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RESEARCH

What is hiding within the moss? Assessing the impact of sheep grazing on bryosphere diversity in Icelandic tundra

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Abstract
Bryophytes are home to an underestimated diversity of macroscopic and microscopic organisms, which are an integral part of the bryosphere. This multitude of species is involved in numerous ecosystem processes such as nutrient cycling. Their role is particularly important in biomes where these processes are fragile, like the Arctic tundra, given recent environmental changes and anthropic pressures. Using environmental DNA methods, we aimed at surveying the diversity and abundance of the bryosphere communities within *Racomitrium lanuginosum*, a common moss species in Iceland, and how they are affected by sheep grazing. We sampled moss from grazed and ungrazed plots at two locations in Iceland and identified all eukaryotes and bacteria within different segments of the moss. Sheep presence influenced the community compositions of protist, bacterial, and fungal communities. Diversity of identified organisms increased down the moss layer, getting closer to the litter and soil, for all organism groups. Additionally, we found that community composition within the moss differed depending on sample location and depth within the moss layer. By screening the major organism groups living inside the moss layer, this study provides novel insights into the spatial distribution of moss-associated organisms within microhabitats.

Keywords EDNA · Arctic tundra · Moss segments · *Racomitrium lanuginosum* · Bacteria, Protists, Fungi, Metazoan communities

Introduction

Bryophytes, mosses in particular, represent an important part of the Earth's biodiversity. While often overlooked, they can host a hidden diversity within the moss layer itself, composed of a large variety of microorganisms, from bacteria to mesofauna. Lindo and Gonzalez (2010) used the term "bryosphere" to emphasize the cryptic diversity of organisms associated with bryophytes and their combined role in ecosystem functioning. Since then, research on the identification of all species living within the moss layer and their participation in ecosystem functions has been increasing but remains insufficient. The overall diversity and roles of each trophic level of the cryptic bryosphere are still to be identified. The major organism groups in the moss layer are bacteria, fungi, microfauna such as tardigrades, rotifers, and nematodes, mesofauna like collembolas and mites and finally macrofauna such as insects (Gerson 1982; Glime 2017; Kauserud et al. 2008; Usher and Booth 1986). Given the architecture of most moss species and the amount of water droplets trapped by moss leaves, the composition of species within the moss layer can sometimes be comparable to aquatic environments (Debroas et al. 2017; Singer et al. 2019). This diversity can also be influenced by a variety of environmental factors such as temperature, moisture, and biogeochemical conditions (Calderón-Sanou et al. 2021; Grau-Andrés et al. 2022; Kardol et al. 2016).

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Tundra ecosystems are subject to extreme conditions that impact vegetation and biodiversity in general (Meineri et al. 2020; Myers-Smith et al. 2011; von Oppen et al. 2024). Mosses, due to their adaptative properties such as poïkilohydry, are often particularly prominent in Arctic vegetation (Longton 1988; Jónsdóttir 2011). The understanding of bryophyte species diversity and their associated roles in the ecosystem is then of first importance for Arctic ecology (Turetsky et al. 2012). The few studies on extreme ecosystems have already shown the presence of an important diversity of organisms within the moss layer, whether it is bacteria, fungi, protists, or metazoa (Câmara et al. 2021; Klarenberg et al. 2022; Ortiz-Rivero et al. 2023). All these organisms form a complex trophic network that may be involved in numerous ecosystem functions such as litter decomposition and nutrient cycling (Turetsky et al. 2012). Changes in these networks will impact ecosystem processes and scale up from local to global scales. Furthermore, community composition within the bryosphere may vary on different spatial scales. Differences in climate and biogeochemical conditions maybe cause bryosphere variation between locations (Jónsdóttir et al. 2005; Van Leeuwen et al. 2018; Klarenberg et al. 2022), while differences in microclimate may shape variation within the moss layer (Ortiz-Rivero et al. 2023). Different moss segments have been shown to host different communities of testate amoebae and of ciliates, with increasing diversity towards the lower segments of the moss (Jassey et al. 2011; Mieczan and Tarkowska−Kukuryk 2014). Today, better knowledge of the diversity and structure of such systems is needed to fully comprehend experimental testing of such hypotheses (Lindo and Gonzalez 2010).

In Iceland, moss-dominated ecosystems such as heathlands, moss lands, and shrublands are widespread (33% cover of Iceland for the "Flokkur 322: Mólendi, mosi og kjarr", CORINE Land Cover 2018), and over 600 different bryophyte species have been identified on the island (Lönnell et al. 2025). The species *Racomitrium lanuginosum* (Hedw.) Brid. is by far the most widespread and common species in most of these ecosystems and has a broad distribution range in the Northern Hemisphere (Hedenäs 2020). This species is considered to be part of the "Large Cushion" bryophyte functional group (Lett et al. 2022), has a low decomposition rate, and displays extremely low nitrogen content compared to other moss species (Lang et al. 2009). Previous attempts at identifying bryopshere diversity inside the *R. lanuginosum* layer in Iceland, using metabarcoding techniques, focused mostly on the bacterial diversity, showing that warming (Klarenberg et al. 2022) and moss functional traits (Klarenberg et al. 2023) were impacting that diversity. A preliminary study using conventional methods of physical extractions showed that *R. lanuginosum* mats host communities of metazoa and protists that respond differently to elevated temperatures (Sæmundsson 2019). The use of conventional and/or metabarcoding techniques in the identification of cryptic biodiversity is currently debated in the scientific community (Geisen et al. 2019). Studies comparing both methodologies tend to indicate that metabarcoding is giving a better estimate of taxonomic richness while conventional methods are more relevant for measurements of abundance and biomass (Santi et al. 2021; Andersson et al. 2023; Weydmann-Zwolicka et al. 2024).

The diversity of Icelandic ecosystems and their associated bryosphere diversity can be influenced by human activity and land use (Mutia et al. 2021). One key component of Icelandic ecosystems is the grazing activity by free-roaming sheep during summer. Relieving ecosystems from sheep grazing can shift plant community composition (Marteinsdóttir et al. 2017), particularly the bryophyte composition and layer depth. Even though ungulate herbivores rarely consume mosses, they impact high-latitude moss communities by trampling and increasing nutrient input (Gornall et al. 2009; Jónsdóttir 1991; Van Der Wal et al. 2004). However, studies focusing on the impact of herbivory on soil microbial communities indicate that communities that have already been developing with the natural disturbance are less likely to be impacted by grazing cessation (Ibañez-Alvarez et al. 2022; Vermeire et al. 2021). Whether that is also the case for the bryosphere remains to be studied.

With this study, we aimed at surveying the diversity and abundance of the bryosphere communities within *Racomitrium lanuginosum* and understanding how sheep grazing cessation is affecting the bryosphere. Furthermore, we asked if this diversity varies both between locations and within the moss layer. We hypothesized that sheep exclusion would induce a shift in the diversity and community composition of organisms living inside the moss layer, by trampling and changing nutrient input. We also hypothesized that different locations would host different bryosphere diversity given differences in climate and vascular plant communities and that small-scale distribution of organisms within the moss layer would be a key component of bryosphere diversity, by potentially displaying higher taxonomic diversity towards the lower segments of the moss layer, closer to the microbial soil communities. To test our hypotheses, we sampled moss from grazed and ungrazed plots at two locations in Iceland. We divided the moss samples into three segments and identified all organisms from bacteria to metazoa using environmental DNA.

Methods

Study sites and experimental design

Moss samples were collected at two different Icelandic study sites, Audkuluheidi and Thingvellir (Fig. 1a). The Audkuluheidi site (65°07′56.2″N 19°40′24.5″ W) is situated in the northwestern central highlands, 480 m above sea level with an average annual temperature of 0.64 °C, average precipitation of 333 mm (based on years 1997–2024, Icelandic Meteorological Office 2024), and a climate classified as oceanic-subarctic-alpine. The dominating vegetation is a dwarf shrub heath with a high abundance of bryophytes and lichens and a total vegetation cover of more than 90%. The most abundant vascular plant species are the dwarf shrubs *Betula nana* L., *Empetrum nigrum* L. and *Salix arctica* Pall., and the most abundant moss and lichen species are *Racomitrium lanuginosum* and *Cetraria islandica* (L.) Ach., respectively. The mean moss layer depth is around seven cm (Figure S1). The soil has a low C/N ratio (around 13) and the average pH is close to neutral (Jónsdóttir et al. 2005). The area has been used as sheep summer grazing rangelands since settlement in Iceland, and during the summer 2016, a grazing exclusion experiment was established (Kushbokov et al. 2023). Six pairs of experimental plots (12 × 12 m) were set up, each pair being separated by more than 100 m and plots within a pair were 4 m apart. For each pair, one plot was fenced using a 1.2 m-high fence to prevent sheep from accessing the plot (hereafter referred to as "ungrazed" plot), and the other one was kept open for control (referred to as "grazed" plot in this study, Fig. 1b).

The second study site is situated within the Thingvellir National Park, in South-West Iceland (64°17′23.1″N 21°05′08.7″ W) at 120 m above sea level, on an 8000-year-old post-glacial lava field. The climate differs from Audkuluheidi and is classified as oceanic-subarctic with a warmer average annual temperature of 4.1 °C (and an average of 11 °C in July) and mean precipitation three times as high, with an annual average of 1214 mm (based on years 1997–2024, Icelandic Meteorological Office). The site is dominated by *Racomitrium* moss heath with low cover of vascular plants (Jónsdóttir et al. 2005), interspersed with

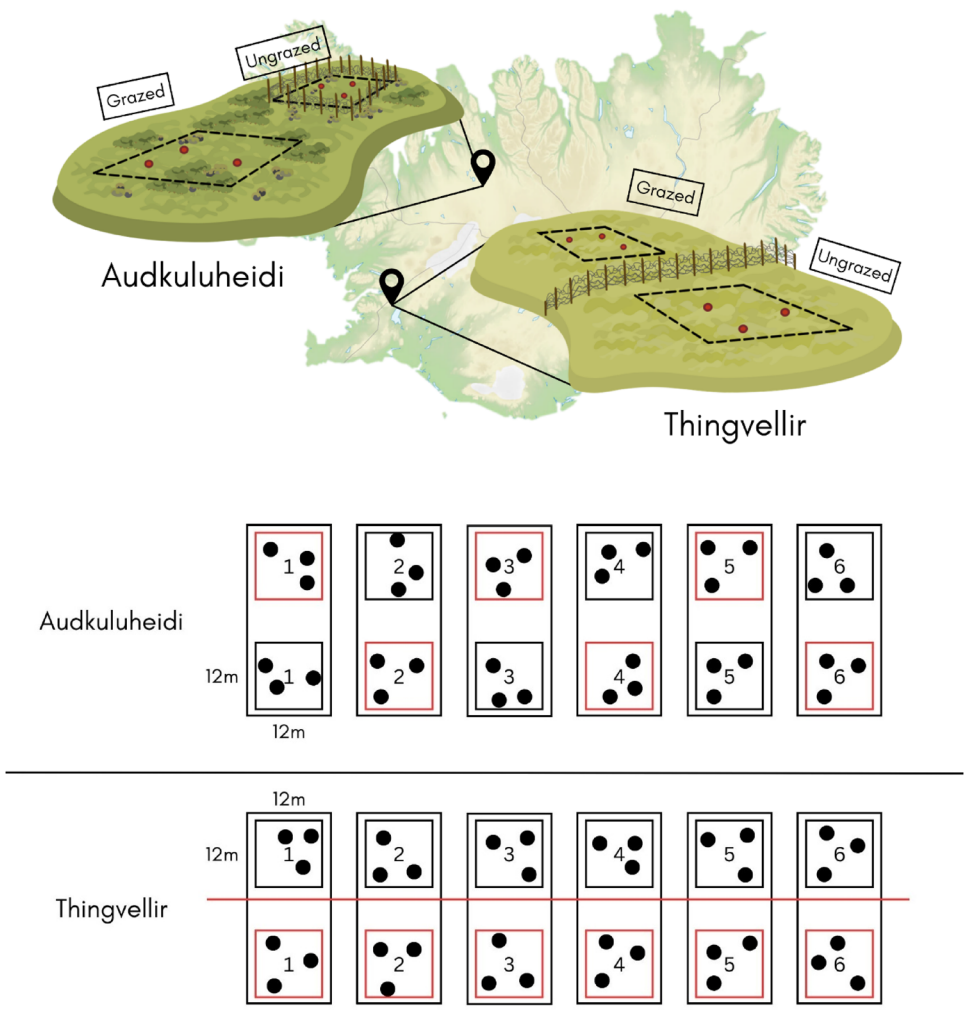


Fig. 1 Schematic representation of the sampling design at both locations. **a** The drawing represents one pair of plots in the two different locations. **b** The full design of 24 plots, with red lines representing sheep exclusion treatment. Upper diagram shows the arrangement of the 12 plots at Audkuluheidi and the lower diagram at Thingvellir. The dots represent the sampling points in the plots. Plot drawings by Léo Rocher

patches of dwarf shrub heath vegetation with taxa such as *Salix* spp., *Carex bigelowii* Torr. ex Schwein and forbs. The moss layer depth ranges from 10 up to 30 cm (Figure S1), the soil is considered nutrient-poor with a relatively high C/N ratio (around 23), and has a pH close to five (Jónsdóttir et al. 2005). A protected area of the National Park has been fenced to prevent sheep grazing since 1928 and since then vegetation and especially patches of birch forest have recovered (Jónasson 1992; Thorsteinsson and Arnalds 1992). We used this same fence to set up a similar sampling design at the Thingvellir site as at Audkuluheidi. Six pairs of 12 × 12 m plots were designated on each side of the fence along a 600 m-long transect, avoiding the patches of dwarf shrub heath and birch forest. The distance between each pair ranged between 50 and 200 m and the distance between plots of the same pair ranged between 34 and 100 m depending on the vegetation patches. The plots on the grazed side of the fence are hereafter considered as "grazed" plots, whereas the plots set up inside the National Park fence are referred to as "ungrazed" plots (Fig. 1b).

To account for potential within-plot variation due to micro-topographical differences, three samples of moss were taken at both locations using random coordinates and then pooled. At each sampling point, a 4 × 4 cm core of the moss mat of around 8 cm deep was cut using a previously cleaned knife with 96% ethanol and 0.2% chlorine. Each sample was taken using a facemask and new nitrile gloves to reduce potential contamination. This sampling effort resulted in 24 samples across the two locations. All the collected moss samples were placed in sterile plastic bags in a cooler in the field and then stored at −20 °C in the lab before DNA extraction.

DNA extractions and sequencing

In the lab, 60 to 90 shoots were taken from each sample. The shoots were then cut into three different parts (hereafter referred to as moss segments in this study): the green living segment (the uppermost one to two cm), the grey middle segment (between two and three cm from the top), and then the brown bottom decaying segment (from three cm from the top to the bottom of the moss layer, Fig. 2). This separation was based on the proportions of alive and dead leaves: the top part containing only alive shoots; the middle one with a mix; and the bottom one with only dead parts of the shoots. This resulted in 72 samples in total. The moss material was then placed into an autoclaved mortar and grounded using liquid nitrogen to create a moss powder to use as material for DNA extractions. DNA was extracted following the NucleoSpin® Soil Extraction Kit (Macherey-Nagel, Germany) in a laminar flow hood cleaned with 0.2% chlorine solution and ethanol. The settings using Lysis Buffer SL1 in combination with the Enhancer SX gave DNA of sufficient quality and quantity for downstream analyses. The purity and quantity of DNA extracted were examined using a NANODROP 1000 (Thermo Fischer Scientific, UK) spectrophotometer for all samples and cross-checked by using a Qubit® 3 Fluorometer Invitrogen, for 12 selected test samples (Thermo Fischer Scientific, UK). A complete description of the DNA extraction can be found in Supplementary Material (Methods S1).

The extracted DNA was sent to NOVOGENE® UK for library preparation and sequencing barcodes of the 16 S rRNA gene (region V3V4) for bacteria, 18 S rRNA gene (region V4) for eukaryotes, and ITS 2 region for fungi, using standard primers (16 S-V34: 515 F: CCTAYGGGRBGCASCAG -and 806R: GGACTACNNGGGTATCTAAT;

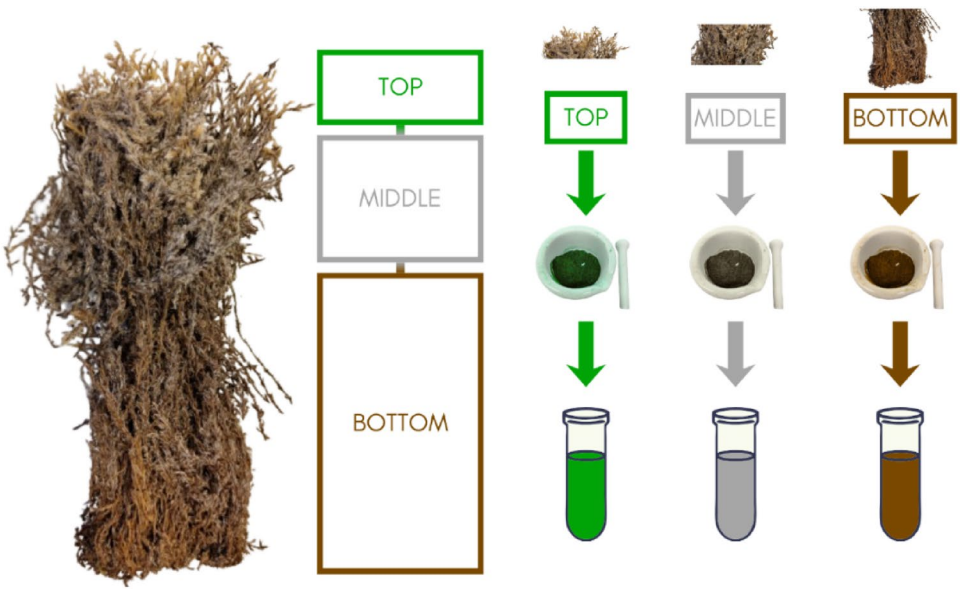


Fig. 2 Separation of the moss samples into different moss segments. The top moss segment was usually around 1–2 cm, 2–3 cm for the middle moss segment, and more than 3 cm for the bottom moss segment

18SV4: 528 F:-, GCGGTAATTCCAGCTCCAA and 706R: AATCCRAGAATTTCACCTCT; ITS2: GCATCGATGAA GAACGCAGC and TCCTCCGCTTATTGATATGC). The 16 S rRNA gene and 18 S rRNA gene were sequenced for the full 72 samples. The ITS2 region was sequenced only on 12 samples, covering all factor combinations, as a specific marker targeting only fungi. Each sample was sequenced at a depth of 100 K.

Data cleaning and statistical analyses

Processing of the read sequences, including demultiplexing and annotation of the sequences was conducted by NOVOGENE using the SILVA 138.1 database for the 16 S and 18 S rRNA genes and the UNITE database v9.0 for the ITS2 region. Operational Taxonomic Units (OTUs) were obtained for the 18 S rRNA gene, by clustering together sequences with 97% identity using Qiime 1 (Caporaso et al. 2010). Amplicon Sequence Variants (ASV) were identified for the 16 S rRNA gene and ITS region, using the DADA2 method (Callahan et al. 2016) by applying QIIME2's classify-sklearn algorithm (Bokulich et al. 2018; Bolyen et al. 2019), a pre-trained Naive Bayes classifier. Based on the classification, abundance tables at the level of kingdom, phyla, class, order, family, genus, and species were generated for each marker.

A table describing the assignment of taxa to each organism group in this study can be found in Supplementary Information (Table S1). The OTUs and ASVs that got assigned incomplete taxonomy for specific level (i.e. "unidentified_X") were considered as NAs for this taxonomic level. Each taxonomical group was analyzed by creating a phyloseq object using the *phyloseq* R package (McMurdie and Holmes 2013). Due to the imprecision of the 16 S and 18 S rRNA genes, the Genus and Species levels were not considered in the analyses. Rarefaction curves were plotted for each eDNA marker using the *rarecurve()* function from the *vegan* package (Oksanen et al. 2001).

Relative abundances of the top 10 most abundant classes for each organism's group were summarized using the *microbiome* package (Lahti and Shetty 2012), with the *aggregate_top_taxa2* and *plot_composition* functions. Given the large number of categories in this study, an UpSet plot was used to display the taxa overlap between the different categories, using the *UpSetR* and *ComplexUpset* packages (Lex et al. 2014; Conway et al. 2017). The top 25 intersections were kept for each organism groups to show the most significant associations. To identify potential associations between taxa from different organism groups, we first performed an indicator species analysis with the *multipatt* function from the *indicspecies* package (Cáceres and Legendre 2009) at the Order taxonomical level. Analyses were conducted separately for each organism group using Location as the grouping factor. This allowed the identification of Orders which were significantly associated with either of the two sampling locations. Pairwise Spearman-rank correlations between the relative abundance of these Orders, transformed with the center-log ratio (CLR) transformation, was calculated to assess their associations using the *rcorr* function from Hmisc package (Harrell and Dupont 2026).

The common alpha diversity indices such as number of observed taxa, Chao1, ACE, Shannon, Simpson and InvSimpson indices, and species evenness were calculated for each organism's group (Metazoa, Protist, Bacteria and Fungi), using the *vegan* package. The alpha diversity indices were all strongly and positively correlated to the Shannon diversity index when looking at the four groups, with Pearson correlation coefficients ranging from 0.65 to 0.95 (Table S2). Due to the high correlation, further analyses were conducted only on the Shannon index. To identify the potential impact of location, moss segments, or sheep exclusion on the alpha diversity, we ran both an analysis of variance (ANOVA) and the linear model $Shannon \sim Location * Moss\ Segments + Location * Treatment + Moss\ Segments * Treatment$, considering pairwise interactions between the three factors. The Moss Segments variable was either used as numerical rank from top to bottom or as categorical to assess which one better explained the variations in the alpha diversity index.

The beta diversity between the different samples was summarized using the Bray-Curtis (BC) dissimilarity with the *vegdist* function in the *vegan* package, and with the UniFrac (UF) weighted and unweighted distances, using the *phyloseq* package, to account for the distances between DNA sequences. To test whether the species community compositions differed between the different locations, moss segments and treatment (grazing and grazing exclusion) and whether these effects are independent, all two-way interactions were tested using a permutational ANOVA (PERMANOVA) on each organism group, based on the *Bray-Curtis* and *UniFrac* distance matrices between communities as $distances\ matrix \sim Location * Moss\ Segments + Location * Treatment + Moss\ Segments * Treatment$. The PERMANOVA was calculated using the adonis2 function from the *vegan* package with 9999 permutations. The Moss Segments factor was again used either as a numerical or a categorical variable in the PERMANOVA and the model with the better fit was selected and presented below. Principal coordinates analysis (PCoA), using the *ordinate* and *plot_ordination* functions from the *phyloseq* package, with the $con.cap = 0$ and $con.size = 1.5$ parameters for the ellipses from the *ggforce* package, were used for visualization of community composition differences according to the PERMANOVA results. R version 4.3.0 (R Core Team 2023)

was used for all statistical analyses and figures presented in this study.

Results

Overview of the bryosphere diversity in the moss layer

The Bacteria group had the highest number of identified taxa, followed by the Fungi group, the Protist one and finally the Metazoa (Table 1). Of the three markers used for the eDNA sequencing, the 18 S rRNA gene gave the largest NA proportions for almost all taxonomic levels (Figure S2). The ITS2 region and 16 S rRNA gene identified taxa more precisely, with almost no NAs in Phylum and Class levels for the Bacteria, and low percentages of NAs for all taxonomic levels for Fungi. Taxonomic resolution for Metazoa was limited, with most OTUs remaining unclassified at the family level (Table 1 and Figure S2). Rarefaction curves showed a plateau for almost all samples, for the three different markers (Figure S3).

In the Metazoa group, the relative abundance of the Arachnida class increased down the moss layer in both locations, from 1% up to more than 25% of the identified taxa in the bottom segment in Audkuluheidi, in contrast with the Bdelloidea and Eutardigrada (Phylum: Tardigrada) classes which decreased from 85 to 30% and from 12 to 5% respectively (Fig. 3a). For the Protists, the relative abundance of the most dominant Class Intramacronucleata (Ciliates) decreased down the moss layer, especially between the top and mid segments (Fig. 3b). The Glissomonadida (Phylum: Cercozoa, Kingdom: Rhizaria) was in contrary of higher relative abundance in the top segment of the moss layer. For the Bacteria, the Cyanobacteriia class was the most important one in the top segment in both locations and almost completely disappeared from the bottom segment communities (Fig. 3c). Finally, for the Fungi, the Agaricomycetes Class relative abundance was much lower in the top segments, compared to the mid and bottom ones (Fig. 3d).

A large number of taxa were shared between the middle and bottom segments in Thingvellir for the Metazoa and Protist groups, followed by a core of taxa present in all samples (Figure S4a and S4b). This was verified for the Bacteria group, with more than 500 taxa present in both middle and bottom moss segments at Thingvellir, followed by taxa specific to the middle and bottom segments from Audkuluheidi (Figure S4c). The core taxa for Bacteria, i.e. taxa present in all the samples, represented 193 taxa. Finally, for the Fungi, the highest intersection was observed for the samples from grazed plots at Audkuluheidi (16 shared taxa), followed by taxa present only in grazed plots at Thingvellir (15 shared taxa, Figure S4d). The core taxa shared between all samples represented only 3 samples.

The correlations between organism groups showed the strongest positive and negative associations between the Echiniscoidea Order (Tardigrada) and Orders of protists and bacteria (Table S3 in Supplementary Information). The Echiniscoidea had strong negative associations with the Reyranellales bacteria (Phylum: Pseudomonadota, correlation = −0.69), Gaiellales bacteria (Phylum: Actinomycetota, correlation = −0.68), but also an association between Heteronematina protist and Dongiales bacteria (Phylum: Pseudomonadota, correlation = −0.68). The Echiniscoidea Order also had the strongest positive associations, with three orders of Bacteria: Chthoniobacterales (Phylum: Verrucomicrobiota, correlation = 0.69); Acetobacterales (Phylum: Pseudomonadota, correlation = 0.67); and HOC36 (Phylum: Proteobacteria, correlation = 0.66).

Impact of location, moss segments and treatment on alpha diversity index

The Shannon diversity index calculated at the OTU or ASV level was overall higher in the bacteria group compared to the three other organism groups, with a median of 5.9 for the Bacteria, 3.2 for the Protist and Fungi, and finally 1.7 for the Metazoa (Fig. 4 and Table S4).

For both Metazoa and Protist groups, the location variable had a significant impact on the diversity index (Table S5). For example, the Shannon index was on average 0.44 higher for the Protist group in the Thingvellir location compared to Audkuluheidi (Fig. 4).

There was a significant increase in the bryosphere diversity from the top to the bottom moss segment, for the Metazoa, Protist and Bacteria groups (Table 2; Fig. 4 and Table S6). The Shannon diversity index increase was always significant from the top to middle, and middle to bottom

Table 1 Number of unique taxa identified at each taxonomic level for the 3 eDNA markers used to analyze the diversity in Racomitrium lanuginosum

Marker	Group	OTUs/ASVs	Phylum	Class	Order	Family
18 S rRNA	Metazoa	348	7	14	21	0
	Protist	1420	23	30	29	21
	Total	1768	30	44	50	21
16 S rRNA	Bacteria	34,950	36	85	192	248
ITS2 region	Fungi	2350	9	33	90	169

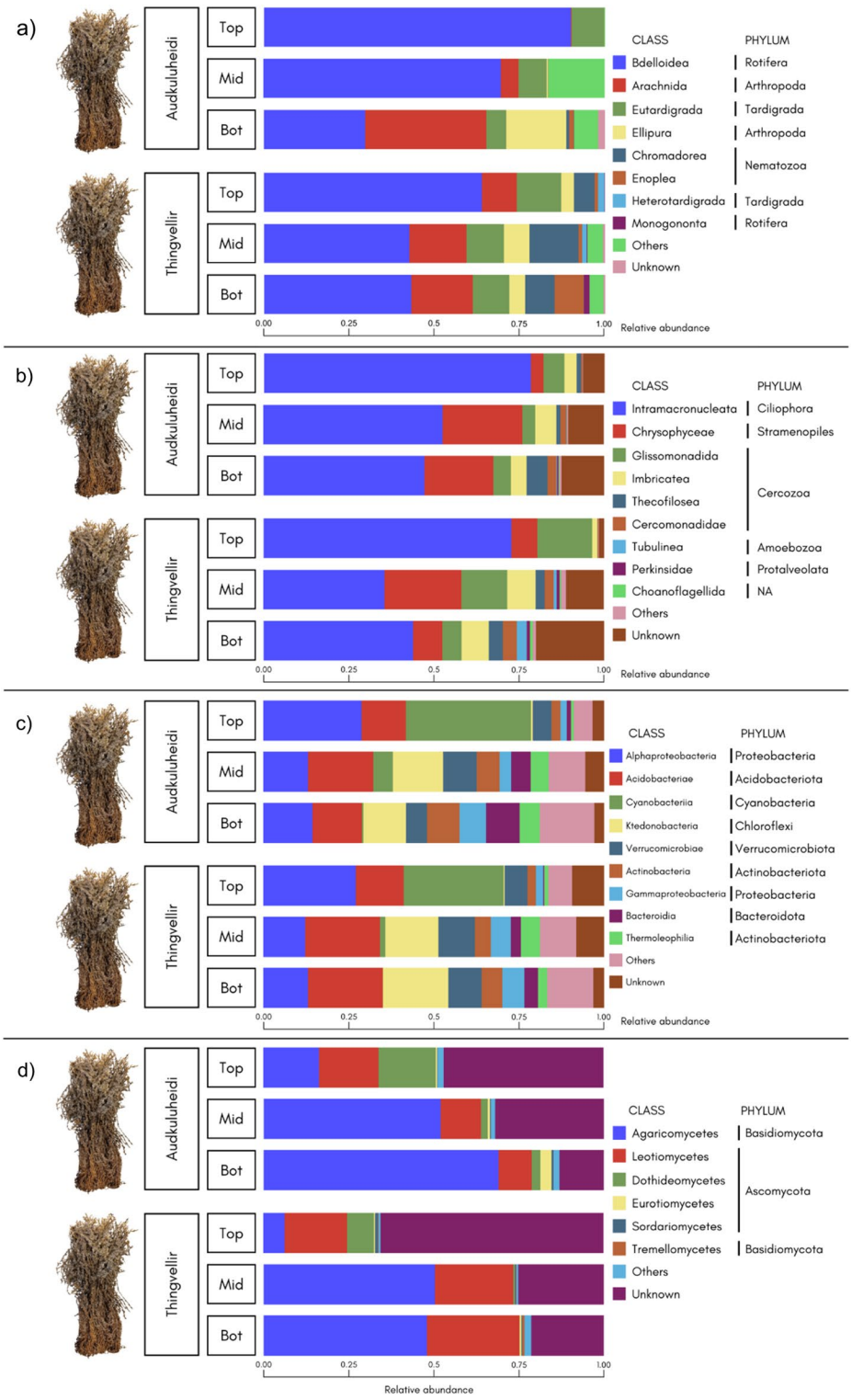


Fig. 3 Relative abundance of the 10 most abundant classes of the studied organism groups at the two locations and within the three segments of the moss Racomitrium lanuginosum, top, middle, and bottom. **a** Metazoa, **b** Protists, **c** Bacteria and **d** Fungi. The colors correspond to the different Classes listed in the legend, with their respective Phylum

segment for those three groups, with an increase of 2.17 on average from the top to bottom segment for the Bacteria group (Fig. 4). The effect of the moss segment on Shannon diversity was by far the most important factor of the three to explain these differences, as seen with the F-values (Table S5).

The location and moss segment interaction were significant for the Metazoa group, due to a significantly higher diversity in the middle segment at Thingvellir, around 0.9, compared to Audkuluheidi (p-value = 0.0001, Table S6). For the Protist group, this interaction was also significant with a significantly higher increase in diversity from the top to

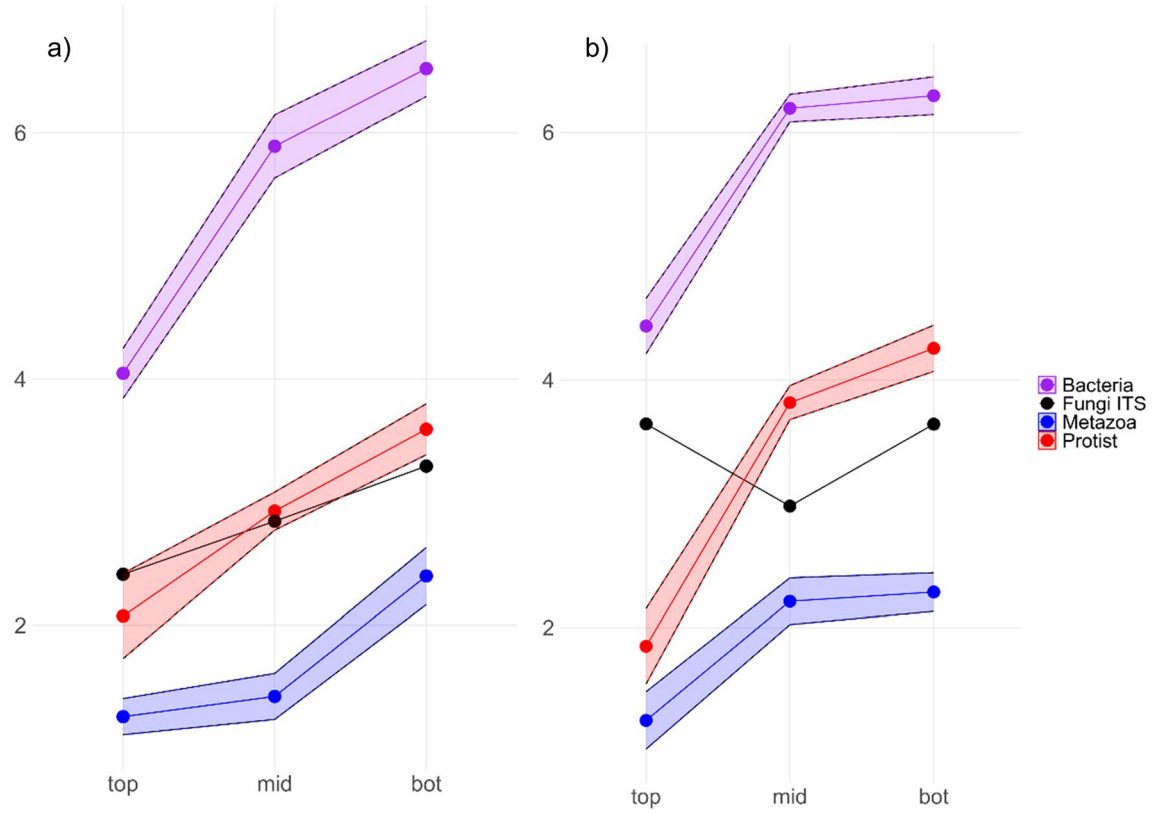


Fig. 4 Shannon diversity index with 95% confidence intervals for each organism group within the different moss layer segments at **a** Audkuluheidi and **b** Thingvellir. Color codes: Metazoa (blue), Protist (red), Bacteria (purple) and Fungi (black) groups

Table 2 Results of the ANOVA analyses on the Shannon alpha diversity index for each of the 4 organism groups (with Moss Segments as categorical)

Groups	Df	METAZOA	PROTIST	BACTERIA	FUNGI
Location	1	6.204 *	17.663 ***	3.267.	10.253.
Moss segments	2	48.898 ***	122.023 ***	241.655 ***	3.565
Location: moss segments	2	10.051 ***	10.381 ***	4.983 **	3.504
Treatment	1	0.059	4.108 *	0.434	1.825
Location: treatment	1	0.001	0.306	11.432 **	5.362
Moss segments: treatment	2	0.433	0.629	0.418	7.258

The F-values are presented with the significance level of the corresponding p-values (. = <0.1, * = <0.05, ** = <0.01, *** = <0.001)

the bottom moss segment at Thingvellir compared to Audkuluheidi (around 0.89, p-value = 0.001). Finally, the Bacteria group showed a smaller increase in diversity from top to bottom and mid to bottom moss segments at Thingvellir, compared to Audkuluheidi (p-value = 0.005 and 0.0145 respectively). There was no significant interaction between the location and the moss segments for the Fungi group.

The sheep grazing exclusion variable (treatment) did not have any effect on the Shannon diversity index for the Metazoa and Fungi groups but significantly affected the Protist group by decreasing the Shannon index of an average of 0.21 when excluding sheep presence (Table 2; Fig. 5). The interaction between location and treatment was also significant for the Bacteria group, with the sheep exclusion increasing the alpha diversity index by 0.32 on average at Audkuluheidi while on the other hand, reducing the alpha diversity index by 0.26 on average at Thingvellir (p-value = 0.0012, Fig. 5 and Table S6).

Impact of location, moss segments and treatment on bryosphere community composition

The PCoA ordinations of the community compositions of the different organism groups explained between 22 and 39.9% of the variation along the first axis and between 11.9 and 20.7% along the second axis (Fig. 6). Both location, moss segments and their interaction had a significant effect on the community composition of the bryosphere in *Racomitrium lanuginosum*, for all four organism groups analyzed, which is visible in the four PCoA plots with a gradient of

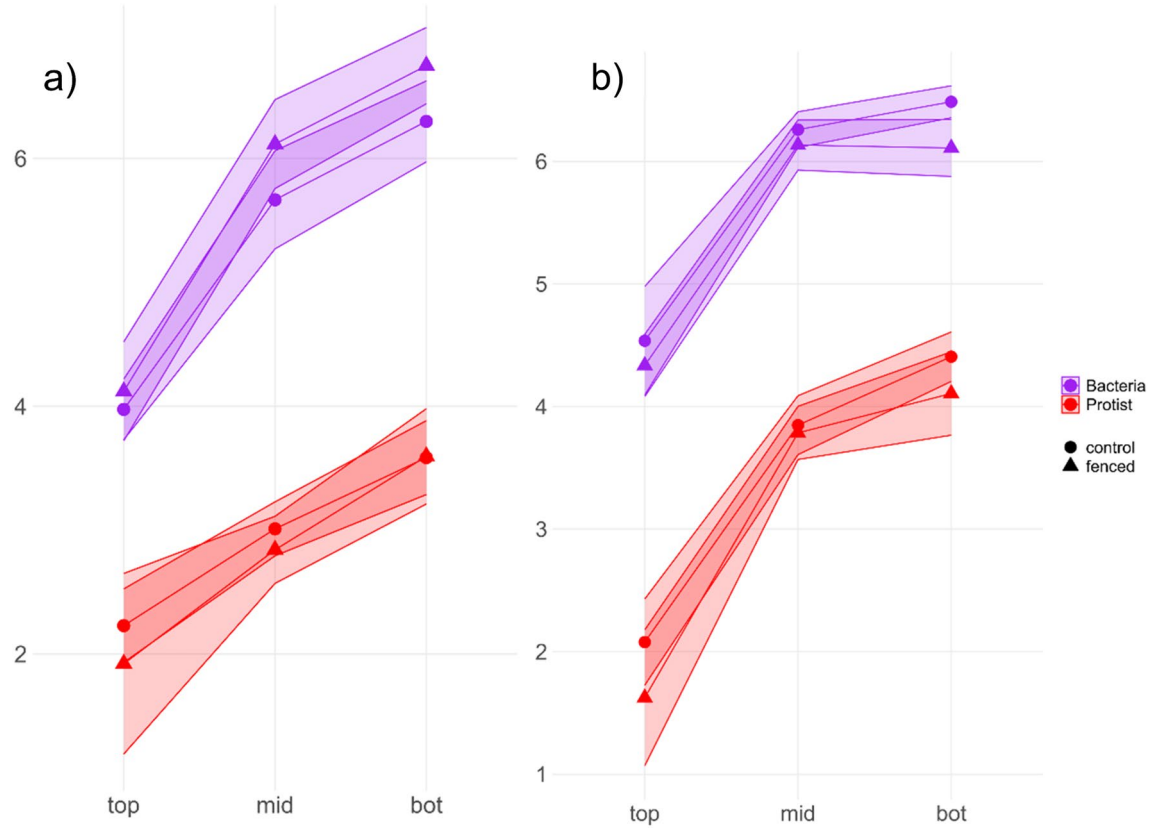


Fig. 5 Shannon diversity index with 95% confidence intervals for each organism group within the different moss layer segments at **a** Audkuluheidi and **b** Thingvellir. Color codes: Protist (red), and Bacteria (purple) groups

communities along the moss layer, and never any overlap between top and bottom segment communities (Table 3; Fig. 6). The significant interaction means that the impact of the moss segments on bryosphere diversity differed between the two locations, which can be visualized for example for the Metazoa plot, with greater differences between top moss segments than bottom segments when comparing the two locations (Fig. 6a). This pattern is reversed for Protists and Bacteria, with an increase in the differences between locations with increased depth (Fig. 6b, c).

The sheep presence (grazed Treatment) did not affect the bryosphere community composition, except for the fungi (p-value = 0.0012; Table S7, and Fig. 7a). However, for both the Bacteria and Fungi groups, treatment significantly interacted with location, meaning that the grazing effect on communities differed between locations (p-value = 0.0353 and 0.0155 respectively; Table S7, and Fig. 7). Similar results were obtained for the analysis of Bray-Curtis and UniFrac weighted distances (Table S8), except for the Fungi group where no significant differences were observed for UniFrac distances. Among the three factors, the moss segment explains most of the variation for the bryosphere community compositions for all organism groups, except Fungi (Table 3). The second most important factor for the community composition is the location, followed by the interaction between location and moss segments, while the treatment had the smallest effect.

Discussion

With this study, our aim was to evaluate the impact of sheep grazing cessation on the overall diversity of organisms living inside the *Racomitrium lanuginosum* layer in two Icelandic locations and identifying potential patterns of vertical distribution of diversity in the moss layer. Sheep exclusion affected overall species' diversity for protist and bacterial taxa as well as bacterial and fungal community compositions. However, the different segments of the moss layer explained the largest proportion of the variations in diversity and community compositions, with alpha diversity gradually increasing with moss depth. Both community composition of species living in *R. lanuginosum* and alpha diversity were different between the two studied locations. Although expected, we are not aware of any previous studies demonstrating this micro-scale differences in organism distribution for all taxa studied. These findings have important implications for the cryptic biodiversity in the

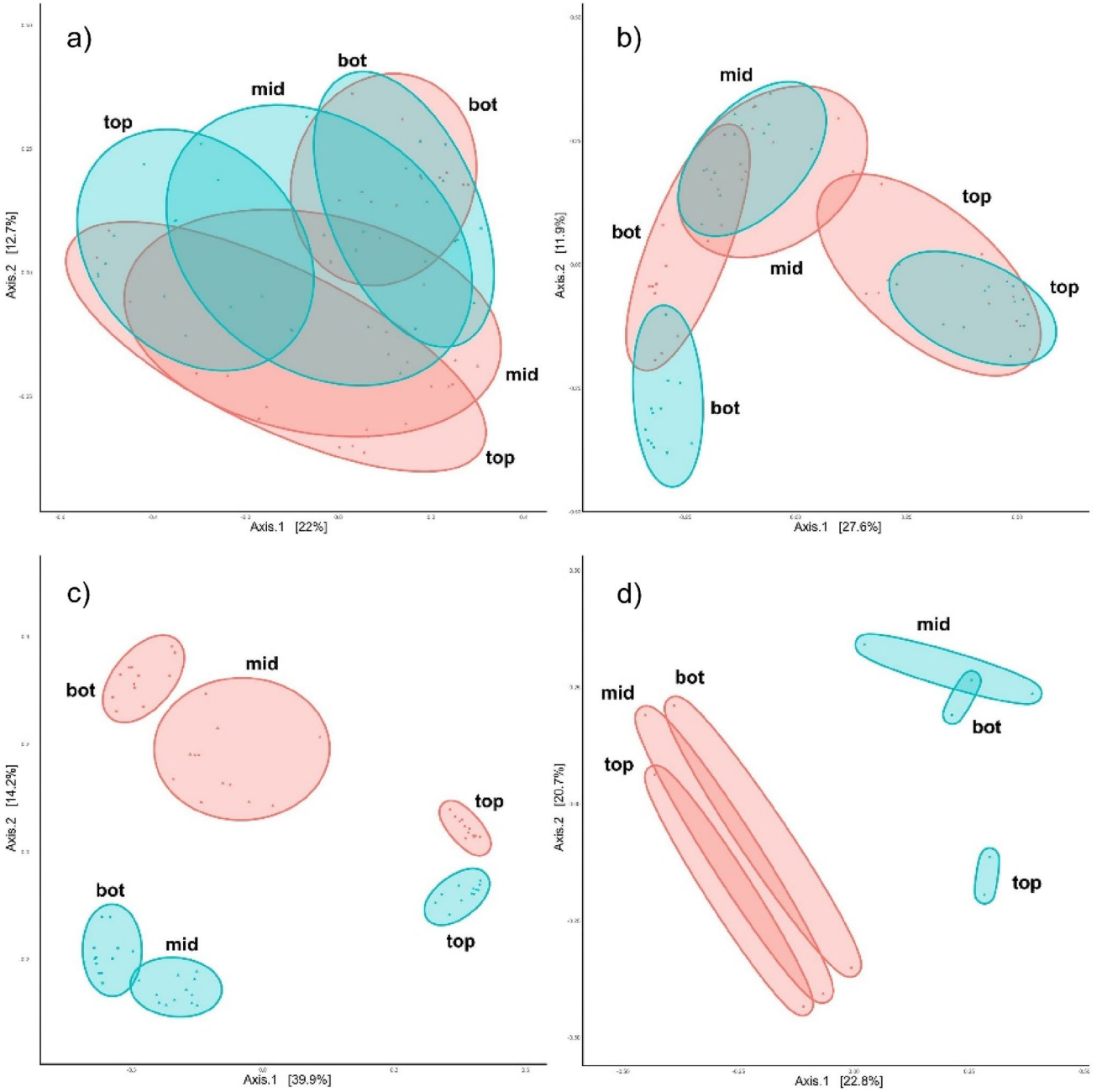


Fig. 6 PCoA plots of the different organism groups within the different moss segments at the two locations, **a** Metazoa, **b** Protist, **c** Bacteria, and **d** Fungi. The color of the ellipses corresponds to the location of the samples (light blue = Thingvellir, pink = Audkuluheidi) and the text to the different moss segment communities. The percentage of variation explained by each axis is indicated

moss layer, especially given the broad distribution range of *Racomitrium lanuginosum* in the Northern hemisphere (Hedenäs 2020).

The specificity of the bryosphere diversity in Racomitrium lanuginosum

In line with previous studies on bryosphere diversity, the *Racomitrium* moss layer hosts a variety of organisms, forming communities primarily shaped by environmental factors related to the depth position within the moss layer (Lindo and Gonzalez 2010; Jassey et al. 2011; Grau-Andrés et al. 2022).

Metazoa

Only a few studies have looked at the metazoan diversity inside the moss layer using metabarcoding techniques and

Table 3 Results of the 3-way PERMANOVA analysis on Bray-Curtis distances for each of the 4 groups

Groups	METAZOA	PROTIST	BACTERIA	FUNGI
Location	5.7392 ***	9.8988 ***	23.8802 ***	4.7221 ***
Moss segments	6.9376 ***	22.0241 ***	47.3860 ***	2.1993 *
Treatment	1.4998	1.5439	1.8507.	3.3036 ***
Location: moss segments	4.4140 ***	4.8864 ***	8.0244 ***	1.7745 *
Location: treatment	1.0024	1.4020	2.4500 *	2.3014 *
Moss segments: treatment	0.9220	1.3516	0.9409	0.9582

Moss Segment was treated as a numerical variable. The F-values are presented with the significance level of the corresponding p-values (. = $p<0.1$, * $p<0.05$, ** $p<0.01$, *** = $p<0.001$). Degree of freedom was 1 for all variables

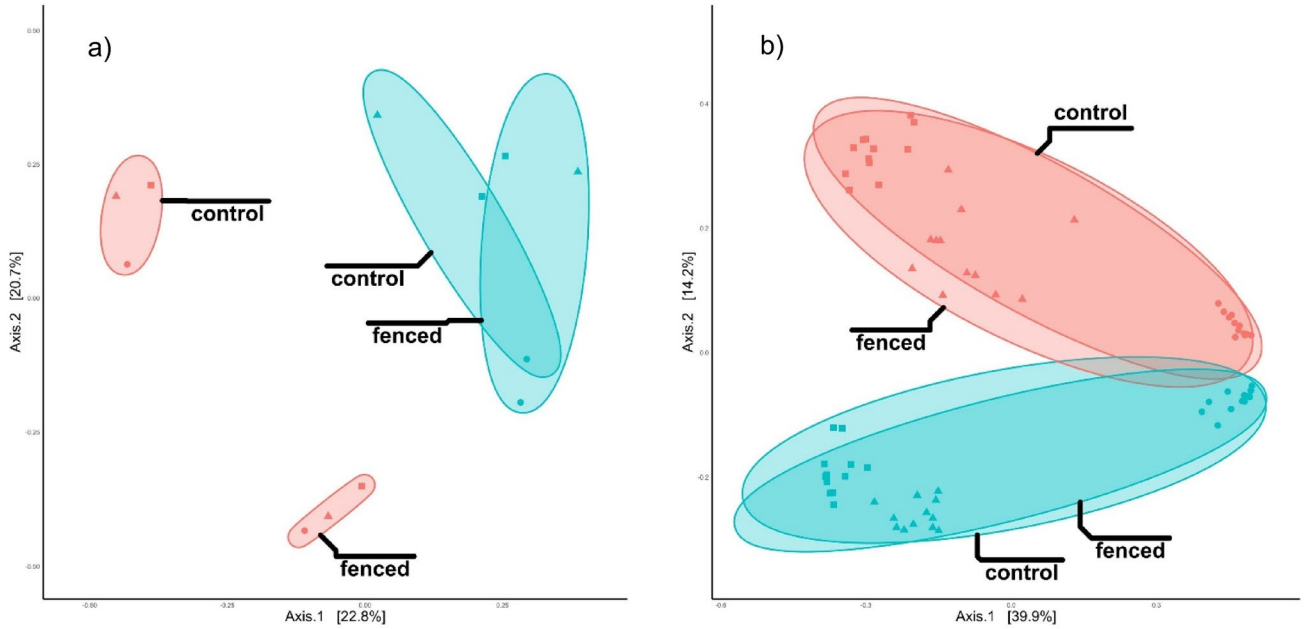


Fig. 7 PCoA plots of the **a** Fungi and **b** Bacteria group within the two grazing treatments (control = grazed; fence = ungrazed) at each location. Color codes: light blue ellipses = Thingvellir, pink ellipses = Audkuluheidi) and treatment (label). Moss segments are indicated with the shapes of the dots (circle = top, triangle = mid, and square = bot). The percentage of the variation explained by each axis is indicated

the 18 S rRNA gene marker. In a single shoot of *Sanionia uncinata* (Hedw.) Loeske in Antarctica, four main phyla were found in similar proportions to our dataset: Arthropoda, Nematozoa, Rotifera and Tardigrada, with 37, 12, 38, and 13% respectively, compared to 23, 7, 57, and 10% in our samples (Câmara et al. 2021). Those metazoan groups are often associated with moss microhabitats, especially in polar regions (Glime 2017; Olszanowski et al. 2018). The moss-dwelling tardigrades Echiniscoidea seem to be an important component of this bryosphere, given the strong correlations between their abundance and the abundance of numerous bacterial and fungal orders. These water bears are known to be associated with various moss and lichen substrates and are well adapted to aquatic environments such as the moist moss layer, which enables them to predate on a variety of taxa and disperse quickly and easily (Glime 2017). Strong positive correlations between orders identified in the moss layer could indicate ecological relationships, like sharing the same ecological niche, as it could be the case between the tardigrades Echiniscoidea and the Rickettsiales bacterial order, which have already been identified as tardigrades' endosymbiont (Vecchi et al. 2018). Conversely, negative correlations described in our study between the same tardigrades and the bacterial order Reyranellales for example, could be related to their distinct preferences of different moss segments, with an upper segment preference for Echiniscoidea against lower segments preference for the bacterial order.

The spatial distribution of metazoan communities found in our dataset is in line with a preliminary study using conventional methods of physical extractions of micro and macrofauna from the moss layer, in the same Thingvellir location (Sæmundsson 2019). A higher abundance of rotifers and tardigrades was found in the upper segment of the *Racomitrium lanuginosum* layer, whereas collembolas were found to be more abundant in the bottom moss segment. This spatial difference, confirmed by our study, may be explained by differences in feeding habitats. Most rotifers

feed on algae and cyanobacteria, and tardigrades tend to feed directly on bryophyte tissue or even other tardigrades, which would then explain their higher abundance in the upper segments of the moss layer (Glime 2017). Collembolas on the other hand feed on fungi, detritus, or even decaying moss, which makes the decaying lower segments of the moss layer a perfect habitat for them (Castaño-Meneses et al. 2004). Those studies show that conventional methods can, and should, be used in combination with metabarcoding methods, in order to fully identify the cryptic biodiversity in the moss layer (Keck et al. 2022).

Protists

The protists proportions found in our dataset matched the bryosphere communities identified in non-Sphagnum and Sphagnum moss species from western Canada, with around 36% of Alveolata, 34% of Rhizaria and 8% of Stramenopiles, against 56%, 27%, and 15% for the same kingdoms in our dataset, respectively (Heger et al. 2018). Both Ciliophora and Cercozoa are phagotrophic taxa that are often found in the moss layer, most likely feeding on bacteria and fungi (Singer et al. 2019). The Chrysophyceae class (Phylum: Stramenopiles) is common in freshwater ecosystems and peatlands and mostly phototrophic, thus contributing to the overall CO_2 fixation by the bryophyte layer (Hamard et al. 2021; Jassey et al. 2022).

One factor explaining the spatial distribution of protists in our dataset is that the bottom segment of the moss layer has an overall higher diversity and hosts a broader range of taxa. In Antarctica, communities of ciliates were identified in the upper and lower segments of the moss layer, with the upper layer being dominated by mixotrophic taxa, while the lower ones showed higher abundances of bacterivorous ciliates (Mieczan and Tarkowska−Kukuryk 2014). In general, the protist communities identified in the moss layer can be compared to aquatic protist communities, which can be explained by the presence of water between *Racomitrium* leaves and in the moss layer (Debroas et al. 2017; Singer et al. 2019). The increasing alpha diversity with sequencing depth for the Protist group is confirming the previous studies showing that those communities are known to contain extensive rare diversity that is difficult to fully recover even with high-throughput sequencing (Burki et al. 2021). Overall, the major ecological patterns observed in the full dataset, including elevated diversity in lower moss segments, remained consistent after standardization of sequencing depth, suggesting that these trends were not solely driven by uneven read counts.

Bacteria

All of the unique phyla identified in both studies by Klarenberg et al. were also found in our samples, indicating a similarity of the bacterial diversity in Iceland using metabarcoding techniques (Klarenberg et al. 2022, 2023). The dominance of Proteobacteria and Acidobacteria phyla found in our dataset is in line with previous studies on bacterial communities within the *R. lanuginosum* layer at the same Audkuluheidi location (Klarenberg et al. 2022), and within both *R. lanuginosum* and *R.ericoides* at an Icelandic glacier forefield (Klarenberg et al. 2023), although in slightly different proportions. This was expected because those two phyla are often linked to dwarf shrub heaths and other tundra ecosystems, such as the Alaskan tundra or the Canadian sub-Arctic, for example (Holland-Moritz et al. 2018; Escolástico-Ortiz et al. 2023). The high Proteobacteria presence could be due to the important labile carbon pool in shrub soils, favoring copiotrophic taxa (Wallenstein et al. 2007), whereas acidic soils and high phenolic compound rates could favor the presence of Acidobacteria, together with the use of nitrite as N source of energy (Kielak et al. 2016; Männistö et al. 2013). Both phyla have been described as important actors of the identified "core microbiome" of soil bacterial communities across the Arctic region and can be considered bioindicators of these ecosystems (Malard et al. 2019). This tendency can also be verified by our UpSet plots (Fig S4), showing shared taxa in each of the samples at both locations. The most abundant genus (*Bradyrhizobium*, Family: Nitrobacteraceae) previously identified in soil communities in the same study site in Audkuluheidi was also found in high abundance within the moss layer in our study, highlighting a connection between the soil and moss microbiomes (Ólafsdóttir 2023).

In our study, the abundance of the Cyanobacteria phylum was highest in the top segment of the moss layer, which is most likely due to their photosynthetic activity and reflects their important role in the carbon and nitrogen cycling inside the moss layer (Escolástico-Ortiz et al. 2023; Holland-Moritz et al. 2021; Rousk et al. 2013). On the other hand, the phylum Chloroflexi, and more precisely the Ktedonobacteria class, had a higher relative abundance within the lower segments of the moss layer, which may be due to their functional role in the degradation of organic matter (Speirs et al. 2019). This supports our hypothesis of the non-homogenous repartition of bacteria and other microorganisms in the moss layer, mostly according to their ecosystem functions (Ortiz-Rivero et al. 2023).

Fungi

The fungal abundance of our data matches the fungal communities identified in a study on *Racomitrium ericoides* in the Icelandic highlands, at the phyla level (Ortiz-Rivero et al. 2023). However, the fungal communities differed at the class level, where the Agaricomycetes class (Phylum: Basidiomycota) represented more than 50% of the relative abundance in our samples, against approximately 20% in the *R.ericoides* moss layer. The relatively abundant presence of Agaricomycetes and Leotiomycetes (Phylum: Ascomycota) in the moss layer and in the tundra in general can be linked to several factors such as the tendency for Agaricomycetes to be involved in mycorrhizal associations (Zhang and Guo 2007), and the tendency of Leotiomycetes to be involved in several fungi-plant interactions like pathogenous relationships, mutualism or even endophytic interactions (Zhang and Wang 2015). Ascomycota is the most abundant phylum in grasslands, while Basidiomycota is more abundant in boreal forests (Jiang et al. 2021). The latter is known to be involved in the decomposition of recalcitrant organic matter (Voříšková and Baldrian 2013).

The reduced relative abundance of Agaricomycetes in upper moss segments likely reflects the oligotrophic, highly exposed, and minimally decomposed nature of apical moss tissue. Lower moss segments provide more stable moisture conditions and greater accumulation of decomposed organic substrates, favoring saprotrophic Agaricomycetes (Lundell et al. 2014). In contrast, the Dothideomycetes class (Phylum: Ascomycota) was more abundant in the top segment than in lower moss segments, which could be due to the presence of other types of plant-associated fungi such as plant-pathogens or even lichenized fungi in this class, most of them known to be extremophiles (Selbmann et al. 2020). On another note, the high percentage of unknown taxa in the fungal dataset, reaching up to 60% of all taxa in the top segment at Thingvellir, is likely due to the incomplete taxonomical descriptions of fungal species in the available databases, especially for the ITS marker, as is most likely the case for plant-pathogenic fungi (Nilsson et al. 2014). It is also common for fungal assessments using eDNA techniques to be influenced by the presence of fungal fruiting bodies that could for example be trapped within the moss layer (Runnel et al. 2024). Because fruiting bodies were not frequently observed at our study sites, we assume that the diversity of fungal readings is mostly from mycelia.

The effect of location, moss segments and treatment on bryosphere diversity

The result of significant increase in alpha diversity indices down the moss layer supports the hypothesis that the repartition of microbial communities in the moss layer is non-homogenous for bryosphere communities, as has been suggested by previous studies (Mieczan and Tarkowska−Kukuryk 2014; Ortiz-Rivero et al. 2023). There may be multiple reasons behind the higher diversity towards the bottom part of the moss layer. One of them could be the closer proximity to the rich soil biodiversity (Calderón-Sanou et al. 2021). However, this cannot be the only explanation since, especially at Thingvellir, some samples were collected on top of lava rocks, with almost no soil horizon. Besides, previous studies suggested little shared taxa between *R. lanuginosum* and its substrate (Escolástico-Ortiz et al. 2023; Klarenberg et al. 2023). This suggests a more limited soil influence on bryosphere communities than expected. Compared to upper moss segments, bottom layers are expected to retain higher moisture levels, accumulate decomposing organic matter, and experience lower environmental fluctuations and sun exposure (Turetsky 2003). These conditions could favor the establishment and persistence of a wider diversity of microbial taxa. Nutrients might also be more available in older moss material and contribute to this verticality of different moss-microhabitats (Yanoviak et al. 2004). In general, according to our study, the bryosphere is a vertically structured ecosystem, displaying environmental gradients down the moss layer, which promote the establishment and growth of specific taxa within each segment.

The higher alpha diversity observed with sheep presence for the Protist group at our study sites could be directly due to trampling, urine and feces deposition by the sheep or indirectly through the vegetation defoliation that may have changed the plant litter quality and quantity. Because some protists are closely related to litter decomposition those mechanisms can impact protist diversity, as previously shown for soil eukaryotes under heavy grazing (Camuy-Vélez et al. 2024). The different impact of sheep presence on fungal and bacterial diversity at the two locations can be due to several factors related to site differences including grazing history. The ungrazed plots at Thingvellir had been protected from sheep grazing for decades while those at Audkuluheidi only since 2016.

The effect of location, moss segments, and treatment on community compositions

In all four organism groups, the two locations presented different communities within the same bryophyte species, which may have been shaped by different ecological factors and their interactions, such as vascular plant community composition and environmental parameters. This indicates that in addition to the structure and architecture of the moss species itself, environmental factors are also important in shaping microhabitats for the bryosphere communities. A

designated study design would be required to distinguish the impact of each ecological factor and its interactions.

At both locations, the top, middle, and bottom moss segment communities were well separated and, most importantly, the top and bottom communities, which are physically separated within the moss layer, never overlapped. This lends support to the hypothesis of a well-defined, stratified community inside the moss layer, shaped by physical, biological, and environmental factors (Mieczan and Tarkowska−Kukuryk 2014; Ortiz-Rivero et al. 2023). However, the significant interaction between location and moss segment for all organism groups reflects moss segment-specific community responses to different climatic conditions. Interestingly, the bacterial top segment communities at the two different locations were more similar to each other than the bottom segment communities, indicating more location-specific diversity in the bottom segments. This pattern being opposite for the metazoan communities may suggest that decomposer communities filled the same roles for ecosystem processes in the bottom segments at both locations, whereas the top segment metazoan communities could be more strongly related to local environmental conditions, such as the quality of the vegetative material for the herbivores. The top segments could even be subjects to more colonization events by mobile arthropods, as previously suggested (Usher and Booth 1986; Yanoviak et al. 2004). Among the four organism groups, the bacterial communities were best separated both by location and moss segment. This could be due to the higher diversity found for the bacteria in general, but also because the bacterial communities may be more dependent on the physical properties of the moss micro-habitats than the other associated organism groups (Bragina et al. 2012). The closer similarity between the middle and bottom segment communities, compared to the top one in almost all groups can also be noted, indicating a specific diversity for the photosynthetic top part of the moss layer.

The grazing treatment (presence or absence of sheep) did not significantly affect community composition for the metazoa and protist groups at both locations. However, as was the case for their diversity, the fungal and bacterial communities were significantly impacted by sheep presence, either directly for the fungal communities or indirectly by the interaction with location for the bacteria. Again, the different fungal communities found under the different treatments could be due to the physical effect of grazing and trampling on the moss layer, by changes in the biogeochemical properties, and litter decomposition processes (Camuy-Vélez et al. 2024). The intensity of grazing has also been proven to impact microbial communities differently, with increased microbial community size under light grazing but decreased size under heavy grazing, which could explain the differences in the impact of sheep presence on bacterial communities for our two different locations (Zhao et al. 2017). Finally, the significance of the sheep presence on the protist communities as seen with the weighted UniFrac distances, taking DNA sequence differences into account, but not with the Bray-Curtis distances, could indicate more closely related protists within one treatment compared to the other.

Conclusion

This study reveals clear patterns of diversity and different community compositions between different locations and different segments within the moss layer using metabarcoding techniques. The main finding is the increasing diversity of organisms down the moss layer for all identified groups, from the metazoan to the bacterial communities. Finally, the sheep presence in the Icelandic rangelands only affects the bacterial and fungal communities, with different species in grazed or ungrazed plots. By focusing on an often-neglected biodiversity of terrestrial ecosystems, we highlight the potential of new environmental DNA techniques to uncover the "non-visible" biodiversity and the importance of increasing our knowledge of this specific diversity for endangered ecosystems. This study is particularly important given the broad distribution range of the studied model moss species across the Northern Hemisphere. Additional effort is required to continue identifying the different organisms living in cryptic habitats and to link their presence with larger ecosystem functions.

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Author contributions BP and ISJ: conceptualization and design of the study. BP, ISJ and SP: investigation, methodology, project administration, resources. BP: data curation, formal analysis, visualization, writing – original draft. ISJ: funding acquisition. ISJ and SP: supervision. All co-authors participated in writing – review and editing and all authors read and approved the final manuscript.

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Data availability The data that support the findings of this study are

available on request from the corresponding author at this DOI: https://doi.org/10.5281/zenodo.15322673. The data are not publicly available due to privacy or ethical restrictions.

Declarations

Competing interests The authors declare no competing interests.

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