitive to climate change, and under ambient CO₂ match our expectations based upon metabolic theory—getting smaller, less round, more metabolically active, and more heterotrophic. However, these trends are reversed by elevated CO₂ concentrations (Figure 3), and the magnitude and direction of these changes are driven by non-linear size dependence (Figure S14). While protist communities converge in taxonomic composition between ambient and elevated CO₂ levels as temperature rises, they diverge in functional trait composition (Figures 3 and 4; Figures S19 and S20), suggesting important but yet unknown effects on peatland function with environmental change.

Protist reductions in volume (Figure 3a; Figure S10) and roundness (Figure 3b; Figure S11) observed under ambient CO₂ 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 organisms 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 (Gillooly et al., 2001). This negative

relationship between size and temperature—the “temperature–size rule” (Atkinson, 1994; Atkinson et al., 2003)—is well documented for both single-celled (Atkinson et al., 2003) and multi-celled organisms (Gardner et al., 2011; Gibert & DeLong, 2014; Verberk et al., 2021), 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 (Figures 3–5). Similarly, shape shifts (e.g., a decrease in roundedness with temperature) likely results from the selection to conserve energy by simultaneously reducing volume (which increases metabolic costs), and increasing surface (which increases resource acquisition; DeLong et al., 2017). Changes in protist shape and size have multiple ecological consequences, including population dynamics and ecological interactions within communities (Gibert et al., 2017; Rocca et al., 2022; Wiczyński et al., 2021), as well as total respiration, which decreases with volume (DeLong et al., 2010). Shifts in size and shape can occur quite rapidly and can be as dramatic as 50% or more (Gibert et al., 2022, 2023). Likely, species that cannot rapidly respond to temperatures are replaced by those that can, leading to high species turnover (Figure 4a).

The increase in the red/green ratio of 70% of size classes in our study (Figure 5b; Figure S13) may be alarming. A decrease in the red/green ratio is associated with decreased autotrophy and, therefore, carbon uptake (Fung Shek, 2015). However, when we normalize the slopes (β coefficients) of our models, the overall increase in heterotrophy is outweighed by a decrease in biomass of heterotrophic

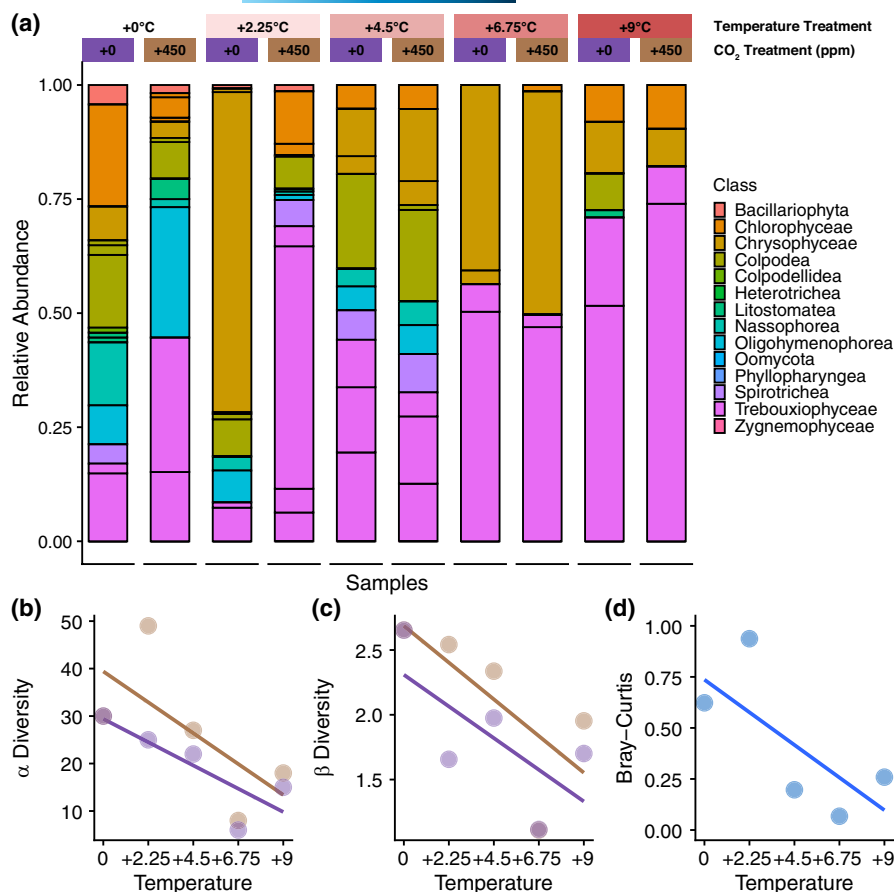


FIGURE 4 Change in (a) protist order relative composition (colored by class) and (b, c) diversity of protist community across temperatures and CO₂ treatments. (a) Protists' order composition (colored by class) shifts both with temperature increase and within CO₂ treatment, with a decrease in (b) total species composition and (c) species diversity at higher temperatures. (d) Species composition becomes more homogeneous—lower Bray-Curtis distance—between CO₂ treatments at higher temperatures, indicating an interactive effect (Pearson's correlation = -0.71).

organisms and an increase in biomass of photosynthetic organisms (Figure S20), likely indicating a net reduction in heterotrophy (Figure 5b; Figures S13, S17, and S18). The results of this increase in the green biomass may be linked to higher abundance (Figure 4a) and shifts in the functional metabolic traits (Figure 5a; Figure S20; Table S4) of photosynthetic clades such as Trebouxiophyceae. In fact, an increase in phototrophic protists with warming—albeit modulated by CO₂—parallel to shifts in their functional traits suggests a change in metabolic activity of the community, a fundamental result in light of the importance of phototrophic eukaryotes for both the peatland C cycle (Hamard et al., 2021; Jassey, Hamard, et al., 2022) and the overall global carbon cycle (Jassey, Walcker, et al., 2022). Coupled with decreases in the abundance of consumers and heterotrophic microbes like Oligohymenophorans and Oomycota (Figure 4a), our findings point to potential shifts in the functional environmental metabolome and carbon cycling of peatlands under climate change (Figure 5b; Figure S20; Table S4).

Counter to our hypothesis, we report surprising reversals in functional trait change with temperature between CO₂ treatments (Figures 3 and 4; Figures S10–S13), suggesting that joint temperature and CO₂ impacts on microbial food webs may be more complex than previously thought (Figure 5; Figures S17 and S18). Interactive effects of CO₂ and temperature have been reported in aquatic phytoplankton systems (Bach et al., 2017; Sommer et al., 2015, 2017). However, these systems only displayed shifts in trait change magnitudes, and not in complete trend reversals, as reported here. Our

results thus emphasize the importance of addressing temperature impacts on microbial organisms in light of simultaneous CO₂ increases. These trend reversals may partially be due to CO₂-induced pH changes in the peat (Dieleman et al., 2015) and/or the stoichiometry of diffusion under higher temperatures and elevated CO₂ (Taucher et al., 2015). Experimental work on ocean phytoplankton showed that rising temperatures and elevated CO₂ can induce a more rapid decrease in community biomass and chlorophyll-*a* concentration (Coello-Camba et al., 2014), and that elevated CO₂ may lessen the demand for a higher surface-to-volume ratio as diffusion becomes easier across the cellular membrane at higher concentrations of dissolved CO₂. These changes may also be due to the increased efficiency of heterotrophic resource acquisition compared with photosynthesis (Clarke, 2017). The observed shift toward heterotrophic resource acquisition under elevated CO₂ conditions (Figure 3d) may lessen the need for energy conservation.

These reported trend reversals under elevated CO₂ may also occur indirectly, through changes in nutrient cycling and mineralization (Rober et al., 2022)—especially of N (Rastetter et al., 1997). Elevated CO₂ often increases the exudation of soluble organic compounds by *Sphagnum* mosses (Fransson & Johansson, 2010)—especially sugars—that contribute to the structure of microbial communities, and can increase the abundance of bacteria and heterotrophic protists (Mitchell et al., 2003). With higher abundances of bacteria, protists may obtain more energy through predation. Petro et al. (2023) recently found similar interactive

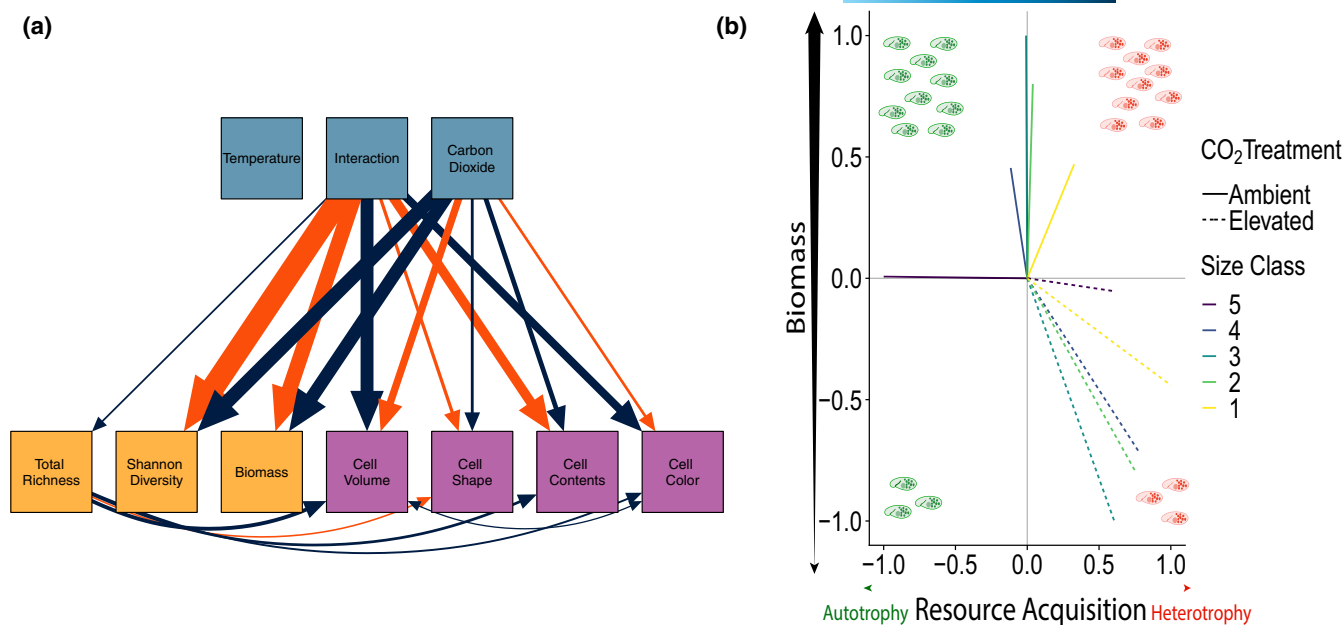


FIGURE 5 (a) Structural equation model (SEM) illustrating the effects of environmental variables and their interaction (light blue) on protist community structure (light orange) and functional traits (purple). Solid lines—shown only the top 30% stronger effects—represent significant effects, with positive 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 CO₂, the increase in respiration is offset by a loss in biomass of these organisms (IV). Given the normalization of difference, slopes ≤ -1 indicate a net reduction in heterotrophy. Under ambient CO₂ 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).

effects of warming and elevated CO₂ on N. Elevated temperature and ambient CO₂ conditions produced the accumulation of nitrogen and a reduction in N₂ fixation, which was reversed under elevated CO₂ conditions. This in turn altered N immobilization by microorganisms, likely leading to a change in microbial food web dynamics. Elevated CO₂ may also indirectly increase nitrogen availability through changes in *Sphagnum* exudation of C and N organic compounds (Bragazza et al., 2013; Carrell et al., 2019)—indirectly influencing N-releasing enzymes (Alvarenga & Rousk, 2021; Rousk, 2022)—or through symbiosis with mycorrhizal fungi (Meier et al., 2015; Uselman et al., 1999).

We found that overall, the trend reversals reported here (biomass, abundance, traits) were size-dependent (Figure S14). 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 (Gibert et al., 2022, 2023; Han et al., 2023; Wiczyński et al., 2021; Yoshida et al., 2003). Our results indicate a significant relationship between proportional changes in traits and size. Larger protists' size (Figure S14a) and shape (Figure S14b) change more than smaller protists per unit volume (Figure S14a,b). Cellular contents follow a sub-linear size dependency (Figure S14c). Because smaller protist phenotypes shift much less and these smaller individuals dominate the relative abundance of the system (Figure 2b; Figure 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 (DeLong & Gibert, 2016; Lühring & DeLong, 2020).

Interestingly, our SEM models (Figure 5a; Figures S16–S18; Tables S2–S4) uncover additional mechanisms through which these trend reversals are possible. For example, our most complex 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 (Figure 5a; Figures S16–S18). While these indirect effects are weaker in magnitude than the direct effects, they also somewhat offset the direct effects through trend reversals (Figure 5a), perhaps shielding the community from even larger community-wide 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 CO₂-enriched world (Amesbury et al., 2019; McPartland et al., 2019; Oliverio et al., 2020). Yet, the simplest SEM model does not retain some of the most salient features of the most complex SEM; that is, the interactive effect of temperature and CO₂. Very large models like the SEMs presented here require inordinately large datasets to fit the data well, so we suspect that, despite our large dataset, the simplest

model is the best model that we can fit well with the data that we have. This does, therefore, not preclude the interaction between temperature and CO₂ contained in the most complex model from occurring in nature, as we show in [Figures 2 and 3](#); rather, the model that contains said interaction may be too complex to fit the data well.

4.1 | 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 that these reported shifts owe to broader non-transient community responses to temperature and CO₂ 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 h at room temperature—could have differentially 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 (De Gruyter et al., 2020; Faillace et al., 2021; Simon et al., 2015), but have, to our knowledge, not been reported in the timescale of days.

Lastly, because our density data was not controlled by total dry moss biomass—known to be positively correlated with microbial abundances (Schroeder et al., 2021)—the observed variability in microbial biomass may be the consequence of climate-induced variability in moss biomass. Indeed, moss biomass has been shown to decrease with warming due to increased evapotranspiration and water limitation (Huang et al., 2021; Jassey & Signarbieux, 2019). If the observed decrease in protist abundance with temperature was due to a decrease in moss biomass alone, we should not observe an increase in abundance in the high CO₂ treatment, which suggests that other processes are at play. Moreover, we have designed our sampling regime so that living and dead moss biomass was consistent across samples and enclosures, so the amount of variation in protist abundances due to differences in the amount of moss biomass should be small, albeit perhaps not null. In fact, the density data recover patterns of temperature and CO₂ 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 congruence across our various data sources, we acknowledge the possibility that these sources of error may have

affected our results, but remain confident that our inferences are robust.

5 | 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 (Jassey et al., 2013). Furthermore, while earth-system models may predict range shifts in *Sphagnum* spp. (Ma et al., 2022), 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 affect the distribution of *Sphagnum* and peatlands in the future (Carrell et al., 2019; Carrell, Lawrence, et al., 2022; Carrell, Velickovič, et al., 2022; Petro et al., 2023). Given the diverse energy acquisition strategies of peatland protists and their predominant distributions in the mid- and high-latitudes of the Northern hemisphere—especially sensitive to climate change (Frolking et al., 2011; Gorham et al., 2012; Limpens et al., 2008; You et al., 2021)—changes in the balance of autotrophy versus heterotrophy, as well as shifts in body sizes and metabolic rates, can have cascading effects on the biological C cycle (Liu et al., 2016; Mitra et al., 2014; Selosse et al., 2017; Wiczyński et al., 2022). The data-driven hypotheses presented here need to be tested further, but are suggestive of important ways in which environmental change may influence and be influenced by rapid microbial responses in density, diversity, and functional trait composition in globally important—and highly threatened—ecosystems such as peatlands.

AUTHOR CONTRIBUTIONS

Christopher L. Kilner: Conceptualization; data curation; formal analysis; investigation; methodology; software; validation; visualization; writing – original draft; writing – review and editing. **Alyssa A. Carrell:** Data curation; formal analysis; investigation; software; writing – review and editing. **Daniel J. Wiczyński:** Formal analysis; writing – review and editing. **Samantha Votzke:** Data curation; investigation; resources; writing – review and editing. **Katrina DeWitt:** Validation; writing – review and editing. **Andrea Yammine:** Data curation; investigation; project administration; writing – review and editing. **Jonathan Shaw:** Writing – review and editing. **Dale A. Pelletier:** Data curation; investigation; writing – review and editing. **David J. Weston:** Data curation; investigation; writing – review and editing. **Jean P. Gibert:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors report no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available on Dryad at <https://doi.org/10.5061/dryad.kprr4xhbx>. Code for all analyses that support the findings of this study are openly available on GitHub at <https://github.com/ClassicCK/Protist-Traits>.

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SUPPORTING INFORMATION

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