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BRIEF REPORT

ENVIRONMENTAL MICROBIOLOGY

Habitat conditions and not moss composition mediate microbial community structure in Swiss peatlands

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Abstract

Peatlands, one of the oldest ecosystems, globally store significant amounts of carbon and freshwater. However, they are under severe threat from human activities, leading to changes in water, nutrient and temperature regimes in these delicate systems. Such shifts can trigger a substantial carbon flux into the atmosphere and diminish the water-holding capacity of peatlands. Microbes associated with moss in peatlands play a crucial role in providing these ecosystem services, which are at risk due to global change. Therefore, understanding the factors influencing microbial composition and function is vital. Our study focused on five peatlands along an altitudinal gradient in Switzerland, where we sampled moss on hummocks containing *Sarracenia purpurea*. Structural equation modelling revealed that habitat condition was the primary predictor of community structure and directly influenced other environmental variables. Interestingly, the microbial composition was not linked to the local moss species identity. Instead, microbial communities varied significantly between sites due to differences in acidity levels and nitrogen availability. This finding was also mirrored in a co-occurrence network analysis, which displayed a distinct distribution of indicator species for acidity and nitrogen availability. Therefore, peatland conservation should take into account the critical habitat characteristics of moss-associated microbial communities.

INTRODUCTION

Peatlands are ecosystems developed under water-saturated, mineral-poor conditions producing a thick carbon-rich humus layer known as peat (Leuschner & Ellenberg, 2017). They cover <3% of the earth's land surface, but store approximately 10% of the global freshwater (Tarnocai & Stolbovoy, 2006) and up to 30% of the world's soil carbon (Limpens et al., 2008). This carbon storage is greater than in any other terrestrial ecosystem (Dise, 2009) and has recently been estimated to be nearly twice as high as previously thought (Nichols & Peteet, 2019). Consequently, peatlands render a net cooling effect on the global climate (Dise, 2009) and are therefore essential players in counteracting climate change.

The largest proportion of peatlands is found in the Northern Hemisphere (Leuschner & Ellenberg, 2017; Tarnocai & Stolbovoy, 2006), where the formation of peat is dominated by *Sphagnum* moss species (Rydin et al., 2006). These species play an outstanding role as ecosystem engineers by creating an acidic, nutrient-poor, cold and anoxic environment (van Breemen, 1995). *Sphagnum*'s porous structure and very slow decay rate contribute greatly to its remarkable ability to retain freshwater and sequester carbon (Bengtsson et al., 2018). This slow decay rate can be attributed to *Sphagnum*'s production of recalcitrant

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compounds (Verhoeven & Liefveld, 1997; Verhoeven & Toth, 1995) and its ability to leach microbe-inhibiting compounds (Chiapusio et al., 2018; Fudyma et al., 2019; Hamard et al., 2019; Sytiuk et al., 2022), which reduce the breakdown of organic matter and thus decomposition.

Even in such harsh abiotic- and antimicrobial conditions, microbes are still able to reside on *Sphagnum* moss and they contribute further to the remarkable carbon storage of peatlands by regulating the ratio between carbon emission and sequestration. For the prokaryotes, such ecosystem functions include anaerobic methanogenic and aerobic methanotrophic microbes that produce and consume methane, respectively (Abdalla et al., 2016), thus maintaining the balance between carbon input and output. Nitrogen-fixing (N_2) autotrophic cyanobacteria and proteobacterial methanotrophs have also been found to provide ammonia (NH_3) to their host (Vile et al., 2014), while mutualistic relationships also exist, such as symbiotic methanotrophic bacteria that feed methane to submerged *Sphagnum* (Raghoebarsing et al., 2005), and bacteria that provide defences against fungal parasites (Opelt et al., 2007; Opelt & Berg, 2004). For the eukaryotes, some examples of their ecosystem functioning abilities have been demonstrated through the microalgae that live on *Sphagnum* moss, which contributes to 10% of the total carbon uptake in peatlands (Hamard, Küttim, et al., 2021). Additionally, although often reduced to a bacterivorous lifestyle, protists occur on multiple trophic levels—from photoautotrophic primary producers over parasites and saprotrophs to predators of all other kinds of microbes (including bacteria, algae, nematodes, fungi and other protists; Geisen, 2016; Geisen et al., 2018). Through predation, they significantly contribute to the nitrogen transfer from their prey to their associated plants (Clarholm, 1985) and can alter the quantity of carbon and nutrients that are cycled (Jassey et al., 2013).

Given that microbes play a central role in maintaining peatland ecosystem functioning, it is important to not only have a detailed taxonomic description of both the prokaryote and microeukaryote composition that inhabits peatlands but to also determine what environmental variables have the largest effect on this composition. In general, the taxonomy of prokaryotes in northern peatlands is well-described (Bragina et al., 2015; Dedysh et al., 2006; Lin et al., 2014), but it is less well-known for eukaryotes, with research mainly focusing on specific groups (e.g., Thormann and Rice, 2007 for fungi; Jassey, Gilbert, et al., 2011 for testate amoebae; but see Lara et al., 2011). This taxonomic composition is affected by peatland moisture level and water chemistry, with wet, nutrient-rich (i.e., groundwater-fed) fens containing more autotrophic—and less heterotrophic—microorganisms compared to ombrotrophic (i.e., rainwater-fed) bogs (Mitchell et al., 2003). Indeed, autotrophic eukaryotes are highly sensitive to water conditions, with only a small percentage present in dry, raised hummocks compared to wet hollows (Mitchell et al., 2003). Shade tolerance is also a key variable in autotrophic community composition, which shifts from eukaryote- to prokaryote-dominated when close to shaded forested areas (Hamard, Céréghino, et al., 2021). Seasonality has also been found to play a role in eukaryote community composition, with Chrysophytes dominating communities in colder conditions, while testate amoebae abundance peaks in the summertime (Lara et al., 2011). Interestingly, although testate amoebae are dominant in the warmer months, they are highly sensitive to experimentally raised temperatures partially due to an increase in *Sphagnum*'s excretion of inhibiting polyphenols into the peatland. This disappearance of the top-predatory testate amoebae from the food web has been found to reduce bacterial abundance, thus indirectly altering ecosystem functioning (Jassey et al., 2013).

These studies provide important insight into the environmental variables that affect different members of the microbial community. However, studying the microbes of both domains together is needed for a holistic view of these systems as functional entities to understand their role in maintaining peatland ecosystems. Prokaryotes and microeukaryotes are linked, most notably through predator–prey interactions, and a shift in the composition of one domain due to an altered environment can have a cascading effect on the members of the other domain. These environment-induced changes in composition can affect the microbial loop and thus the ecosystem functioning of a peatland. Prokaryotes may however have a different response to environmental variables than eukaryotes, making it necessary to examine which variables are most important for each domain separately.

This study aims to provide a comprehensive overview of microbial peatland life that targets both prokaryotes and microeukaryotes and to determine which environmental variables are most important for shaping their composition. To this end, mosses of five *Sphagnum*-dominated northern peatland sites in West Switzerland were collected to identify pro- and eukaryotic microbes via metabarcoding regions of the 16S- and 18S rRNA genes. We standardized our sampling within and across peatlands by examining only the microbes inhabiting the upper layer of *Sphagnum* moss that was located on raised hummocks containing the carnivorous plant *Sarracenia purpurea* L. We restricted our sampling to these sites and late summer because we also investigated microbial communities living inside the leaves of *S. purpurea* and were interested in these microorganisms as potential colonizers into newly opened leaves (Korn, 2021).

We used environmental descriptors for habitat conditions, moss species composition and geographic distance between sites to determine their potential

TABLE 1 Expectations of how the measured environmental variables would affect microbial community structure. Note that *habitat conditions* are obtained from Ellenberg's indicator values of the surrounding vegetation.

	Variable	Unit	Expectation
Auxiliary variable	Sampling intensity	g	Higher sampling intensity leads to more discovered taxa and thus a shift in communities
Environment	Geographic distance	m	Geographically closer communities are more similar
	Altitude	m	Composition differs along the altitudinal gradient
	Canopy cover	%	Organisms show solar radiation preferences
	Moss composition	Unitless	Organisms show adaptations towards the local composition
Habitat condition	Light	Unitless	Organisms show solar radiation preferences
	Temperature	Unitless	Organisms show preferences for average temperature
	Humidity	Unitless	Organisms show preferences for water saturation
	Soil reaction	Unitless	Organisms show preferences for acidity
	Nitrogen	Unitless	Organisms show preferences for nitrogen availability

influence on the microbial community composition. We hypothesize that both the pro- and eukaryote communities will differentiate along habitat conditions. Since acidity is known to drive microbial community composition (Lauber et al., 2009), we predict that communities will be mostly influenced by the pH of the local habitat, but that temperature will also play a large role (Dupont et al., 2016; Griffiths et al., 2011; Jassey, Chiapusio, et al., 2011). Details on the expected responses to the different factors are given in Table 1. We further hypothesize that moss composition will also affect microbial taxonomy because different moss species excrete different levels of soluble antimicrobial phenols (Bengtsson et al., 2018).

EXPERIMENTAL PROCEDURES

Sampling design and handling

The five sample sites (LT—Les Tenasses, LM—Les Mosses, LV—Les Veaux, Les Embreux, CB—Champ Buet; Table S1, Figure S1) are located in Western Switzerland, ranging in altitude from 605 to 1443 m above sea level with between-site distances ranging from 2.9 to 96 km. All sites are raised bogs, except the fen CB (BAFU, 2017a, 2017b). The main difference between these two peatland types is that fens are minerotrophic (i.e., groundwater-fed) with variable pH and nutrient content, while raised bogs are ombrotrophic (i.e., rainwater-fed) and thus, acidic and oligotrophic ecosystems (Leuschner & Ellenberg, 2017). The individual samples in each site were located in close vicinity (maximum 50 cm) to *S. purpurea* plants growing in drier hummock conditions. This standardization across field sites made our samples environmentally comparable despite the basic difference between bogs and fens. Note that site selection was constrained by site availability and the presence of *S. purpurea*, which generated an imbalance in the number of fens versus raised bogs. Consequently, the effect of altitude is confounded with peatland type and should be considered with caution. On the 20–24 August 2018, four 'sectors' within each site were determined along a 20–30 m transect. Two replicates of approximately 40 mL of moss were collected from each sector using sterilized forceps ($n = 5$ sites $\times$ 4 sectors $\times$ 2 sample replicates per sector $= 40$ samples). The stemlets were plucked from the top layer of the peatland, the euphotic zone, at a maximum depth of 10 cm from the surface. The samples were placed in 50 mL macrocentrifuge tubes (two per sector) and were immediately chilled on ice. They were returned to the laboratory within several hours, where they were prepared for storage on the collection day. These samples were used for metabarcoding the prokaryote and microeukaryote community composition inhabiting the peatlands. For moss species identification, additional mosses were collected in the same manner. These mosses were air-dried and identified following Frey et al. (2006) and Laine et al. (2018) using the nomenclature from www.swissbryophytes.ch (accessed September 2018).

Genomic DNA (gDNA) was harvested by first soaking the mosses in approximately 5–15 mL sterile, nuclease-free water (AMRESCO, DEPC treated), depending on their water content, and vortexing for 30 s. After briefly vortexing a second time, the mosses were gently pressed with a pistil to obtain 2 mL of water. This process was repeated one additional time so that approximately 4 mL of water was available per sample. For a subset of samples, a 50 μL aliquot of this water was checked with a compound microscope (Olympus CKX41) to verify that protists and bacteria were present. The water from all samples was then centrifuged (12,000 *g* for 10 min at 4°C), and the supernatant was discarded. The resulting pellets were frozen at −20°C as DNA storage. To account for the relationship between moss quantity (i.e., habitat size) and the number of recorded taxa of microorganisms,

the mosses were dried and weighed. The resulting dry weight value was used as a proxy for *Sampling intensity*, which was incorporated as an auxiliary variable. All material and surfaces in the field as well as the laboratory were wiped with 10% NaClO and were sterile, DNAse/RNAse- and pyrogen-free.

DNA extraction and sequencing

The gDNA was extracted from the samples with the Qiagen DNeasy PowerSoil DNA Isolation Kit. DNA concentration was measured with the Qubit 1× dsDNA HS assay on a Qubit 2.0 Fluorometer; if the concentration exceeded the kit's range, the Qubit dsDNA BR assay was employed. Two primer pairs were used: one targeting the prokaryotic V4 16S region (515FB/806RB, Caporaso et al., 2018) and one targeting the eukaryotic V4 18S region rRNA gene (574*_f/1132r, Hugerth et al., 2014). The gDNA samples were sequenced at Genome Québec (Montreal, Canada) on an Illumina MiSeq in paired-end mode with a read length of 250 bps. The PCR conditions can be found in Table S2. To quantify optimal sequencing depth, which is related to community complexity, two randomly selected samples were run at a sequencing depth of 100 k reads/sample for both primer pairs first. Based on the rarefaction analysis of these samples, we concluded that a coverage of about 100 k reads/sample was adequate, and the remaining samples were sequenced accordingly in two sequencing runs for each primer pair. Each sample was added twice to double the sequencing depth of 65 k reads/sample. Due to low read numbers, all 16S samples were re-sequenced and then pooled sample-wise. Due to low read quality, four 18S samples were re-sequenced and pooled sample-wise.

Each machine run was accompanied by two positive and one negative control (i.e., 10 mM Tris from PowerSoil C6) to supervise the performance of the MiSeq machine. The positive controls were pro- and eukaryote mock communities, one undiluted and one diluted 1:5. The 16S mock community contained 11 bacterial species (4 g-positive, 7 g-negative), while the 18S mock community contained nine eukaryotes. The selected pro- and eukaryotes used in the mock communities represented different clades of common environmental taxa (see Table S3 for exact composition). The 16S and the 18S rDNA sequences of the mock community species differed by more than 5% to allow validation of the amplicon sequence generation.

Environmental descriptors

We used *habitat condition* (i.e., a composite variable compiled from *light*, *temperature*, *humidity*, *soil reaction* and *nitrogen*), *canopy cover* and between-site *distance* and *altitude* as the environmental descriptors to determine their potential influence on the microbial community composition in the five sites. Note that *soil reaction* is a proxy for the soil acidity of the site. Water table depth was not considered since the sampling was standardized to drier hummocks across sites.

The canopy cover, which was used as a proxy for radiation intensity (Bianchi et al., 2017), was measured via photographs taken with a hemispherical lens (Canon EOS 5D Mark II with an 8 mm fisheye lens). To this end, one hemispherical photo on the sector level was taken. To standardize the compass direction, the bottom of each photo was pointed to the geographical north. The photos were first binarized and converted to PNG with GIMP 2.10.22 (GIMP Development Team, 2020) with manually optimized threshold values, then cropped to the circular outline of the fisheye photo before calculating the pixel distribution with a bash script.

The geographical positions of the sectors were precisely determined by marking their location on a 25-cm resolution orthophoto and then digitized with QGIS 2.18.28 (QGIS Team, 2018) on an aerial photograph (map source: http://geodata4edu.ethz.ch/). Then, the coordinates were imported into R (R Core Team, 2020) and reduced to 1D via multidimensional scaling (MDS, $GOF = 0.900$).

The *habitat condition* (*light*, *temperature*, *humidity*, *soil reaction* and *nitrogen*) of the peatland was derived via Ellenberg's indicator values (EV)—habitat condition indices for Central European flora (Ellenberg et al., 1992; Ellenberg & Leuschner, 2010)—from records of the surrounding vegetation. These EVs (vegetation data source: InfoSpecies.ch) were extracted for the vascular plants and mosses that were previously recorded to be located in the peatlands used in this study and from the mosses sampled for species identification. Non-peatland species and trees were excluded from this list. The vascular plant list that was extracted from InfoSpecies.ch represented overall plant species identity at the site level. As the aim of our study was to examine the environmental variability between the different sites and not within site variability at the microhabitat scale, the use of EVs was sufficient.

The structure of moss composition at each site was described with a non-metric multidimensional scaling (nMDS) with Bray–Curtis dissimilarities (vegan 2.6-4 package; Oksanen et al., 2019). A single dimension was sufficient to capture this structure. The coordinates of the sites were used as quantitative composition descriptors in the structural equation model (SEM).

Analysis

All analyses were run on R 4.3.2 (R Core Team, 2020) or as specified otherwise.

Amplicon sequence variants construction

Primers were removed from the amplicon reads with cutadapt (Martin, 2011) and dada2 1.28.0 (Callahan et al., 2016). Subsequently, the reads were filtered for quality, paired-end reads were merged based on their overlap and amplicon sequence variants (ASVs) were constructed with dada2. ASVs were classified with the RDP classifier (Wang et al., 2007) based on the SILVA 138 reference database (Quast et al., 2012). For the eukaryotes, the amplicons were too short for the paired-end reads to overlap, thus, they were simply concatenated. This procedure is justified after stringent quality filtering (Hugerth et al., 2014).

Sequences and affiliated taxonomy were handled with the R package phyloseq 1.44.0 (McMurdie & Holmes, 2013). Sequences that remained unidentified at the domain or phylum level were discarded (3.58% of prokaryote and 18.33% of eukaryote ASVs). The remaining ASVs were transformed to relative abundance and filtered for low-percentage-abundance taxa ($<1 \times 10^{-6}$ sample-wise). To reduce spurious ASVs, sequences were aggregated on the genus level. These ASVs were then filtered for non-target organisms (for the prokaryotes: mitochondria and chloroplasts [3.39%]; for the eukaryotes: vertebrates, plants, molluscs and non-crustacean arthropods [18.03%]).

Microbial diversity and community composition

To analyse the difference in α-diversity (i.e., ASVs agglomerated on genus level) between sites, a one-way ANOVA was conducted with α-diversity as the response variable, and site as the explanatory variable. The analysis was done separately for prokaryotic- and eukaryotic α-diversity. The data were controlled for a normal distribution with a Q-Q plot (Figure S3). To test for differences in mean α-diversity between the fen site and the four raised bog sites, Tukey's 'Honest Significant Difference' (Tukey's HSD) was applied as a post-hoc test to obtain multiple pairwise comparisons between the site-level α-diversity of each domain. A significance level of $\alpha = 0.05$ was chosen. To examine for differences in community composition within and between sites, a nMDS was created using Bray–Curtis dissimilarities (vegan 2.5-6 package (Oksanen et al., 2019)) on natural logarithm $+1$ transformed ASV data. These dissimilarities were then reduced into two dimensions; the coordinates on the first axis of each, the pro- and the eukaryotes were used as response variables in the SEM.

Structural equation modelling

A SEM was used to infer the causal relationships (Pearl, 2012) of the environment on the pro- and eukaryote microbial community composition in the peatland mosses. The geographic variables were *distance* between sites and sectors and *altitude*; the local variables were *canopy cover* and the four EVs for *light*, *temperature*, *humidity* and *soil reaction* (acidity). The EVs were summarized in a composite variable (*habitat condition*) to obtain the collective effect of these abiotic factors (Grace & Bollen, 2008). We included *moss composition*, which was at the same time dependent on the previous variables and was influencing the pro- and eukaryote composition. Moss composition at each sector was expressed as the coordinates of the first axis of a nMDS analysis. Additionally, the pro- and eukaryotic communities were inserted to account for the interaction between them. Finally, *sampling intensity* was used as an auxiliary variable affecting pro- and eukaryotic composition to account for sampled habitat size. The EVs for *nitrogen* were excluded from the model due to their high correlation with *soil reaction* ($r = 0.97$; Figure S2). The specific hypotheses linking environmental variables and the composition of both microbial communities are listed in Table 1 and are visualized in Figure S4.

The SEM was obtained by first standardizing the numeric variables. The composite variables for both of the response variables—the pro- and eukaryote communities—were then manually calculated with lavaan 0.6-17 (Rosseel, 2012). For this, the EVs were regressed on both response variables to obtain their individual responses to the composite variable *habitat condition* in a saturated, unstructured path model. The obtained scores were multiplied with the original values of each indicator and then these values were summed to form the composite scores. The SEM was fitted with blavaan 0.5-3 (Merkle & Rosseel, 2015), running four chains with a warm-up of 10,000 and sampling for 10,000 iterations. Results were plotted with semPlot 1.1.6 (Epskamp, 2019) and refined with Inkscape 1.2.2 (InkscapeProject, 2020).

Indicator species

We chose to determine the indicator species for acidity to use in our co-occurrence analysis because acidity is known to have a large influence on microbial community composition, and it is an essential environmental difference between fens and raised bogs (Leuschner & Ellenberg, 2017). The indicator species were calculated using the R package indicspecies 1.7.14 (Cáceres & Legendre, 2009) with 999 permutations on a significance level of $\alpha = 0.05$ based on the EV *soil reaction*. In analogy to the EVs (Ellenberg et al., 1992), samples with a mean *soil reaction* value below two were classified as 'strong-', those above two and up to four as 'medium-' and those above four and up to five as 'moderate-' acidity. Taxa that occurred in less than eight samples (which refers to a minimum occurrence of

20% of all samples) were excluded from the analysis. Only taxa with correlations ≥0.7 were considered as indicator species (Table S4).

Co-occurrence analysis

Co-occurrence networks were calculated to evaluate the potential interactions between and within the prokaryotes and eukaryotes in the sampled peatland mosses. For this, only taxa that occurred in at least eight samples were considered. We are aware of the risk of over-interpretation with co-occurrence networks (Goberna & Verdú, 2022); in our case, we believe that it is sensible to use this method since we are dealing with well-defined communities inhabiting a restricted and well-defined ecosystem type. Spearman's rank correlation coefficient ρ was then calculated using the R package HMisc 5.1-1 (Harrell Jr et al., 2020) for all possible pairs of taxa. p-values were adjusted for multiple testing using Benjamini–Hochberg's false discovery rate approach (Benjamini & Hochberg, 1995). These pairs were subset to $\rho > 0.7$ (moderate to strong association) and p-value < 0.001. From these data, a graph was created and annotated with metadata using the R package igraph 1.5.1 (Csardi & Nepusz, 2006). The graph was then exported to Cytoscape 3.10.1 (Shannon et al., 2003) for visualization with the network properties, indicator species and Louvain communities.

To understand how the co-occurrence network was linked to the environment, we analysed with Mantel tests how this network matched the EVs (*light*, *temperature*, *humidity* and *soil reaction*) measured at the sampling sites. If an environmental variable plays a role, co-occurring taxa are expected to have similar optima for that variable. Consequently, we measured the optimum of each environmental variable for each taxon; for this, we used the average of each variable weighted by taxa abundance at each site. Then, we measured the Euclidean distance between the optima of all pairs of taxa, yielding a distance matrix for each of the four EVs. Additionally, we examined if moss composition played a similar role (do co-occurring prokaryotes and eukaryotes share similar moss species?). We measured each moss taxon for its 'optimum' based on the nMDS coordinates of the moss communities and computed a fifth distance matrix for the moss. We used Mantel tests to relate the five distance matrices in environmental and moss optima with Spearman's rank correlation co-occurrence matrix, which describes the network. To match the structure of the SEM, we used *temperature* and *altitude* as covariables for the partial test. The analyses were performed with the function *mantel* of the R package ecodist (Goslee & Urban, 2007).

RESULTS

Habitat conditions and moss composition

The sites were similar in terms of *light*, *temperature* and *humidity*, but differed strongly in *soil* reaction (acidity) and *nitrogen* (Figure 1). As expected, the fen CB had the highest levels of *soil reaction* and *nitrogen*. In total, nine moss species were identified from the collected samples, with five species belonging to the peat mosses (*Sphagnum*; Table 2). The moss species composition converged well into 1D in the nMDS (stress = 0.045), whereby the raised bogs and the fen were well separated, with moss composition in CB different than the other four sites (Figure 2). The most commonly identified peat moss was *S. magellanicum* agg., which is a species complex that includes *S. divinum* and *S. medium* (Hassel et al., 2018). It was present in every sector of all four raised bogs but was absent from the fen CB. The second most frequently observed peat moss was *S. angustifolium*, which was also present in all raised bogs, but absent from some sectors and from the fen CB. While *S. magellanicum* agg. and *S. angustifolium* were found exclusively in the raised bogs, *S. subnitens* and *Calliergonella cuspidata* were restricted to the fen CB. Two species, *S. capillifolium* and *S. rubellum*, were found only in LT (Table 2).

Microbial diversity and community composition

At the global scale, there was a greater diversity of prokaryotes compared to eukaryotes in the moss microbial community (i.e., 662 and 469 genera, respectively). The mean prokaryotic α-diversity (i.e., average diversity from all eight samples within a site) was distinctly the highest in the low altitude fen CB ($\overline{x} = 252.0$), followed by LE ($\overline{x} = 212.1$), LM ($\overline{x} = 195.3$), LV ($\overline{x} = 184.4$) and LT ($\overline{x} = 178.6$; Figure S5). Similarly, mean eukaryotic α-diversity was highest in CB ($\overline{x} = 146.9$), followed by LT ($\overline{x} = 118.5$), LM ($\overline{x} = 117.0$), LE ($\overline{x} = 105.9$) and LV ($\overline{x} = 99.5$; Figure S5). The sample quantiles roughly matched the theoretical quantiles (Figure S5), and consequently, the ANOVA followed by Tukey's HSD was calculated: the difference in mean α-diversity between the fen and the raised bogs was statistically significant for all site pairs for prokaryotic α-diversity, but only for two site pairs for eukaryotic α-diversity (namely, CB-LT and CB-LM; Table S5).

Overall, most prokaryotes belonged to the bacteria, with the archaea being a minority that occurred only in one sample of the CB site (Figure 3). Bacteria were represented in 30 different phyla, whereby the Proteobacteria were by far the most abundant in terms of sequence abundance. Within the Proteobacteria, the

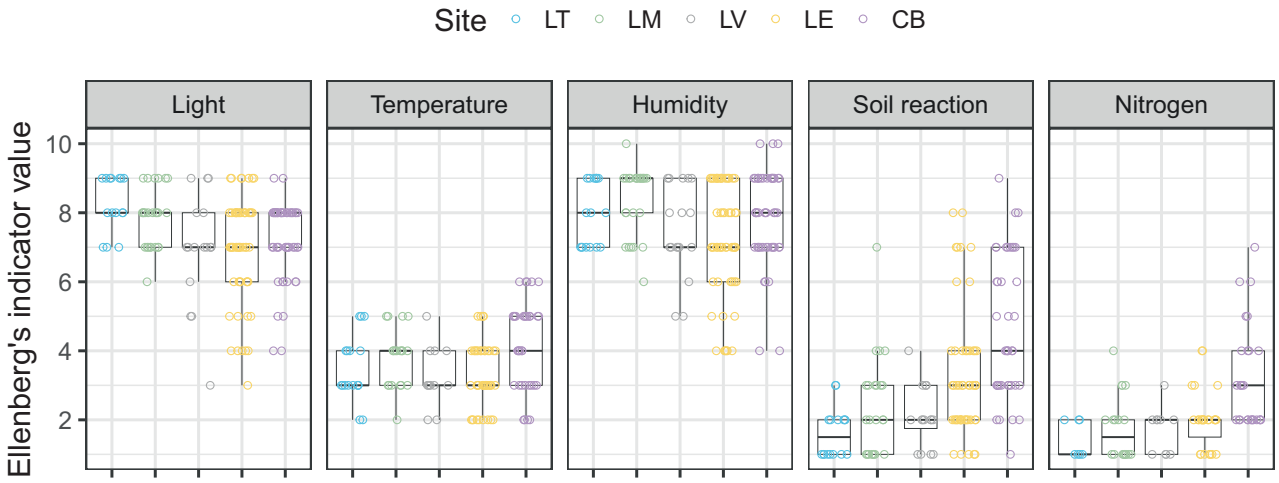

FIGURE 1 The Ellenberg's indicator values were retrieved from previously recorded plants and mosses (data source: InfoSpecies.ch) for each site. *Light* medians 7 and 8 referred to 'half-' and 'half to full light' indicator plants; *temperature* medians 3, 4 and 5 to 'cold to cool-', 'cool-' and 'cool to moderately warm' indicator plants; *humidity* medians of 7, 8 and 9 to 'humid-', 'humid to wet-' and 'wet' indicator plants; *soil reaction* medians—that should not be confused with pH—of 2, 3 and 4 to 'strong acid to acid-', 'acid-' and 'acid to moderately acid' indicator plants, respectively; *nitrogen* medians of 2 and 3 to 'extreme nitrogen shortage to nitrogen shortage-' and 'nitrogen shortage' indicator plants (Ellenberg et al., 1992). CB, Champ Buet; LE, Les Embreux; LM, Les Mosses; LT, Les Tenasses; LV, Les Veaux, which are organized by high- to moderate Ellenberg Value for *soil reaction*.

TABLE 2 Identity and occurrence of the sampled moss species within each sector of each site. Sites are organized from low altitude (left) to high altitude (right), with CB being the fen site and LV, LE, LT and LM being the raised bog sites.

Site	LT				LM				LV				LE				CB			
Species|sector	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Aulacomnium palustre											x				x		x			
Calliergonella cuspidata																				x
Sanionia uncinata															x					
Sphagnum angustifolium		x			x	x		x	x	x	x	x			x	x				
Sphagnum capillifolium	x	x		x																
Sphagnum magellanicum agg.	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x				
Sphagnum rubellum			x	x																
Sphagnum subnitens																	x	x	x	x
Straminergon stramineum														x						

Acetobacteraceae, Caulobacteraceae and Burkholderiaceae were the most abundant families across all sites (Table S6). For the eukaryotes, there were 44 phyla present, with algae represented by five phyla, and eight fungal and seven metazoan phyla were found (Figure 3). The most abundant phylum was Nematozoa, followed by the Arthropoda, Ascomycota and Ochrophyta, respectively (Figure 3, Table S6). Within each of these phyla, the most abundant ASVs were matched to nematodes from the class Chromadorea, an unknown Arthropoda, fungi from the class Dothideomycetes (Genus Leptosphaeriaceae) and a flagellate protist from the class Chrysophyceae (Genus Chromulinales_fa). Additionally, fungus from the class Agaricomycetes, green algae from the class Chlorophyceae and protozoa from the class Cercozoa were highly abundant. These highly abundant eukaryotes were present in all sectors of all sites (Table S6) and covered several trophic levels (autotrophs, mixotrophs, heterotrophs, bacterivores and decomposers).

For both the pro- and eukaryote communities, the sites were well separated in the 2D nMDS (Figure 4; prokaryotes: stress = 0.126, eukaryotes: stress = 0.190). The sites were all well-separated with only one exception (i.e., the two samples of prokaryotes in sector '1' from the site LM). The fen CB was separated along the first axis from the raised bogs for the prokaryotes and along the second axis for the eukaryotes. This separation may be due to the Dinoflagellate genera, in which 12 of the 20 genera were found only in the fen site (Table S6).

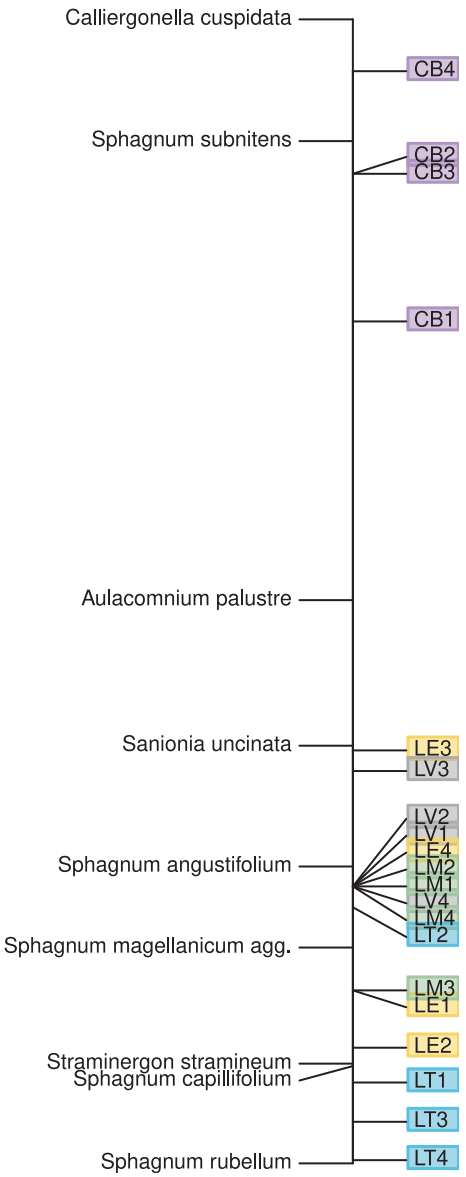

FIGURE 2 A one-dimensional nMDS plot of the moss species composition sampled within each of the four sectors (labelled as numbers) of each site (denoted by colours and labelled as the raised bogs LT (Les Tenasses), LM (Les Mosses), LV (Les Veaux), LE (Les Embreux) and the fen CB (Champ Buet). Species scores are computed as the average of the site scores where they are present. The sites separated well according to habitat type, with the raised bogs more similar in moss composition compared to the fen.

Structural equation modelling

The SEM converged well, with all $\hat{R}$—a convergence diagnostics of a Markov chain Monte Carlo (Gelman & Rubin, 1992)—equal to 1.000. The posterior predictive *p*-value was 0.363. The geographical and environmental descriptors (*distance*, *altitude and canopy cover*) had a strong effect on the microbial communities; however, this effect was mediated through the composite *habitat condition*. The direct effect of these descriptors on the microbial communities was close to negligible, especially for the eukaryotes. For the prokaryotes, *altitude* and *canopy cover*—and to a lesser extent also *distance*—had an intermediate direct effect on community composition. Still, the habitat condition composites had by far the strongest influence on both the eukaryote and prokaryote communities (Figure 5).

For the prokaryotes, all EVs made an intermediate contribution to the composite *habitat condition*, while the individual contribution of the EVs was rather strong for the eukaryotes. *Light* was the most influential composite component for the prokaryotes, while it was *soil reaction* and *temperature* for the eukaryotes. *Moss composition* itself was strongly influenced by *altitude* and *canopy cover*. It showed a significant effect on the prokaryotes, but its effect on the eukaryote community composition was rather weak. *Moss composition*'s correlation with *habitat condition* was low, indicating that both variables were well described by the environmental variables. The interdependency of the two domains on each other was negligible. In addition, the auxiliary variable *sampling intensity* had a negligible effect on microbial composition (Figure 5). As *nitrogen* was excluded from the model due to its high correlation with *soil reaction*, all statements about the latter are valid as the same for the former.

Indicator species of acidity and co-occurrence network

Of the 194 taxa identified as indicator species of acidity, 109 were indicators of moderate, 40 of medium and 45 of strong acidity (Table S4). This distinction was in line with the identified moss species at each site, which were tolerant to different levels of acidity (Figure 2). In the co-occurrence network (Figure 6), the 670 nodes (taxa) were connected by 6965 edges, with 6895 positive and 70 negative relationships. The average degree (i.e., the average number of links per node) was 20.8, whereby the degree ranged from 1 (116 nodes) to 100 (65 nodes).

The core of the network was a dense cluster of highly connected nodes composed, which contained all recorded domains (archaea, bacteria and microeukaryotes). Adjacent was a rather loose subnetwork, which contained the majority of all indicator species of acidity (92.3%). They contained phototrophs (e.g., Nostocaceae, *Chrysamoeba*), mixotrophs with several taxa belonging to the dinoflagellates, and chrysophytes (e.g., *Peridinium*, *Uroglena*, Dinoflagellata; *Ochromonas*, *Chromulina*) and heterotrophs (e.g., Comamonadaceae, Monhysterida, *Sporichthya*; *Colpodidium*, Chromadorida, *Conexibacter*, *Chitinimonas*, *Parachaela*). Throughout the entire network, the most abundant taxa were an unidentified member of the candidate phylum Candidatus Eremiobacterota (previously WPS-2), an Acetobacteraceae, *Poterioochromonas* and *Acidocella*. A nematode from the order Triplonchida was also highly abundant but was found in its own sub-network.

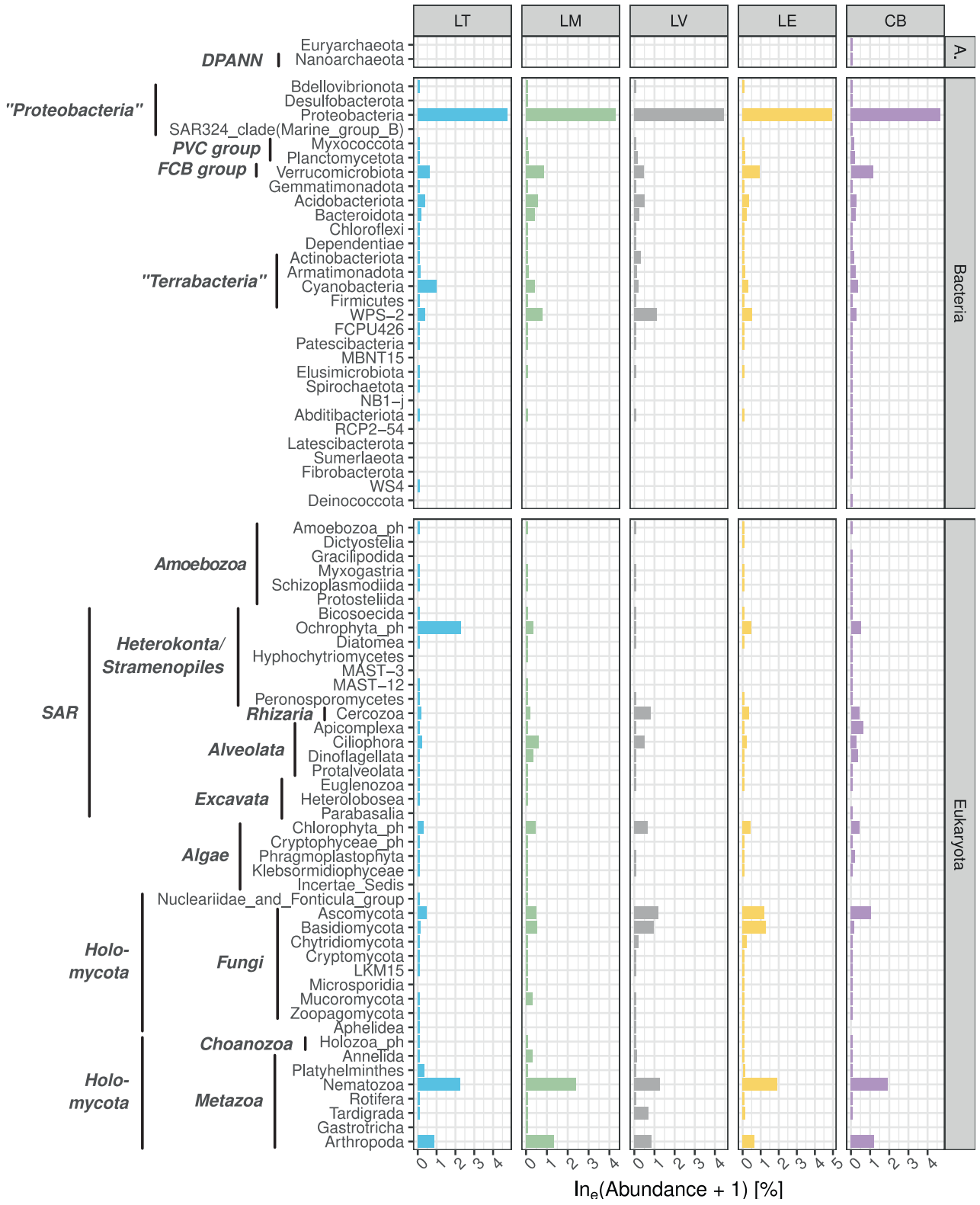

FIGURE 3 The relative abundance of the moss microbial phyla within each site. A, archaea. Relative total abundances <0.01 within a site and >0 were replaced with 0.01 for visualization reasons. CB, Champ Buet; LE, Les Embreux; LM, Les Mosses; LT, Les Tenasses; LV, Les Veaux.

The Mantel tests between the Spearman co-occurrence matrix and the optima's distance matrix of the four EV variables confirmed the important role of acidity but also revealed a strong connection with light. Specifically, the Mantel tests yielded the strongest influence on *light* ($r = -0.37$), followed by *humidity*

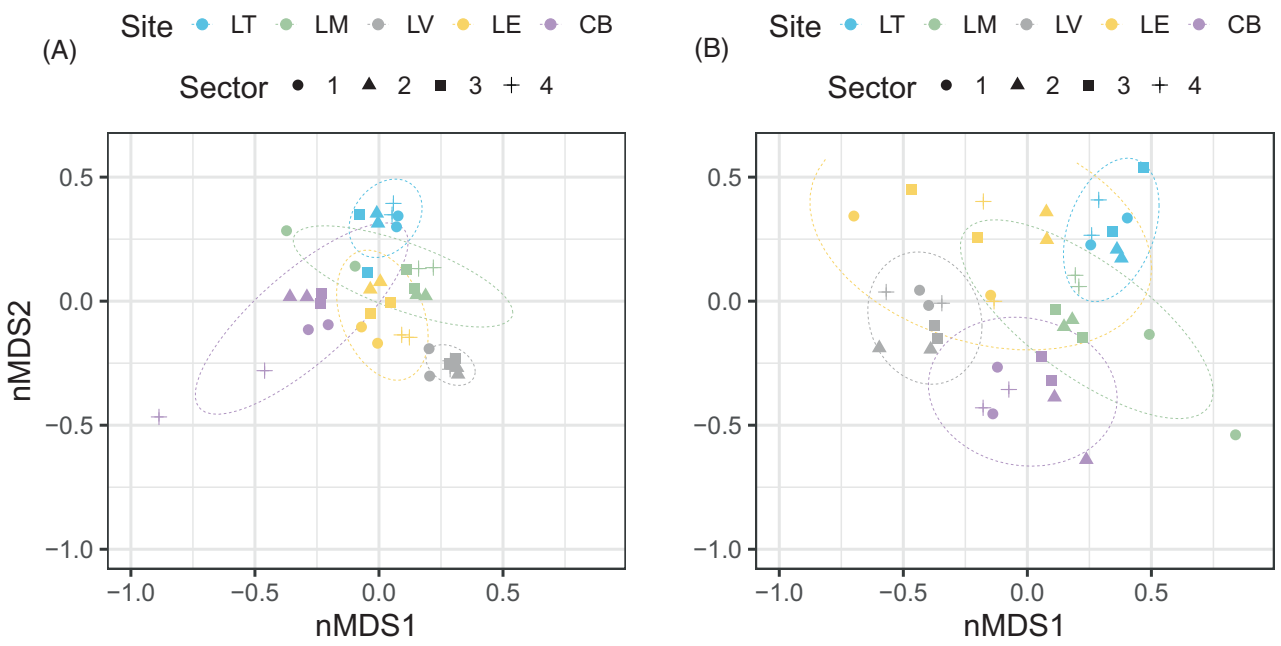

FIGURE 4 NMDS biplots representing the similarity in (A) prokaryote- and (B) eukaryote community composition reduced to 2D across sites and sectors. Sites are denoted by colours and the four sectors within each site are denoted by shapes. Ellipses delineate the 95% CIs of each site. CB, Champ Buet; LE, Les Embreux; LM, Les Mosses; LT, Les Tenasses; LV, Les Veaux, which are organized by high to moderate Ellenberg Value for *soil reaction*.

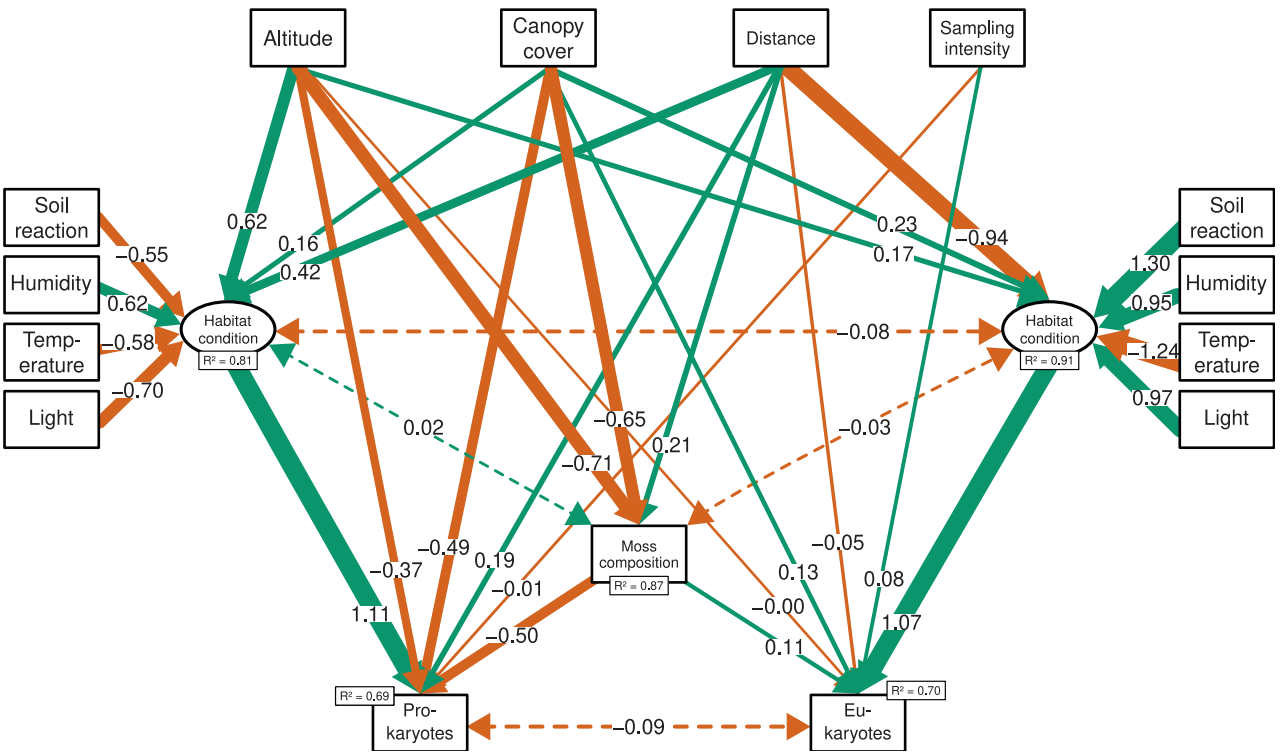

FIGURE 5 Path diagram of the SEM determining which variables influenced pro- and eukaryote community composition. The graph was assembled from the computation of the composite *habitat condition* with lavaan and the subsequent SEM with blavaan. Ellipses indicate composite variables; they were calculated for each of the response variables separately to account for their individual effect. *Nitrogen* was excluded from the composite variable due to its high correlation with *soil reaction*. Numbers on the arrows denote path coefficients (regression estimates), the thickness of the arrows denotes the strength of a relationship, while the arrow colour denotes the sign of the relationship (green for positive and brown for negative). Dashed arrows represent correlations between variables.

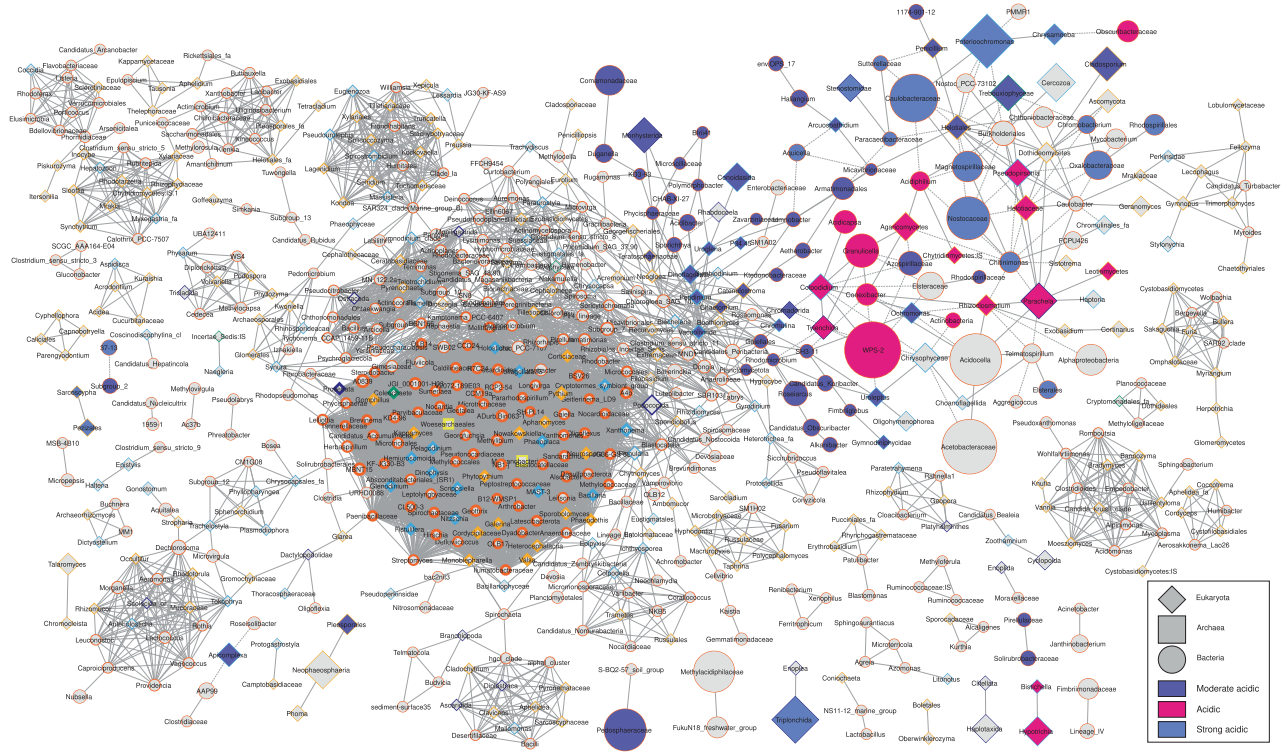

FIGURE 6 Co-occurrence network for the pro- and eukaryotic taxa that occurred in at least eight moss samples with correlation $\rho > 0.7$ and Benjamini–Hochberg false discovery rate corrected *p*-value < 0.005. Node size is scaled by abundance and edge width by correlation strength. Node outline is scaled by the degree (number of edges) of a node. Node outline colours denote the taxonomic affiliation (yellow—archaea, red—bacteria, orange—fungi, blue—protists, green—algae, dark violet—micrometazoa) (*Parachela* is a typo in the SILVA 138 database and refers to the tardigrade *Parachaela*).

($r = -0.26$), *soil reaction* ($r = -0.23$) and *temperature* ($r = -0.21$). All Mantel coefficients *r* were significant at a *p*-value < 0.001. Interestingly, the simple Mantel test for the co-occurrence matrix and distances in moss composition yielded a statistically highly significant result ($r = -0.23$, *p*-value < 0.0001); however, this effect strongly decreased with the partial Mantel test accounting for *altitude* and *canopy cover* ($r = -0.014$, *p*-value = 0.06), which matches the result of the SEM analysis.

DISCUSSION

Despite their importance in global carbon and freshwater storage of peatlands, moss-associated microbes are strikingly understudied, even in comparison to some much less accessible ecosystems, such as the deep-sea floor (Rusin, 2016). This work focuses on a well-defined microhabitat type, namely the top layer mosses in peatland hummocks harbouring *S. purpurea* plants, but is comprehensive in targeting both pro- and microeukaryotes. Our results contribute to the pioneering sequencing works on peatland microbes (Bragina et al., 2012; Bragina et al., 2014; Bragina et al., 2015; Dedysh et al., 2006; Lara et al., 2011; Lin et al., 2014; Opelt et al., 2007; Singer et al., 2019; Wicaksono et al., 2021). Herein, we provide an overview of the main microbial lifeforms in this microhabitat and their potential interactions, as well as demonstrate the driving environmental factors of community structure.

We sampled the mosses and their associated microbial community in two different peatland types stretching across an altitudinal temperature gradient, where the sites' pairwise distances ranged from 2.9 to 96 km. As expected, we found a clear distinction in moss composition in the fen compared to the raised bogs that followed a gradient of acidity and nitrogen availability (Figure 1 and Table 2). The two mosses that occurred exclusively in the fen were *Sphagnum subnitens* and *Calliergonella cuspidata* (Table 2): the first typically grows in poor fens, but also in oligo- to mesotrophic habitats (Daniels & Eddy, 1990; Frahm & Frey, 1987), and the second is found in marshes and wet meadows, but also on wet stems and rocks (Frahm & Frey, 1987). On the contrary, the two most common species that were absent in the fen are typical raised bog Sphagna: *Sphagnum magellanicum* agg. is a wide-spread raised bog species that typically grows on hummocks, while *S. angustifolium* grows in drier areas and edges of raised bogs (Frahm & Frey, 1987). Yet, despite these differences in moss composition

between the two habitat types, both the pro- and eukaryote community compositions were site-specific, with the microbial community composition different even among the four raised bog sites (Figure 4).

As expected in microbial studies, our study found that the phyla-level composition for both the pro- and eukaryotes was highly similar among all five sampled peatlands (Figure 3). However, the communities clearly diverged at the genus level, indicating community divergence and specialization to the habitat at a lower taxonomic level. Interestingly, the prokaryotic communities in the fen differed from those in the raised bogs, whereas this difference was less apparent for the eukaryotic communities (Figure 4). Using SEM, we found that the microbial community composition was primarily driven by the *habitat condition*, a composite variable derived from the surrounding peatland vegetation representing the collective effect of *soil reaction* (and thus also *nitrogen*), *light*, *temperature*, and *humidity* through Ellenberg's indicator values (Figure 4). EVs, which are widely used in vegetation science to assess and, in particular, compare multiple plots (Diekmann, 2003), translate a given plant species composition into long-term ecological values. By capturing a broader range of environmental states, they offer a greater reflectance of general conditions compared to point measurements. The latter are more accurate for fine-scale evaluation of environmental conditions but are more prone to issues with incomplete sampling (Ewald, 2003). The agreement of EVs with measured values is sufficiently high (Schaffers & Sýkora, 2000, Szymura et al., 2014). One drawback, though, is that they are not independent of environmental variables – mean EVs are derived from species composition that is influenced by the environment. As we mainly used the EVs of vascular plants and mosses to draw conclusions about microbial communities, there is no risk of circular reasoning.

The *moss composition* and the geographical- and environmental- descriptors (*distance*, *altitude*, and *canopy cover*) had a low- to negligible- influence on the community composition. These general environmental descriptors influenced the microbial composition of peatlands indirectly by affecting *habitat conditions*. The *habitat condition* of a site was found to be equally important for both the pro- and the eukaryote composition, whereas their most important component was *light* for the pro- and *soil reaction* and *temperature* for the eukaryotes. *Soil reaction*, and thus, also nitrogen, in particular, had opposite effects on the eukaryotes and prokaryote composition. In general, pH is regarded as a strong predictor of bacterial communities (Lauber et al., 2009), likely due to its direct and indirect influence on the ionic state that modulates the availability of many inorganic ions and metabolites, and thus plays a crucial role in nutrient uptake for prokaryotes (Schlegel & Jannasch, 2013). Variables related to pH and nutrient availability have also been found to explain peatland prokaryotic community composition in other studies (Bragina et al., 2012; Lin et al., 2014).

As prokaryotes are in general distinctly more species-rich than eukaryotes (Locey & Lennon, 2016; Pawlowski et al., 2012), the α-diversity of all sampled sites followed this pattern (Figure S3). The observed α-diversity patterns of pro- and eukaryotes were nearly congruent for all sites. The fen CB was the most diverse site for both domains. Due to the low pH and nutrient scarcity, raised bogs are generally considered species-poor, often containing specialized organisms for both flora (Leuschner & Ellenberg, 2017) and fauna (Desrochers & van Duinen, 2006). Regarding the present results, this pattern seems also to hold for microbes. Likewise, other studies have found fewer bacteria species on an oligotrophic *Sphagnum* species compared to a mesotrophic one (Opelt et al., 2007). In our study, similar to (Wicaksono et al., 2021) who sampled from the full vegetation of Austrian-raised bogs, the Proteobacteria were much more abundant compared to other studies conducted in bogs (Bragina et al., 2015; Lin et al., 2014). This result seems plausible because the dominant moss species in our study, *S. magellanicum* agg., generally exhibits a rather congruent phyla-level bacteria composition (Bragina et al., 2012).

The most striking prokaryotic taxonomic difference between the present study and others was the consistent occurrence of archaea in both Austrian (Bragina et al., 2015) as well as Minnesotan bogs (Lin et al., 2014), while this domain was negligible in the present study although the primers we used were able to detect them (Caporaso et al., 2018). Archaea are known to inhabit deeper, anoxic peatland layers in higher densities than the surface (Dedysh et al., 2006; Lin et al., 2014). It is plausible that this domain was missing in our study because sampling was restricted to the euphotic surface zone, with all samples taken above 10 cm depth.

In comparison to prokaryotes, microeukaryotes are substantially understudied in all kinds of environments (Debroas et al., 2017), which also applies to peatlands (Graham et al., 2017; Lara et al., 2011). One of the first extensive studies on peatlands that used a molecular identification approach revealed a vast diversity of different microeukaryotes in a raised bog pool (Lara et al., 2011). Moreover, since the study was published, reference databases have steadily been increasing in both quality and quantity (Glöckner et al., 2017). As in the present study, Singer et al. (2019) found that microeukaryotic communities of temperate fens and raised bogs separated well; however, taxonomic differences between the habitat types remained unresolved in their case. That study found a composition that was dominated by osmo-, mixo- and phagotrophs, while phototrophs and parasites played a subordinate role.

More than 50% of all reads were assigned to fungi, with the other abundant taxa belonging to the Chrysophyceae, Apicomplexa, Ciliophora, and to a lesser extent, to the Chlorophyta, Cercozoa and Cryptophyta (Singer et al., 2019). Overall, these results only partly matched the taxonomic composition in our study. In particular, the difference in fungal richness and abundance might be explained by the more stringent resolution and ecological filter criteria that were applied in the present study. Additionally, Singer et al. (2019) sampled temperate peatlands in multiple months (i.e., June, July and October), which could result in an overall larger taxonomic richness compared to the single August time point in our study.

Instead of following an altitudinal temperature gradient, the *habitat condition* of the five peatlands followed a gradient of acidity and nitrogen availability (*soil reaction* and *nitrogen*)—a phenomenon that is typical for pedobiomes (Leuschner & Ellenberg, 2017). This feature was also reflected in the distribution of indicator species of acidity across the three main clusters of the microbial co-occurrence network. These three clusters contained pro- and eukaryotic taxa from the five peatlands, each with different functional groups of photo-, mixo- and heterotrophs. This gradient of acidity was observed also in the connectivity between the clusters, with high connectivity along the gradient from moderate- to acid- to strong acidity, but low connectivity between moderate and strong acidity (Figure 6). In the SEM analysis, light availability was also found to be linked to our communities. This factor was less studied than acidity for moss microbiomes (but see e.g., Hamard, Küttim, et al., 2021), but its importance in aquatic systems has been highlighted (Charvet et al., 2014; Ruiz-González et al., 2013). Interestingly, the contribution of light was also revealed by the Mantel tests. With these tests, we related the co-occurrence network to the ecological niches of the species through their optima for the environmental variables. The fact that the results of the Mantel tests closely matched those of our SEM analysis provides an argument for their combined use. The SEM treats the communities through their positions on ordination axes and explores the relationships of explanatory variables with path-analytical models of various types, from simple linear regressions to mixed-effects models (Lefcheck, 2016). The co-occurrence network offers a representation of the species in the communities and of their potential roles (in our case, their sensitivity to acidity), which opens the possibility of gaining a deeper understanding of community organization.

CONCLUSION

This work, targeting both pro- as well as microeukaryotes, provides an in-depth overview of microbial lifeforms in peatland hummocks and their co-occurrence. Importantly, our results highlight that the light availability, temperature, humidity and soil acidity of Central European peatlands are the main direct drivers for microbiome composition. Thus, structural modifications of the habitat are likely to shift peatland microbiome composition and should be incorporated into management plans. For example, commonly applied land 'amelioration' techniques, such as drainage or reclamation, massively impact the vegetation. This change in vegetation will have downstream effects on microbial communities that ultimately may interfere with the carbon retention of peatlands. The composition of the pro- and the eukaryotes was both strongly affected by *habitat conditions*, although by different strengths of the environmental components, highlighting the importance of investigating both domains to understand the structure and functioning of these microbial communities.

AUTHOR CONTRIBUTIONS

Rachel Korn: Conceptualization; investigation; writing – original draft; methodology; validation; visualization; writing – review and editing; formal analysis; project administration; data curation. **Christian Berg:** Methodology; writing – review and editing; data curation; validation. **Louis-Félix Bersier:** Conceptualization; funding acquisition; writing – review and editing; formal analysis; supervision; resources; visualization. **Sarah M. Gray:** Conceptualization; investigation; writing – review and editing; visualization; project administration; data curation; supervision. **Gerhard G. Thallinger:** Writing – review and editing; methodology; formal analysis; supervision; data curation; visualization; validation.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All code including raw data tables is available on GitHub: https://github.com/bathyscapher/peatland-microbes, and raw sequences are publicly available in the NCBI database under BioProject PRJNA733308:

https://www.ncbi.nlm.nih.gov/bioproject/PRJNA733308. The FASTAs with either an M or a Q at the 7th position are the sequences used for this project (e.g., CB1N01**Q**r_16S_R1.fastq.gz).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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