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How cushion plant communities structure nival soil biodiversity: A metabarcoding study in the French Alps

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ABSTRACT

In the challenging environmental conditions of high elevation ecosystems, cushion plants create micro-climatic and fertile shelters which host a vast diversity of organisms. Yet, the taxonomic diversity of these hosts remains poorly described, and to what extent cushion plants structure these communities remains unclear. We sampled soils beneath six different species of cushion plants, along with bare-ground controls, across two different elevation gradients in the French Alps. We used environmental DNA metabarcoding to investigate the effect of different species of cushion plants on the α and β diversity of fungi, bacteria, eukaryotes, and for the first time in these ecosystems, unicellular eukaryotes and soil worms. Cushion plants hosted a surprisingly large diversity of organisms, from bacteria to mites and collembolans, forming rich and complex ecosystems. α-diversity between cushion plant and bare soil samples differed only for fungi, with communities partly structured by the cushion plant species' identity. The effect of cushion plant species on composition and β-diversity of eukaryotic and fungal communities surpassed the environmental effect, while it equaled the site effect for bacterial communities. These results highlight the key role of biotic interactions in shaping the composition of high elevation communities, and clarify the role of cushion plants as engineer and foundation species in these harsh environments. By sheltering highly diverse communities at such high elevation, cushion plants may play a prominent role in the ecological assembly of these diverse, yet poorly known, ecosystems.

Introduction

High altitude ecosystems are characterized by some of the most extreme environmental conditions on earth, like those prevailing in the European Alps above 2800 m a.s.l. (Lineweaver & Chopra, 2012). Dramatic frost events, drastic variations of temperature and humidity, intense solar radiation, fierce winds and shallow soils are among the key factors constraining life at high elevations (Körner, 2021). These severe conditions make the high alpine zone one of the margins of life on earth, where few organisms seem able to thrive (Crawford, 2008; Körner, 2011; Mani, 1968). In particular, the harsh abiotic conditions encountered at high elevation have triggered the diversification of a unique flora (Qian et al., 2021; Smyčka et al., 2022). One of the most striking morphological features of plants living at high elevations is the cushion life-form, an evolutionary convergence response towards cold and dry conditions (Boucher et al., 2012) which repeatedly emerged in many distinct plant lineages and various arctic and alpine regions of the globe (Boucher et al., 2016; Xu et al., 2019). Cushion plants mainly occur in vertical cliffs and steep screes (Fig. 1 A) in high mountain ecosystems (Aubert et al., 2014), where they appear to play a crucial role in maintaining biodiversity (Reid et al., 2010).

Cushion plants are primarily characterized by a densely stemmed, low stature canopy, resulting in a half-dome or flat-mat stature, which efficiently buffers temperature and humidity variations that are typical of alpine environments (Cavieres et al., 2007; Fischer & Kuhn, 1984). Owing to these particular morphological features, cushion plants are often described as ecosystem engineers (sensu Jones et al. (1994)) because they durably alter several local environmental parameters, including the soil beneath them (Chen et al., 2015b; Mihoč et al., 2016).

In addition, cushion plants shelter a variety of organisms. They may enhance the local plant diversity in high altitude communities (Chen et al., 2015a; Sklenář, 2009) and enlarge the elevation range of some plants (Badano et al., 2006; Raath-Krüger et al., 2019), through a mechanism described as facilitation (Brooker et al., 2008). As islands of

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Fig. 1. Illustration of some of the cushion species sampled in the present study and their typical environment. A) High alpine cliff (circa 3000m) with a ridge hosting numerous cushion plants from various species (*Saxifraga exarata, Saxifraga oppositifolia, Silene acaulis*. B) *Androsace alpina*, C) *Silene acaulis*, D) *Saxifraga oppositifolia*, E) *Saxifraga exarata*.

more favorable micro-environments, cushion plants also serve as a refuge for organisms beyond the plant kingdom. They favor fungi and microbes (Rodríguez-Echeverría et al., 2021; Roy et al., 2013), which in turn serve as resources for higher trophic levels such as mites (Minor et al., 2016) or arthropods (Molina-Montenegro et al., 2006), whose diversity and abundance are also enhanced by cushion plants (Hugo et al., 2004; Molenda et al., 2012). Cushion plants are thus foundation species since they contribute to the formation of unique ecological communities within otherwise harsh high-alpine environments (Kikvidze et al., 2015; Wang et al., 2021). Various compartments of the communities hosted by cushion plants, such as fungi, microbes or arthropods, have already been characterized. However, these assessments often lack integration across compartments. Additionally, the presence and diversity of other taxa, such as unicellular eukaryotes or worms, inside cushion plants have not been evaluated so far. Consequently, we lack a comprehensive description of the communities sheltered by cushion plants that would encompass most taxonomic groups simultaneously.

An interesting fundamental question raised by cushion plants is whether and how the identity of foundation species influence and shape associated communities. This was previously observed for tropical bromeliads, where the tanks of coexisting plant species host different algae and micro-invertebrate communities (Carrias et al., 2014; Marino et al., 2013), but also in carnivorous pitcher plants, where different species harbor distinct bacterial communities (Chou et al., 2014). Intra-specific phenotypic variation of cushion plants influences the richness and composition of the facilitated plant communities, as demonstrated in *Geum rossii* cushions (Michalet et al., 2011), or increased richness in denser cushions of *Festuca gautieri* (Al Hayek et al., 2015). At the inter-specific level, different cushion plant species facilitate the establishment of distinct plant communities (Hupp et al., 2017; Liu et al., 2023; Sklenář, 2009), owing to variations in the shape or density of cushions, but also indirectly through changes in soil conditions induced by different plant species (Chen et al., 2015a; 2015b). Furthermore, phenotypic variations within cushion species of *Silene acaulis* and *Thylacospermum caespitosum* (Liu et al., 2023; Roy et al., 2013; 2018) have been shown to alter soil bacterial and fungal communities. We thus expect that different cushion plant species may shelter contrasted communities (unicellular eukaryotes, fungi, arthropods, worms, bacteria), an hypothesis that remained untested so far.

Despite previous evidence documenting species-specific effect of cushion plants on associated soil communities, we still lack a comprehensive, cross-kingdom, description of communities hosted by cushion plants, that is including bacteria, fungi, as well as macro- and micro-invertebrates. In this study, we considered six species of cushion plants from the western European Alps and used environmental DNA (eDNA) metabarcoding to target eukaryotes, fungi and bacteria from soil sampled beneath cushions or from bare ground. We aimed at better documenting the biodiversity associated with alpine cushion plants, and understanding the effect of plant identity on hosted communities, while accounting for environmental factors potentially confounding their engineering effects. To address these objectives, we tackled the following questions:

- Question 1. Are communities found inside cushion plants more diverse than those in bare soil?
- Question 2. Do different cushion plant species harbor contrasting diversities of soil organisms?
- Question 3. What is the relative contribution of cushion plant identity, sampling site and elevation to the community structure of soil organisms?

Materials and methods

Sampling sites and study species

Sampling took place in July 2019 in the northern French Alps, in the Vanoise and Mont Blanc mountain ranges, near the Pointe de Bellecote (45.27 N, 6.75 E) and Col du Passon (45.97 N, 6.98 E), respectively. In the following, these two sampling sites will be referred to as ”Bellecote” and ”Passon”. At each site, soil samples were collected at three distinct sampling plots, spaced approximately 150 meters apart in elevation. The Bellecote plots ranged from 2950 m to 3200 m, and were located on dolomite for the two higher plots, and a mixture of dolomite and quartzite for the lower one. The Passon plots ranged from 2850 m to 3300 m on an homogeneous substrate made of gneiss or mylonite. In each plot, we collected soil samples from underneath cushion plants along with one control sample taken from unvegetated, bare ground, several meters away from any visible plant. These controls are referred to as ”bare soil” throughout this work. A total of 44 soil samples were

Table 1
Summary of the species and number of individual plants sampled in each site.

Species	Family	Passon	Bellecote	Total
Androsace spp	Primulaceae	4	2	6
Cherleria sedoides	Caryophyllaceae	2	4	6
Saxifraga bryoides	Saxifragaceae	4	2	6
Saxifraga exarata	Saxifragaceae	2	2	4
Saxifraga oppositifolia	Saxifragaceae	2	4	6
Silene acaulis	Caryophyllaceae	4	6	10
Bare soil		3	3	6
Total		21	23	44

taken from two sites and three study plots per site, corresponding to 38 cushion plants from six distinct species (four of them are illustrated in Fig. 1 B, C, D, E), and to six bare soil samples, i.e, one bare soil sample per plot, three per site (Table 1). Within study plots, the number of sampled cushion plant species ranged from two to five, excepting one study plot where only one cushion plant species occurred (*Androsace saussurei*). 21 samples were taken on the Passon site, and 23 on Bellecote (Table 1). The *Androsace* species sampled belonged to *Androsace alpina* and *Androsace saussurei*, in Bellecote and Passon sites respectively. These two species are very closely related, sharing highly similar vegetative morphology and leaf traits, and were long mistakenly identified as a single species due to their cryptic morphological differences. It was only a few years ago that genomic analyses clearly delimited them as distinct species (Boucher et al., 2021). Due to this cryptic nature, we postulate that their effect on ecosystem structure should be similar. To allow inter-site comparisons, *Androsace alpina* and *Androsace saussurei* are thus grouped in the following parts, and referred as *Androsace spp*.

Environmental DNA

Molecular analysis

For each sample, approximately 15 grams of soil was collected using tools previously sterilized with alcohol and passed through a flame before use. The samples were immediately placed in a sterile container with dried silica gel to ensure DNA preservation until extraction in the lab. For cushion plants, soil was taken from beneath the cushion's canopy. DNA was then extracted in the lab following the procedures described in Taberlet et al. (2012). Before PCR amplification, DNA extracts were diluted ten times. DNA amplification was carried out in a final volume of 20 μL containing 2 μL of DNA sample, 10 μL of AmpliTaq Gold 360 Master Mix 2X (Applied Biosystems^{TM}, Foster City, CA, USA), 2 μL of primer mix (5 μg mol^{-1} each initial concentration) and 0.16 μL of Bovine Serum Albumin (20 μg mL^{-1}, Roche Diagnostic GmbH, Mannheim, Germany). A combination of two eight base-long tags attached to the 5' end of each primer was used to assign each amplicon to its sample. For each DNA sample, PCR were performed on four replicates. DNA contained in samples were amplified with two universal markers targeting eukaryotes (euka02, targeting 18S rRNA gene) and bacteria (bact01, targeting 16S rRNA gene), and with a clade-specific marker for fungi (fung02, targeting ITS1) (Taberlet et al., 2018). The result of the amplification process was checked using capillary electrophoresis on a QIAxcel System (Qiagen GmbH, Hilden, Germany). PCR products were mixed in an equi-volume way (15 µl each) and purified using MinElute Purification kit (Qiagen). Purified products were pooled before sequencing. Sequencing libraries were built for each DNA marker with the METAFAST protocol. Depending on the DNA marker used and the expected length of barcode sequences, NextSeq (euka02) or Miseq (bact01, fung02) Illumina platforms were used for sequencing. Library construction and sequencing were done by Fasteris (Geneva, Switzerland). Negative blank controls of extraction and PCR, as well as positive PCR controls were processed and sequenced along with the biological samples (Zinger et al., 2019).

Bioinformatics

Sequences from the different libraries were pre-processed using the OBITools pipeline (Boyer et al., 2016). Forward and reverse paired-end reads were assembled based on their overlapping 3'-end sequences, and then were demultiplexed and dereplicated. Sequences with low paired'end alignment scores were discarded, as well as singletons, sequences containing ambiguous bases and PCR errors using the obiclean command. Molecular Operational Taxonomic Units were built by clustering sequences at a 97% similarity threshold within libraries using the SUMACLUST algorithm (Mercier et al., 2013; https://git.metabarcoding.org/obitools/sumaclust/). Taxonomic annotation for the ribosomal universal markers of Bacteria and non-metazoan Eukaryotes were performed on Operational Taxonomic Units (OTUs) using the SILVAngs pipeline (https://ngs.arb-silva.de/silvangs/, version r138.1, Quast et al., 2013). To improve the taxonomic annotation of metazoan eukaryotes, assignation with the highest identity score between SILVAngs and ecotag result were retained. For the fungal markers, the ecotag command from the OBITools was used, and marker-specific databases built with the ecoPCR program (Ficetola et al., 2010), from the EMBL database release 136.

Taxonomic annotations with >90% identities were retained. Cross-sample contaminations, reagent contaminants and spurious PCRs were removed on the basis of negative blank controls, sequences retrieved from unused tag combinations, and PCR replication level using the R package *metabaR* (Zinger et al., 2021). After curation, each sample with more than 2 valid PCRs replicates were pooled together by averaging the read number per OTUs. For each samples, OTUs not present in more than one PCR replicate or representing less than 10 reads within a sample were removed. OTUs present in only one sample were removed as well. No rarefaction of the number of reads was performed, as it tends to remove rare but ecologically important OTUs (McMurdie & Holmes, 2014). To avoid contamination of the generalist markers by the surrounding plants, all OTUs related to plant and chloroplast genomes were removed from the dataset deriving from the eukaryotes and bacteria markers, respectively.

The euka02 generalist marker amplifies a large spectrum of organisms, including fungi, which we also amplified using a more specific ITS marker (fung02). Hence, we removed all OTUs assigned to fungi from the euka02 primer. In all following analyses, "Eukaryote" thus refer to Metazoa and Protists.

Statistical analyses

Effect of cushion plant presence and species identity on α-diversity

The diversity of OTUs for each sample was estimated using Hill numbers (H^q) (Hill, 1973). Varying the value of the q parameter allows to compute different diversity metrics, with H^0 = richness, H^1 = exponential of the Shannon entropy (Shannon index hereafter), H^2 = the inverse of Simpson index (Simpson index hereafter) along varying values of the q parameter. As q value increases, the weight of low abundance OTUs decreases versus OTUs with high abundance. In the following, tables only report results using Shannon index (H^1). This index is chosen because it is well adapted to the study of diversity in a metabarcoding context, striking a balance between assigning less significance to rare OTUs, that might result from spurious sequences or contaminants, and effectively identifying diversity patterns (Alberdi & Gilbert, 2019; Calderón-Sanou et al., 2020). However, analyses using H^0 and H^2 were also performed to obtain a complete diversity profile and gain further insights into the abundance structure of the data (Calderón-Sanou et al., 2020).

For each metabarcoding primer, i.e. for eukaryotes, fungi and bacteria, we performed the following set of analyses using linear models (LMs) and log-transformed response variables.

To answer Question 1 (Q1) and evaluate the effect of cushion presence on soil diversity, we used LMs with log-transformed soil diversity as

the response variable against two sets of predictors. First, soil diversity was regressed against the presence or absence of a cushion plant (Predictor "Cushion presence"), without considering the species of the cushions. Then, to gain a deeper understanding of the effect of different cushion plants on diversity, we fitted diversity against cushion species (Predictor "Cushion species"), with bare soil as reference level. This allowed to compare diversity responses between bare soil and each cushion species. Site and elevation were used in each regression, in an additive combination with the cushion presence or species variable.

To answer Question 2 (Q2), bare soil samples were removed from the analysis to focus exclusively on the effect of cushion species on diversity. A third LM thus tested the effect of the identity of the cushion plant (Predictor "Cushion species") on log-transformed soil diversity. Type II Analysis Of Variance (ANOVA) was performed of these models to evaluate the inter-specific differences in diversity between cushion plants.

In all models, the additive combination of site and elevation predictors was tested in addition to the cushion-related predictors. Elevation was centered on zero and scaled to unity, and then set as a continuous predictor. For all models, we performed visual checks and tests using the *DHARMa* package (Hartig & Lohse, 2022) to ensure homoscedasticity and normality of the residuals.

Considering the low sample size and the unbalanced sampling design, we performed a power analysis to check whether these parameters could have impacted our results. For Eukaryotes and Bacteria, we used a balanced sampling design with one bare soil per cushion plant and generated data based on the observed standard deviation of each

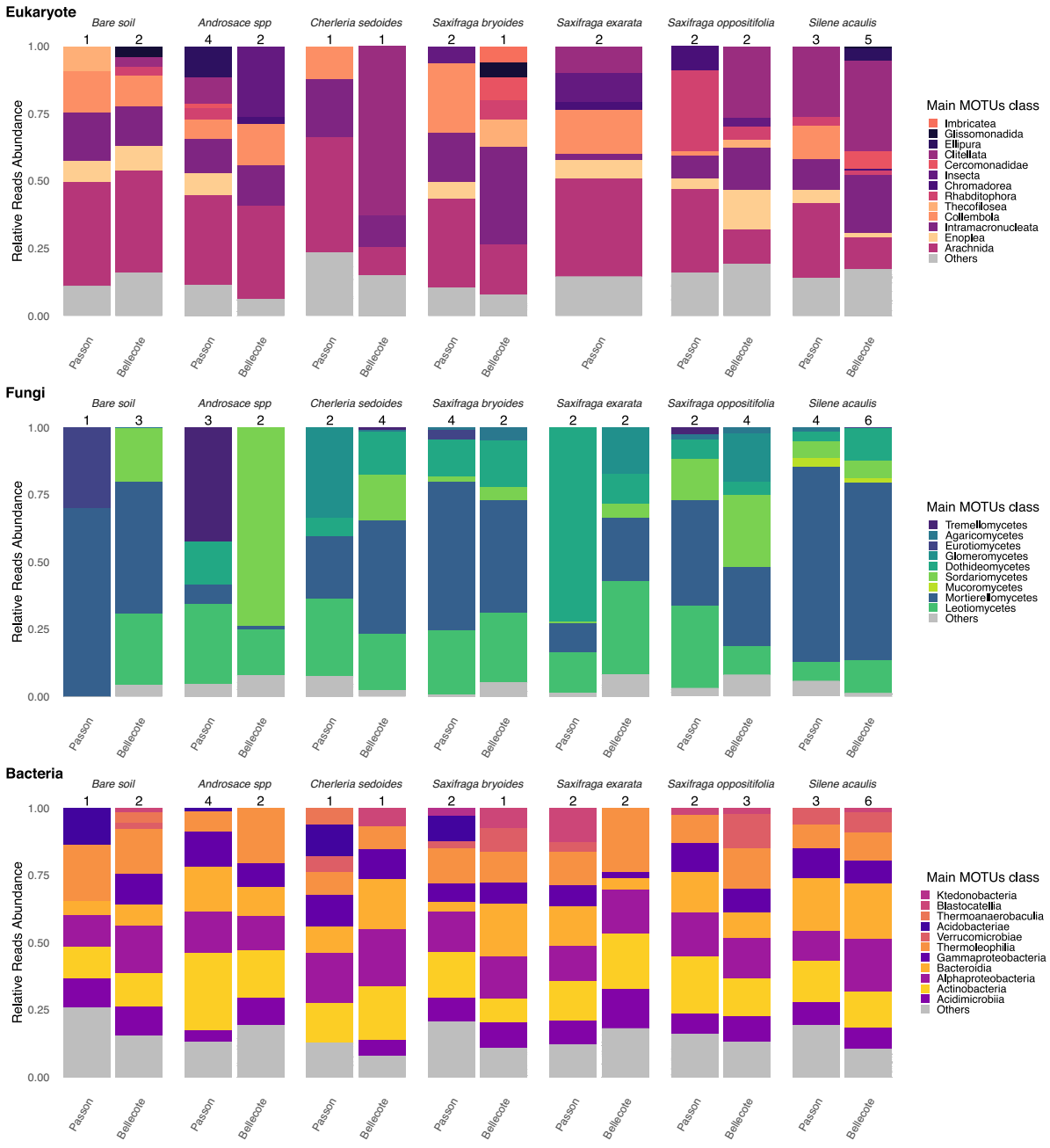

Fig. 2. Relative Reads Abundance (RRA) after filtering of OTUs of the three groups of organisms studied (Eukaryotes (Metazoa and Protists), Fungi and Bacteria) for each cushion plant species and bare soil, and per site. Number of samples for each class are given on top of the bars. All OTUs assignments are shown at the the class level, and are averaged over all samples for a given association of species and site. OTUs representing less than 5% of the total number of reads were aggregated to the "Others" category (in grey).

category (bare soil and cushion plants) while varying the effect size (difference between bare soil and cushion plants means), as well as the sample size, from 10 to 200 samples. In each configuration, we computed the statistical power for 1000 simulations.

Influence of cushion plant species and environmental variables on β-diversity

To test how soil community composition responded to different cushion plant species and environmental variables (Q3), we performed permutational multivariate analysis of variance (PERMANOVA; Anderson (2001)) to study patterns of community compositions. Community matrices were transformed using Hellinger formula, i.e square-root of the Relative Reads Abundance (RRA) (Laporte et al., 2021), and Bray-Curtis dissimilarities were computed between all pairs of samples and for each primer. Matrices were permuted 999 times to assess the significance of all three predictors : cushion species, site and elevation. The reported R^2 values were obtained from type II ANOVA, which are unaffected by the order of variables in the formula. Hence, the sum of all variable's R^2 does not equal the full model R^2, which is better expressed as $R^2_{model} = 1 - R - 2_{\text{Residuals}}$. This analysis was implemented using the *adonis2* function from R package *vegan* (Oksanen et al., 2022).

For visualization purpose, Non-metric Multi-Dimensional Scaling (NMDS) were built on the same dissimilarity matrices that were used for PERMANOVA. Factorial predictors were highlighted by constructing ellipses for cushion species and site effects, while a 2D smooth surface was projected into the NMDS space to depict the effect of elevation. All these analyses were done using the R package *vegan* (Oksanen et al., 2022).

We ran all analyses using the R software 4.2.1 (R Core Team, 2022). All values of OTUs diversity are given as mean ± standard deviation, unless stated otherwise.

Results

Following the bioinformatics pipeline, a total of 439, 180 and 1411 OTUs were identified for the euka02, fung02 and bact01 primers respectively (see Appendix A: Table 1). The transformed relative abundances of different OTU classes found in our samples are displayed per cushion species and per sites in Fig. 2. The most represented classes among all samples were Arachnida (n_{reads} = 59331, 27% of total reads count), Intramacronucleata (n_{reads} = 32493, 14%) and Clitellata (n_{reads} = 31602, 14%) for eukaryotes, Mortierellomycetes (n_{reads} = 24180, 34%), Leotiomycetes (n_{reads} = 12226, 17%) and Dothideomycetes (n_{reads} = 9143, 12%) for fungi, and Actinobacteria (n_{reads} = 16787, 17%), Alphaproteobacteria (n_{reads} = 14407, 15%) and Bacteroidia (n_{reads} = 13801, 14%) for bacteria.

Among eukaryotes, metazoans represented about 30% of all OTUs (n_{OTUs} = 131) and 70% of the reads (n_{reads} = 159620), the other taxa being microscopic and unicellular eukaryotes (see Appendix A: Fig. 1. Among metazoans, about 57% of the organisms were identified as Arthropoda (n_{OTUs} = 75, n_{reads} = 90002), which included Arachnida (mostly mites), Collembola and Insecta. The second biggest group of metazoans were worms (Annelida, Nematoidea and Platyhelminthes) which represented 38% of the OTUs(n_{OTUs} = 50) and 43% percent of the reads (n_{reads} = 68889).

Effect of cushion plant presence on α-diversity (Question 1)

Based on Shannon index, the presence of cushion plants induced a 2.13 times increase in soil diversity of fungi when compared to bare soils ($H^1_{In;\ Fungi} = 10.4 \pm 5.9$; $H^1_{Out;\ Fungi} = 4.5 \pm 2.6$; p-value = 0.036; Table 2), but did not impact soil diversity of eukaryotes and bacteria. Fungal communities were particularly increased beneath *Cherleria sedoides* ($H^1_{Che\ sed;\ Fungi} = 15.9 \pm 5.2$), *Saxifraga bryoides* ($H^1_{Sax\ bry;\ Fungi} = 11.2 \pm 5.9$) and *Saxifraga exarata* ($H^1_{Sax\ exa;\ Fungi} = 13.0 \pm 8.7$), when compared to bare soil.

Site had an almost significant effect (p-value = 0.061, Table 2) on the diversity of bacterial communities, with a greater diversity in Passon ($H^1_{Passon} = 209.6 \pm 58.7$) compared to Bellecote ($H^1_{Bellecote} = 164.1 \pm 56.7$).

Power analysis showed that within the confidence interval of the observed effect size, high statistical power could be achieved. However, due to the high natural statistical dispersion of our data, these simulations indicated that our sampling design could only detect statistical differences between bare and plant soil samples for high expected statistical effects and much larger sample sizes. Under current conditions, it would indeed be challenging to detect a statistical effect, even with increasing sample size (see Appendix A: Fig. 2).

The other two diversity metrics (i.e. richness and Simpson index) depicted a similar dynamic for eukaryote and bacteria, where low abundance species were site specific (H^0 : p-value$_{Site;\ Bacteria}$ = 0.048; H^0 : p-value$_{Site;\ Eukaryote}$ = 0.046; see Appendix A: Table 2), but were barely significant to non-significant for Shannon diversity and Simpson inverse (Fig. 3A left and right; see Appendix A: Table 3). This underlines that the spatial variation in bacterial and eukaryotic community diversity was primarily driven by less abundant OTUs. Likewise, the most cushion-specific fungis were rare OTUs (Fig. 3A center; see Appendix A: Table 2 and Table 3). While all cushion species increased richness comparatively to bare soils, only *Cherleria sedoides* did so when considering Simpson index (see Appendix A: Table 4 and Table 3).

Effect of plant species identity on α-diversity (Question 2)

Cushion species identity did not impact Shannon index diversity, and thus for all communities considered, although ANOVA results show a very low p-value for fungi (H^1 : p-value$_{Cushion\ species;\ Fungi}$ = 0.07; Table 4). Only *Cherleria sedoides* species showed higher diversity compared to other cushion species (H^1 : p-value$_{Che\ sed;\ Fungi}$ = 0.038; see Appendix A: Table 8). Similar p-values were observed for the effect of elevation on eukaryotes (H^1 : p-value$_{Elevation;\ Eukaryota}$ = 0.06; Table 4) and site on Bacteria ((H^1 : p-value$_{Site;\ Bacteria}$ = 0.07; Table 4)). The different cushion plant species thus had very low to no influence on soil diversity.

Table 2
Predictors estimates from Linear Model regression for the effect of cushion presence, site and elevation on Shannon Index, for each metabarcoding primer. Levels of Cushion Presence predictors indicate effects in bare soils (*Out*) or beneath cushion plant soil (*In*). *P*-values in bold indicate a significant (< 0.05) effect.

	Eukaryote			Fungi			Bacteria		
Predictor	Beta	95% CI	*P*-value	Beta	95% CI[1]	*P*-value	Beta	95% CI[1]	*P*-value
Cushion presence									
Out									
In	0.8	0.33, 1.9	0.59	2.13	1.05, 4.31	**0.036**	0.95	0.62, 1.45	0.81
Site									
Passon									
Bellecote	1.28	0.74, 2.19	0.36	1.24	0.82, 1.89	0.30	0.79	0.61, 1.01	0.061
Elevation	0.84	0.64, 1.11	0.21	0.88	0.71, 1.09	0.23	0.95	0.84, 1.08	0.46
No. Obs.		28			41			32	
R^2		0.105			0.172			0.158	

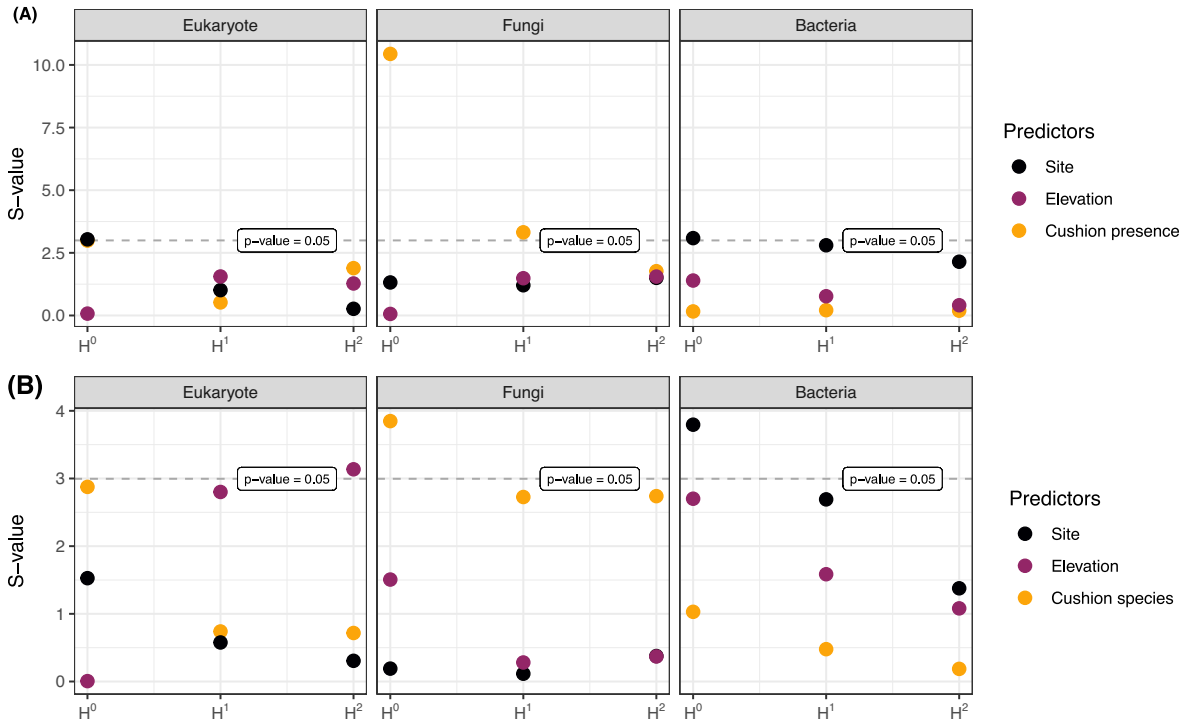

Fig. 3. A) Effects of cushion plant presence, site and elevation on α-diversity. B) Effects of cushion plant species, site and elevation on α-diversity of cushion plants. The significance of predictors is expressed using S-value (S-value = -$\log_2$(p-value)). The grey dashed line displays the $\alpha = 0.05$ value. Values above this line are considered significant. For q = 0, H^0 = richness, for q = 1, H^1 = exponential of the Shannon Entropy, and for q = 2, H^2 = inverse of the Simpson Index. Greater values of q give less weight to rare OTUs.

Table 3
Linear Model regression for the comparison between bare soil and cushion plants effect on Shannon Index. Shannon index was modelled against cushion species, site and elevation on Shannon Index. Reference level of Cushion Species predictor was set to *Baresoil* to allow for diversity comparison between bare soil and each cushion species. *P*-values in bold indicate a significant (< 0.05) effect.

	Eukaryote			Fungi			Bacteria		
Predictor	Beta	95% CI	*P*-value	Beta	95% CI	*P*-value	Beta	95% CI	*P*-value
Cushion species									
Bare soil									
Sil aca	0.68	0.25, 1.84	0.43	2.03	0.96, 4.26	0.061	0.9	0.55, 1.45	0.64
Che sed	0.78	0.2, 3.02	0.70	3.96	1.69, 9.27	**0.002**	0.74	0.38, 1.45	0.37
Sax bry	0.78	0.23, 2.65	0.67	2.62	1.12, 6.15	**0.028**	0.95	0.52, 1.74	0.87
Sax opp	1.41	0.45, 4.42	0.53	2.16	0.99, 4.74	0.054	1.2	0.7, 2.05	0.49
And sp	0.65	0.22, 1.96	0.42	1.11	0.48, 2.54	0.80	0.97	0.56, 1.67	0.91
Sax exa	1.06	0.26, 4.31	0.94	2.82	1.18, 6.7	**0.021**	0.97	0.55, 1.72	0.91
Site									
Passon									
Bellecote	1.32	0.72, 2.43	0.35	1.13	0.75, 1.69	0.55	0.8	0.6, 1.05	0.10
Elevation	0.82	0.59, 1.16	0.25	1.02	0.79, 1.3	0.90	0.92	0.79, 1.08	0.32
No. Obs.		28			41			32	
R^2		0.257			0.390			0.262	

Richness of fungal communities was, however, structured by cushion species, with *Cherleria sedoides* and *Saxifraga oppositifolia* hosting the richest communities ($H^0_{\text{Che sed; Fungi}} = 33.5 \pm 10.8$), $H^0_{\text{Sax opp; Fungi}} = 30.2 \pm 9.2$) while *Androsace spp.* had the poorest fungal community ($H^0_{\text{And sp; Fungi}} = 13.4 \pm 6.8$). Site significantly influenced bacterial richness ($H^0_{\text{Passon; Bacteria}} = 439 \pm 123$; $H^0_{\text{Bellecote; Bacteria}} = 320 \pm 116$; $p\text{-value}_{\text{Site; Bacteria}} = 0.022$; Appendix A: Table 6), emphasizing the weight of the lesser abundant but site-specific OTUs. Simpson index showed that eukaryote diversity decreased with elevation (see Appendix A: Table 10).

Influence of cushion species, elevation and site on community composition (Question 3)

All predictors contributed to explain the spatial structure of OTU community composition, though in varying proportions (Table 5). Altogether, cushion species, site and elevation contributed to explain around 40% of the composition of these different communities ($R^2_{Euka} = 0.41$; $R^2_{Fung} = 0.39$; $R^2_{Bact} = 0.46$).

For eukaryotes and fungi, cushion species was the main predictor, explaining 19% and 18% of community composition, respectively ($R^2_{\text{Species; Euka}} = 0.19$; $R^2_{\text{Species; Fung}} = 0.18$; Table 5). The fungal communities hosted by *Androsace* spp., *Saxifraga bryoides* and *Silene acaulis* appeared well segregated (Fig. 4A, D), although the ones hosted by

Table 4
Results of the type II ANOVA showing the effect of cushion plant species, site and altitude on soil organisms MOTUs diversity (Shannon entropy), as modelled using Linear Model with log-transformed response variables. Results are shown for each primer used.

Primer	R^2	Predictor	Sum Square	Df	F-value	*P*-value
Eukaryote	0.267	Cushion species	2.08	5	0.94	0.48
		Site	0.15	1	0.35	0.56
		Elevation	1.78	1	4.03	0.06
		Residuals	7.50	17		
Fungi	0.278	Cushion species	4.19	5	2.35	0.07
		Site	0.01	1	0.02	0.89
		Elevation	0.04	1	0.10	0.76
		Residuals	10.32	29		
Bacteria	0.252	Cushion species	0.44	5	0.71	0.62
		Site	0.46	1	3.71	0.07
		Elevation	0.21	1	1.71	0.20
		Residuals	2.58	21		

Table 5
Results from the PERMANOVA analysis for each group of organisms (Eukaryotes (Metazoa and Protists), Fungi and Bacteria) realised with *adonis2* functions from the *vegan* package. Significant predictors are highlighted in bold with their statistics. PERMANOVA were ran with 9999 iterations for each group.

Primer	Predictor	Df	SumOfSqs	R^2	F	*P*-value
Eukaryote	**Cushion species**	4	1.28	0.19	1.28	**0.040**
	Site	1	0.60	0.09	2.40	**0.002**
	Elevation	1	0.55	0.08	2.22	**0.002**
	Residual	16	4.00	0.59		
	Total	22	6.77	1.00		
Fungi	**Cushion species**	5	2.36	0.18	1.76	**0.001**
	Site	1	1.32	0.10	4.93	**0.001**
	Elevation	1	0.50	0.04	1.88	**0.014**
	Residual	29	7.77	0.61		
	Total	36	12.76	1.00		
Bacteria	**Cushion species**	4	0.97	0.17	1.55	**0.011**
	Site	1	1.04	0.18	6.67	**0.001**
	Elevation	1	0.30	0.05	1.94	**0.028**
	Residual	20	3.12	0.54		
	Total	26	5.74	1.00		

Silene acaulis were mixed with those of *Saxifraga oppositifolia* and *Cherleria sedoides* (Fig. 4D).

The proportion of community variance explained by sampling site was about twice as low as the variance explained by cushion species, for both Eukaryotes and Fungi ($R^2_{Site;\ Euka} = 0.09$; $R^2_{Site;\ Fung} = 0.10$; Table 5). Eukaryote communities were not clearly segregated between Passon and Bellecote (Fig. 4B), whereas there was a clear split between the fungal communities of Passon and Bellecote (Fig. 4E). For eukaryote communities, elevation explained an amount of variance comparable to the one of sampling site ($R^2_{Elev;\ Euka} = 0.08$). For fungi communities, elevation, however, explained much less variance than sampling site ($R^2_{Elev;\ Fung} = 0.04$; Table 5). The bigger spread of fungi communities observed in Passon, but not in Bellecote (Fig. 4F), may be due to the higher elevation range among sampling sites at Passon (elevation range = 450 m) in comparison with Bellecote (elevation range = 250 m).

For bacteria, the main predictor of community variation was site, followed by cushion species ($R^2_{Site;\ Bact} = 0.18$; $R^2_{Species;\ Bact} = 0.17$; Table 5). Sites were clearly segregated by the NMDS analysis (Fig. 4H), but this separation was less pronounced between cushion species. The NMDS showed that *Androsace* spp. and *Saxifraga bryoides* hosted distinct bacterial communities, while those hosted by *Silene acaulis* and *Saxifraga oppositifolia* largely overlapped (Fig. 4G). Elevation poorly explained the composition of bacterial communities ($R^2_{Elev;\ Bact} = 0.04$; Table 5).

Discussion

Our study investigated the crucial influence of cushion plants on the diversity and composition of high alpine soil communities through a cross-kingdom approach targeting a large spectrum of organisms from various life domains such as eukaryotes, fungi and bacteria. To our knowledge, this is the first study combining several foundation plant species from distinct phylogenetic lineages and such a comprehensive description of communities. We demonstrate that alpine cushion plants host complex and diverse communities, with organisms occupying several trophic levels, from micro-organisms to large predators such as Acari or Arachnids. Our results thus provide novel insights into the assembly and structure of natural communities thriving in high alpine environments, and into the structuring role of cushion plants in these harsh abiotic conditions.

Environmental DNA metabarcoding (Taberlet et al., 2018) is now considered a very powerful tool to study biodiversity, especially in extreme and remote environments (Fraser et al., 2018; Krah & March-Salas, 2022) where field sampling is challenging. eDNA studies co-occurring DNA sequences in environmental samples, but can be fooled by mechanical or biological spreading of DNA sequences by highly dispersive organisms, thus blurring the observed signal (Beng & Corlett, 2020; Lunghi et al., 2022). Matching the observed sequences to actual organisms is another challenge of eDNA, and requires well documented reference databases that are certainly lacking in the case of high-elevation ecosystems. Moreover, generalist primers associated to short barcodes such as the euka02 (100 to 150 bp length) lack resolution to lower taxonomic levels, calling for the use of more resolving primers targeting selected groups. How to relate sequences to species-level groups has been a long-lasting debate in bio-molecular studies, with the original clustering approach into OTUs being now challenged by the use of Amplicon Single Variants (ASVs; Callahan et al., 2016). ASVs have been widely adopted in microbiology, but the intra-specific genetic variability of Metazoa tend to over-estimate diversity based on ASVs (Brandt et al., 2021).

Despite these shortcomings, eDNA studies have yielded very insightful results on high-elevation ecosystems, enhancing our knowledge of cliff ecosystems (Krah & March-Salas, 2022), soil trophic networks (Calderón-Sanou et al., 2023) or species colonization processes on glacier forelands (Guerrieri et al., 2022).

Environmental DNA is especialy interesting to the study of extreme and remote environments, where field sampling is challenging (Fraser et al., 2018; Krah & March-Salas, 2022), as it captures a snapshot of communities while reducing the time spent in the field. Difficulty of access to sampling location and logistical constraints indeed hampered our ability to collect a large number of samples. Sampling soil beneath cushion plants is not without damage for the plant structure, while cushion plants are long-living perennial plants of patrimonial value. We thus wished to collect representative samples with minimal disturbance to high-alpine plant communities. We are convinced that the sample design allows a comprehensive study of the soil diversity hosted by alpine cushion plants, though with a limited appraisal of the diversity compared to bare soil.

Diversity inside cushions compared to bare soils (Question 1)

Contrary to our expectations, we found mild evidence of diversity increase related to cushion plant presence. Only fungal communities were influenced by the presence of cushion plant species, which increased diversity for both richness and Shannon index (Fig. 3, Table 2. This result matches previous studies on fungal communities, showing various responses to the presence of cushion plants. *Silene acaulis* decreased their α-diversity when measured by Simpson Index (Roy et al., 2018), whereas plants of the *Azorella* genus had phylum-specific effects on fungi (Rodríguez-Echeverría et al., 2021). Conversely, Wang et al. (2020) did not detect any effect of *Thylacospermum caespitosum* cushions

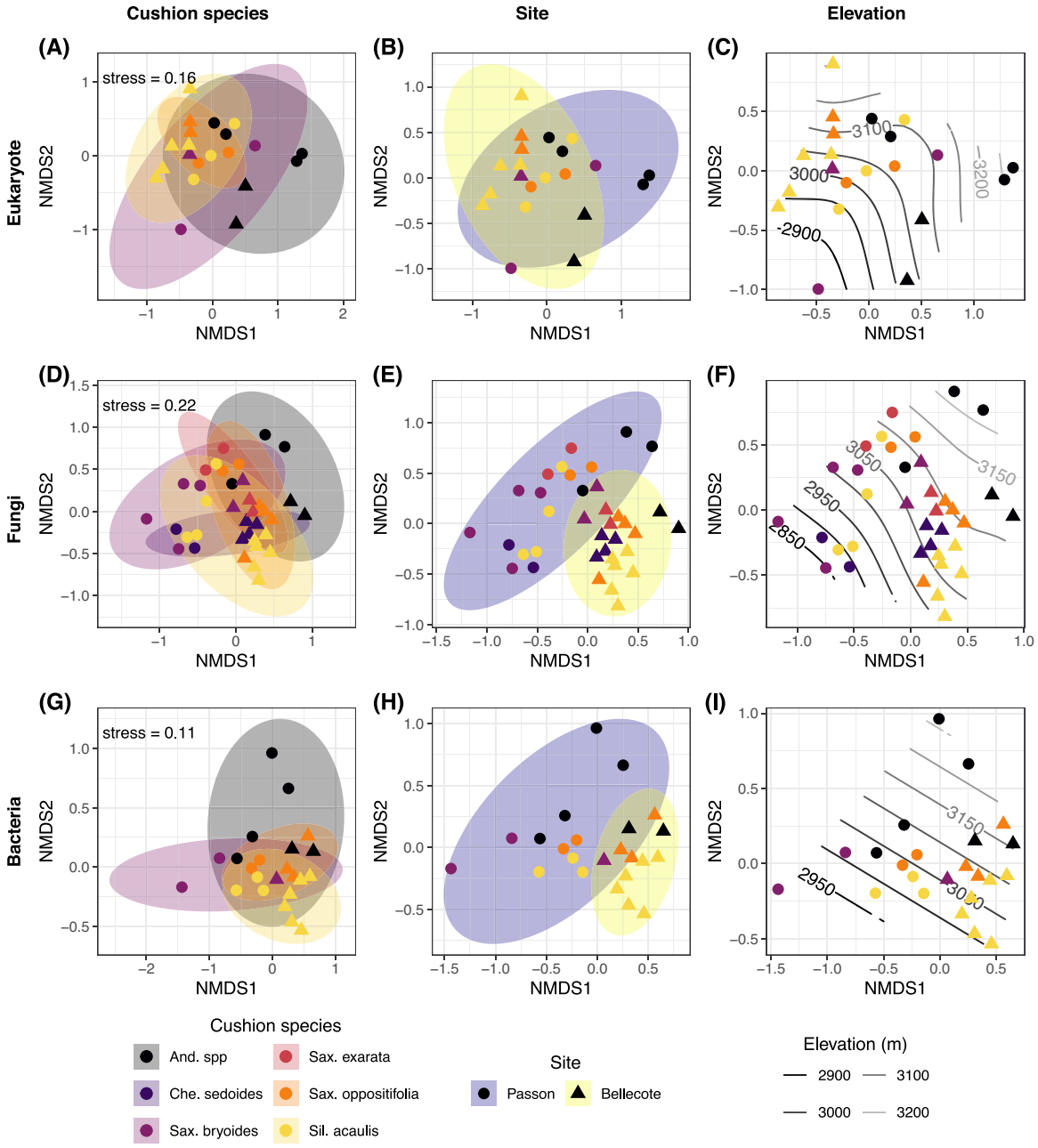

Fig. 4. First and second NMDS axis of the dissimilarities between the soil communities of cushion plant samples for Eukaryotes (A, B, C), Fungi, (D, E, F) and Bacteria (G, H, I). For each ordination, stress indice is reported in the left (A, D, G) plot. One factor influencing community dissimilarity is highlighted in each column : Cushion species (A, D, G), site (B, E, H) and elevation (C, F, I), from left to right. Plotted factor result from significant PERMANOVA analysis. For Eukaryotes (Fungi excluded) and Bacteria, only four cushion species were considered (*Silene acaulis, Saxifraga bryoides, S. oppositifolia* and *Androsace* species. Ellipses and smooth surfaces were obtained using the R package *vegan* (Oksanen et al., 2022). Abbreviations used in the figure : *And.* = *Androsace, Che.* = *Cherleria, Sax.* = *Saxifraga, Sil.* = *Silene.*

on fungal diversity, and in similarly isolated cliff habitats, the presence of plants did not impact fungal richness (Krah & March-Salas, 2022).

Bacterial and eukaryote diversity in high alpine soils were mainly driven by site effect. Local variation of soil characteristics and abiotic parameters are known to have a greater influence on bacterial richness than the presence of cushion plants (Rodríguez-Echeverría et al., 2021; Roy et al., 2013; Wang et al., 2020). Furthermore, the two sites studied here differed in their geology and hence pH, as Passon's soil are acidic (gneiss) while Bellecote's are both acidic (quartzite) and basic (dolomite), and pH is known to be a strong driver of bacterial diversity (King et al., 2010). For eukaryotes, this result is in stark contrast with the previous studies on large invertebrates which reported cushion plants as hosts to many species (Chen et al., 2021; Ľuptáčik et al., 2021; Molenda et al., 2012; Molina-Montenegro et al., 2006). In the present study, few eukaryote taxa (Eutardigrada, Sminthurididae) were specifically found beneath cushions, whereas others were in fact detected in both cushion plants and bare soil samples, such as protists, nematodes, springtails, arachnids or insects. Although little is known on the unicellular eukaryotes dwelling in high elevation ecosystems (Giachello et al., 2023), it was nevertheless demonstrated that they colonize alpine soils through wind dispersal (Mazel et al., 2022). This might explain, at least partly, why the diversity of eukaryotes did not seem to be generally enhanced inside cushion plants.

The number of bare soil sampled in the present study may be considered to be very low, with only one bare soil for about ten cushion plant samples, when compared to similar studies using a one-to-one design (e.g. Michalet et al., 2024; Roy et al., 2013). Our bare soil samples might thus not be representative enough of the diversity of high-alpine ecosystems considering their environmental heterogeneity at small spatial scale (Donhauser & Frey, 2018; Zinger et al., 2009). Our results are thus to be considered a proof-of-concept or preliminary, and are calling for further validation at a larger spatial scale. However, we noticed during sampling that bare soils were devoid of aboveground vegetation cover but still contained root systems, sometimes extending several meters away from any visible plant. Consequently, bare soils exhibited significant biological activity even at high elevations, which may seem counter-intuitive but supports our findings that bare soil may also host significant biological diversity.

Differences in α-diversity between cushion species (Question 2)

Only the richness of fungal communities was affected by the identity of the cushion plant species (see Appendix A: Table 4), although we detected a marginal effect of the cushion species identity on the Shannon diversity (Table 3 and Fig. 3). Differences in fungal diversity between cushion plants was previously shown on mycorrhizal and endophytic fungi in the Arctic, with high diversity under *Silene acaulis* and low diversity under *Saxifraga oppositifolia* (Abrego et al., 2020). In the present case, only the *Cherleria sedoides* species hosted increased richness than other species. However, fungal communities differed phenotypes within single species in alpine environments (Liu et al., 2023; Roy et al., 2013; 2018; Wang et al., 2020). Such intra-specific variation might buffer inter-specific diversity differences. These results are a first step in our understanding of the role of inter-specific variations in engineer species. These findings should be interpreted with caution, as the small sample size considered here might hamper the generalisation of our results to other ecosystems. These findings confirm that different foundation species may have different effects on the α-diversity of fungi in the ecosystem they create, and this raises the question of what plant traits may explain such disparate effects on the assembly of soil fungi communities.

Effects of cushion species identity on β diversity (Question 3)

Cushion plants share a common general organisation, but they differ in many functional traits, such as stem density, canopy compactness, chemical composition, and lineage history. As four to six different species of cushion plants were sampled within study sites, our results provide novel insights into how foundation species structure associated communities and promote diversity. Depending on the taxa considered (eukaryotes, fungi and bacteria), cushion plant species identity explained 17–19% of variation in the composition of soil communities. This highlights the paramount importance of the engineer effect of cushion plants, especially for eukaryotes and fungal communities (Table 5). The biotic effect appeared to be stronger than climatic (elevation effect, 5–8%) and geographic (site effect, 9–18%) effects. Due to the limited sample size considered here, and while we believe our findings to accurately depict the influence of engineer species diversity on high-alpine biodiversity, we must limit the generalization of these results to similar comparable ecosystems.

Elevation is commonly confounded with several environmental variables such as temperature, precipitation, UV radiation and snow cover duration. Although elevation is a fundamental climatic factor in alpine environments, it is not the only indicator of climatic variation as the site effect probably correlated with additional environmental factors. However, soil communities located in similar elevations had comparable community composition, especially in the case of eukaryotes (Fig. 4). Since the Permanova analysis revealed that cushion species identity explains much more variation than elevation, the effect of elevation visible in Fig. 4 is likely due to the correlation between elevation and species identity (for instance, *Androsace spp.* were only found in the higher plots of both sites), but also to the differences in elevational ranges sampled. Site captured the effect of bedrock as well as disparities in regional species pools or soil history, but also wider regional climatic variation, e.g. the Passon site is wetter and endures longer snow cover than Bellecote. Additional environmental parameters, such as yearly snow dynamics or soil chemical composition, may deserve a finer description as they are very likely contributing to the composition of soil communities.

All three predictors together explained about 40% of the variation in soil community composition (Table 5), which is comparable to other published studies (Roy et al., 2018; Wang et al., 2020). The strongest effect of cushion plants was found for eukaryote and fungi ($R^2 = 0.19$ and $R^2 = 0.18$ respectively), and the estimated statistical effect was larger than the sum of the variance explained by site and elevation together. Whereas eukaryotes α-diversity did not differ between cushions, the composition of these communities was mainly explained by the identity of the cushion plant (Table 5). Since cushion plants may have the ability to create species-specific habitats (Hupp et al., 2017), some organisms are undoubtedly favored by the specific conditions of a given cushion. Therefore the cushion plant community is likely to exert a major shaping force upon the entire ecosystem structure. For instance, fungi are often related to particular plant species and even to intra-specific lineages (Roy et al., 2018). As an example, *Silene acaulis* and *Cherleria sedoides*, two species belonging to the Caryophyllaceae family, share a very dense above-ground vegetative structure, and influence community diversity in a similar way (Fig. 4). The presence of specialized mycorrhizal and endophytic fungi (Molina-Montenegro et al., 2015) may also drive the effect of cushion species identity on fungal community composition.

In the case of bacteria, the effect of cushion species was as strong as the effect of site ($R^2 = 0.17$, Table 5). This suggests that the influence of plant identity on bacteria communities is larger than expected, since soil properties are generally overwhelming drivers of bacteria community structure. For instance, Wang et al. (2020) found that phenotypic variation in *Thylacospermum caespitosum* contributed to 17% of the variance in soil bacteria communities, against 69% explained by sampling sites. Our results hence provide the additional evidence that bacterial communities at high elevation also strongly respond to biotic interactions with larger organisms such as plants, and not only to the abiotic conditions of their living environment, soil in this case.

Potential consequences of cushion species diversity at the ecosystem-level

The importance of the inter-specific diversity of engineer species on community composition has been highlighted for cushion plants (Liu et al., 2023; Roy et al., 2018; Wang et al., 2020) as well as for other foundation species (Largaespada et al., 2012; Nicastro et al., 2020). Engineer and foundation species typically modify their environment, but even closely related organisms sometimes show discrepancy betweeen the characteristics of their respective engineered space (Momberg & le Roux, 2020) and its associated diversity (Badano et al., 2006; Roos et al., 2022). In the case of engineer organisms exerting a strong effect on their environment, biotic filtering thus becomes a very important driver of community structure (Thakur & Wright, 2017). In fact, the presence of engineers may imply both biotic and abiotic filters by creating a feedback between one another, which in turn impacts the structure, the stability and the diversity of the community, even when engineer organisms are scarce (Yeakel et al., 2020). The diversity of engineer species thus contributes to maintain biodiversity, especially in highly constrained environments such as high-alpine ecosystems.

Conclusions

The role of cushion plants as ecosystem engineer and foundation species, but also their diversity, contribute to shape the communities of various groups of taxa, from bacteria to micro-invertebrates. Cushion plants created shelters for life in the extreme conditions found on mountain tops, and probably shaped the history of these ecosystems over a long period of time. If engineer species are shaping their environment, their diversity is structurally important to maintain biodiversity, especially in environments such as high alpine ecosystems which are structured by these engineer species. As high-alpine environments undergo profound changes leading to massive glacier retreat and the colonization of alpine ecosystems into formerly glaciated areas (Bosson et al., 2023), it becomes critical to understand the assembly dynamics of these ecosystems. It is necessary to investigate the functional differences between cushion plants species, the environmental parameters that they both withstand and contribute to change, and their effects on soil communities, to better understand mechanisms of community assembly and ecosystem functioning in high-alpine ecosystems.

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CRediT authorship contribution statement

Keyvan Dumas: Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing, Visualization. **Alexy Rosa:** Formal analysis, Writing – review & editing. **Glenn Yannic:** Supervision, Validation, Writing – review & editing. **Christiane Gallet:** Supervision, Writing – review & editing. **Irene Calderon-Sanou:** Data curation, Software, Writing – review & editing. **Clément Lionnet:** Data curation, Software. **Ludovic Gielly:** Investigation, Writing – review & editing. **Wilfried Thuiller:** Conceptualization, Funding acquisition, Writing – review & editing. **Sébastien Lavergne:** Conceptualization, Supervision, Writing – review & editing, Investigation. **Sébastien Ibanez:** Conceptualization, Investigation, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at 10.1016/j.baae.2024.08.002

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