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Soil filtration-sedimentation improves shelled protist recovery in eukaryotic eDNA surveys

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Abstract

A large part of the soil protist diversity is missed in metabarcoding studies based on 0.25 g of soil environmental DNA (eDNA) and universal primers due to ca. 80% co-amplification of non-target plants, animals and fungi. To overcome this problem, enrichment of the substrate used for eDNA extraction is an easily implemented option but its effect has not yet been tested. In this study, we evaluated the effect of a 150 μm mesh size filtration and sedimentation method to improve the recovery of protist eDNA, while reducing the co-extraction of plant, animal and fungal eDNA, using a set of contrasted forest and alpine soils from La Réunion, Japan, Spain and Switzerland. Total eukaryotic diversity was estimated by V4 18S rRNA metabarcoding and classical amplicon sequence variant calling. A 2- to 3-fold enrichment in shelled protists (Euglyphida, Arcellinida and Chrysophyceae) was observed at the sample level with the proposed method, with, at the same time, a 2-fold depletion of Fungi and a 3-fold depletion of Embryophyceae. Protist alpha diversity was slightly lower in filtered samples due to reduced coverage in Variosea and Sarcomonadea, but significant differences were observed in only one region. Beta diversity varied mostly between regions and habitats, which explained the same proportion of variance in bulk soil and filtered samples. The increased resolution in soil protist diversity estimates provided by the filtration-sedimentation method is a strong argument in favour of including it in the standard protocol for soil protist eDNA metabarcoding studies.

KEYWORDS
Chrysophyceae, environmental DNA, filtration, protists, soil, testate amoebae

1 | INTRODUCTION

Soils contain a huge, but mostly unknown diversity of life (Bastida et al., 2020; Fierer & Jackson, 2006; Singer et al., 2021; Tedersoo et al., 2014; Wu et al., 2011). The taxonomic deficit is highest for microbial groups (Decaëns, 2010) and especially protists (eukaryotic microbes excluding Fungi; Chao et al., 2006, Geisen et al., 2018). Filling this knowledge gap requires a major effort combining molecular and morphological approaches (Heger et al., 2014). The density of soil protists varies considerably depending on their size and habitat type (Foissner, 1999). Larger soil protists, such as some testate amoebae (i.e. polyphyletic group of amoeboid protist enclosed in

a test), occur at much lower density (e.g. 10–10^4 ind.g^{-1}; Ehrmann et al., 2012) than smaller groups, such as heterotrophic flagellates (e.g. 10^4–10^6 ind.g^{-1}; Foissner, 1999). Thus, classical volumes used for soil eDNA studies (i.e. 0.25 g) may lead to under-estimates of diversity and high perceived randomness of occurrences in ecological studies. Soil samples, even sieved at the standard 2 mm mesh size, still contain a high proportion of minerals or relatively coarse organic matter, leaving only a very small proportion of particles with the size of larger micro-organisms. The proportion of these elements, however, varies strongly among soil samples and therefore so does the volume of useful material for eDNA extraction. Extracellular DNA may partly compensate for this bias, but properly assessing this requires experiments (e.g. mock communities), which are not applicable for most protists which are hard or impossible to grow in culture.

Protist communities in soils are mainly characterised by eDNA metabarcoding amplifying the V4 or V9 hypervariable regions of the 18S rRNA gene of most eukaryotes using general eukaryotic PCR primers (Mahé et al., 2017; Ramirez et al., 2014). One of the main, and often ignored, issues associated with the use of these general primers is the co-amplification of non-target Embryophyta, Fungi and Metazoa. These three groups regularly represent the majority of the sequencing output and, as a result, only the most abundant (i.e. predominantly the smaller) protists may be recovered. Indeed, comparative analyses have shown that a much higher diversity is covered in the same samples when using specific primers as compared to general primers (Lentendu et al., 2014). However, due to the large phylogenetic diversity of protist clades, tens of specific primers and PCR reactions would be necessary to cover the full protist community of a single sample, making the specific primer approach impracticable for large-scale surveys. Thus, the current gold-standard may fail to uncover the full diversity of protists, possibly partly undermining attempts to study protist alpha diversity and ecology in terrestrial environment (e.g. biogeography, niche delineation, etc...; Geisen et al., 2017).

To assess more efficiently protist diversity in soil, intracellular DNA can be targeted by isolating protist cells. Single-cell sorting has been successfully used to isolate protists for metabarcoding analyses in aquatic samples (Li et al., 2017; Shi et al., 2011). However, this approach is impractical for soil protists due to the high masking effect of mineral particles and organic debris (Lentendu, 2015). Enrichment of cells with density-gradient centrifugation allows to mitigate this issue for abundant eukaryotic microbes (Lentendu et al., 2013), but this approach does not work for shelled protists as their gravimetric density is too close to that of soil particles. Far from increasing the representativity of shelled protists, it would favour mainly bacterial, fungal and naked protist (e.g. flagellates, naked amoebae, ciliates) cells. In all enrichment strategies, however, the main drawback is the reduced resolution in the least abundant taxa which mostly correspond to larger primary or secondary predators. Alternatively, sieving efficiently removes coarse (>150 μm) soil particles in order to maximise the density of protists in the <150 μm fraction of the sample. This corresponds to the classical preparation of soil and mosses to isolate testate amoebae for microscopy, which is based on a filtration-sedimentation protocol (Booth et al., 2010; Hendon & Charman, 1997). While this enrichment method is successful for microscopy of testate amoebae, its impact on the whole soil protist community, as characterised by total (i.e. intracellular and extracellular) eDNA metabarcoding, remains unknown. The absence of filtration prior to soil microbial molecular analyses is even more surprising when knowing the systematic filtration and size fractionation used in marine and freshwater molecular microbial ecology (de Vargas et al., 2015; Debroas et al., 2015).

In this study, we assessed the impact of the filtration-sedimentation preparation on the recovery of the protist community using V4 18S HTS, as compared to the standard sieved bulk soil preparation. The main hypothesis was that filtration-sedimentation would increase the relative proportion of testate amoebae and other shell forming or larger micro-organisms, while reducing the relative proportion of filamentous organisms which are expected to be retained by the 150 μm mesh size filter. As metabarcoding data are compositional by nature, we also expect a small reduction in relative proportion distributed over all other targeted organisms. We, however, hypothesised that, except for testate amoebae and other shell-forming micro-organisms, the diversity and the general structure of the whole protist community would remain unchanged between filtration-sedimentation and bulk soil. The additional hypothesis is that non-target macro-organism groups (plants and animals) would be reduced in relative abundance by the filtration-sedimentation due to the exclusion of most of their tissue debris by the filtration. In order to test these hypotheses, soil samples were collected in contrasted forest and alpine grassland habitats in multiple regions of the world, and the protist communities were compared between traditional bulk soil DNA extract and DNA extracted after filtration-sedimentation.

2 | MATERIALS AND METHODS

2.1 | Sampling

Soil samples were collected at 24 sampling sites located in four regions (La Réunion, France; Hokkaido, Japan; Sierra Nevada, Spain; Alps, Switzerland) between June and September 2019 (Table S1). In each region, samples were collected in three forests and three alpine grasslands. Each soil sample was composed of at least 10 ca. 100 g sub-samples of litter and topsoil material collected so as to cover all micro-habitats within each sampling site. The sampling site area was ca. 5 × 20 m in forests and 5 × 10 m in the alpine grasslands. Soil samples were kept at 4°C for a maximum of 2 days before further processing.

2.2 | Soil processing

Soil samples were thoroughly mixed and sieved using a 5 mm and then a 2 mm mesh size. Approximately 1 g of fresh sieved soil was

placed into 2 mL tubes containing 1 mL of LifeGuard Soil preservative solution (Qiagen). These samples are referred to as *bulk soil*. Ca. 20 g of sieved fresh soil was then used to produce *filtered* samples using the following filtration-sedimentation protocol: (i) the soil was placed in a 500 mL bottle with twice the volume of distilled water and shaken vigorously for 30″; (ii) the soil slurry was poured into a 150 µm mesh size filtration tower (8 cm diameter and 11 cm tall screw cap polypropylene jar with both ends cut out and the 150 µm mesh size filter maintained by the screw cap lid ring; Figure S1), on top of which a standard tea strainer was place to retain the largest particles; (iii) soil particles retained in the tea strainer and the 150 µm filter were thoroughly washed with distilled water using a wash bottle in order to allow small particles to flow through the filters; (iv) all water and soil material passing through the filter was collected in a beaker and was left to sediment for at least 30 min; (v) 1 mL of the sediment was collected using a 1 mL pipette (cutting off 3–5 mm at the end of the tip to avoid clogging), and placed into 2 mL tubes containing 1 mL of LifeGuard Soil preservative solution. All bulk soil and filtered samples were kept refrigerated during transportation and placed into a freezer upon arrival in the laboratory until further processing.

2.3 | Soil chemical analyses

Sieved soil samples were dried for 5–7 days at 40°C. Five grams of soil was mixed in distilled water with a 1:2.5 (wt/vol) ratio to measure pH (Metrohm pH 621, Metrohm). Residual humidity was determined after heating soil samples at 105°C for 24 h. Soil organic matter content was measured by loss-on-ignition at 450°C using a muffle-furnace (Nabertherm). The bioavailable phosphorus was determined by colorimetry (Olsen, 1954). Soil organic carbon and nitrogen were measured by a CHN analyser (FLASH 2000, Thermo Fischer Scientific). Comparisons of the measured variables between the habitats and regions are provided in Figure S2.

2.4 | Molecular analyses

The preservative medium was removed by centrifugation (2500 *g* for 5 min) and 0.25 g of soil or soil-filtered extract was used for DNA extraction following standard instructions of the PowerSoil Pro DNA extraction kit (Qiiagen). The DNA concentration was quantified by Nanodrop and was used as template for PCR at a concentration of 5–10 ng/µL. The V4 region of the 18S rRNA gene was amplified using the primer pair TAReuk454FWD1 and TAReukREV3 (Stoeck et al., 2010). The PCR reactions consisted of 0.3 µM of each primer, 0.3 mM of DNTPs, 2.5 mM of $MgCl_2$, 1 X of buffer, 0.5 $U.\mu L^{-1}$ of GoTaq® hot start polymerase (Promega) and 1 µL of DNA template prepared in a final volume of 20 µL. An 8-nt barcoding sequence and a 0–4-nt heterogeneity spacer were attached to the 5′-end of each forward and reverse primers. The PCR thermocycling conditions were as follows: an initial step at 95°C for 5′, followed by 35 cycles consisting of 30″ at 94°C, 45″ at 47°C and 1′ at 72°C and a final step at 72°C for 10′. Amplification successes were controlled on agarose gel in the presence of positive and negative controls. Triplicate PCR products were pooled and the DNA concentration was quantified using a QuBit fluorometer (Thermo Fisher Scientific). Equimolar pools of up to 160 samples were prepared and sent to a sequencing facility (ID-GENE). Pools were further prepared into libraries using the Illumina TruSeq DNA PCR-Free kit and sequenced on an Illumina MiSeq with v3 chemistry and 600 cycles (2 × 300; Illumina Inc.). The samples used in this study are part of three distinct Illumina runs.

2.5 | Bioinformatic processing

Raw sequencing reads were analysed using DeltaMP v0.5, an automatic pipeline to leverage HPC computational power for metabarcoding bioinformatic analyses (Lentendu, 2022). Raw reads were demultiplexed with Cutadapt v2.10 (Martin, 2011) by first separating reads by orientation while detecting reads starting with the forward or the reverse primer in the R1 libraries and conversely in the R2 libraries, and then by matching and removing barcodes at the 5′-end allowing until two mismatches. The primers were then stripped from the 5′-ends allowing up to three mismatches. The maximum expected error values along the sequence length were estimated with VSEARCH v2.13.6 (Rognes et al., 2016). A trimming parameter optimisation was conducted for each library and each orientation in each run, in order to keep the maximum number of reads per sample (but at least 90% of demultiplexed reads in each sample, with 10% of sampled allowed below this threshold), searching for the longest stretch of nucleotides of at least 230 nt, and at least 500 nt cumulative for both libraries, with the lowest maximum expected error of at least 4 over the truncated length. Reads were then dereplicated at the sample level and error rates were determined for each combination of libraries, orientations and runs separately using the DADA2 R package V1.14.1 (Callahan et al., 2016). Amplicon sequence variants (ASV) were called using the DADA2 command dada, with the pool option set to true. The ASVs of run 1 and run 1 and 2 were used as prior knowledge to call ASVs with the dada command for run 2 and run 3 respectively. This ensures that abundant ASV in samples of the first runs was correctly detected in the following runs, even if they have low abundance. Pairs of reads were merged using the DADA2 command mergePairs with minimum overlap of 10 nt and a maximum of two mismatches. The paired reads originally with the reverse primer at the 5′-end of R1 library were reverse-complemented using the SeqinR R package command c2s. Chimeras were removed using the DADA2 command removeBimeraDenovo, with the pool option set to true. ASVs were assigned to a consensus taxonomy among best matches of the PR2 database (Charif & Lobry, 2007; Guillou et al., 2013) using the VSEARCH global pairwise alignment algorithm.

2.6 | Statistical processing

2.6.1 | Tag-jump control

In order to control for tag-jumps (Schnell et al., 2015), eight positive controls of DNA extracted from two cultivated algae species (*Desmodesmus maximus* and *Pediastrum duplex* with contamination of *Filamoeba nolandi*) were included in each sequencing run. These species originated from freshwater and are unlikely to be found in soil samples, so that any occurrence of an ASV assigned to these three species in soil sample libraries was considered as tag jump. For each of these positive control ASVs, a multiple linear model was drawn to model the maximum number of reads in a given soil sample (i.e. originating from tag-jump) by the total number of reads of this ASV over all samples and its maximum number of reads in a single sample. The 99.9% upper confidence interval of predicted values with this model was used as threshold to identify read occurrences of any ASV likely originating from tag jump (Figure S3). For each ASV, read counts below the predicted threshold were set to 0 and these removed reads were re-assigned to the other samples with valid occurrences following a probability distribution based on their read counts. The efficiency of the method was controlled by the removal of tag-jump ASVs in the positive and negative control samples.

2.6.2 | Statistical analysis

In order to test for enrichment in testate amoebae and other taxa in filtered samples as compared to bulk soil samples, differential abundance tests were conducted using ANCOM-BC v 1.2.2 (Lin & Peddada, 2020) at the ASV level as well as at different taxonomic levels (e.g. species, genus, family, higher clades). This approach takes into account the compositional bias of the data (Gloor et al., 2017). Significant log-fold changes between bulk soil and filtered samples were assessed with two-sided Z-tests in each region and habitat. Multiple test *p*-values were corrected with the 'BH' method (Benjamini & Yekutieli, 2001). As a matter of comparison, significant differences in reads relative abundance were computed with a simple Mann–Whitney test after square-root transformation and re-scaled to real read counts, summing to the median of per samples read counts in all samples, as also recommended for a simpler alternative to tackle the compositional bias issue (Gloor et al., 2017).

Alpha diversity indices in the form of Hill's numbers with exponent 0, 1 and 2 (i.e. richness, exponential of Shannon index and inverse Simpson index) were computed with the vegan R package v2.5-7 (Oksanen et al., 2021). The richness index showed a linear correlation with the square root transformed read counts per sample (ANOVA: $R^2 = .24$; p-value $= 2e^{-4}$; Figure S4). In order to remove this sequencing depth bias, only the residuals of the linear model were analysed, which are the residual variances in richness not explained by the sequencing depth bias (Hiiesalu et al., 2014). Richness residuals and Hill numbers 1 and 2 were centred-scaled on a −1 to 1 range to allow for comparability between indices. Mann–Whitney tests were used to compare unfiltered and filtered sample's alpha diversity indices overall as well as in each habitat and country separately. Beta diversity analyses were based on Bray–Curtis dissimilarity indices of the Hellinger-transformed protist ASV matrix. A non-metric multidimensional scaling was used to visualise the change in community structure between filtered and bulk soil samples, while significant changes were tested with PERMANOVA. The protist ASV matrix was further split between filtered and bulk soil samples and constrained redundancy analyses were conducted separately on these two datasets in order to compare the best model explaining changes in community structures.

3 | RESULTS

3.1 | Enrichment in shelled protists

To understand the effect of filtration-sedimentation on the observed soil eukaryotic community composition, enrichment at different taxonomic levels was tested. Among the 3743 ASVs occurring in at least three samples, only 11 ASVs were found to have significant differential abundance between bulk soil and filtered treatments over all samples (Figure 1 top). Of these, 10 were enriched in filtered samples, including four Euglyphida testate amoebae, and one was enriched in bulk soil samples. Only one ASV, identified as the yeast predator ciliate *Pseudoplatyophrya nana*, had significant depletion in filtered samples.

When ASVs were aggregated to genus level (Figure 1 bottom), 26 genera had significant differential abundance between bulk soil and filtered samples. Of these, 15 were enriched in filtered samples, notably, four testate amoebae (three Euglyphida: *Assulina*, *Euglypha* and *Trinema* and one Arcellinida: *Cryptodifflugia*), one Chrysophyceae and two Rotifera genera. To the contrary, three fungal (*Archaeorhizomyces*, *Glomus*, *Kurtzmanomyces*) and three Variosea (Amoebozoa) genera were depleted in the filtered samples. Multiple other protist genera displayed significant enrichment in filtered samples among the Cercozoa, Ciliophora, Chlorophyta, Dinoflagellata and lobose Amoebozoa, but also significant depletion in filtered samples among Cercozoa, Ciliophora, Cryptophyta and Nematoda.

For most ASVs and genera, there were no significant log fold changes between filtered and bulk soil samples. However, strong tendencies appeared for multiple clades: testate amoebae, Chrysophyceae, Rotifera and Tardigrades showed systematic higher relative abundances in filtered samples. These general tendencies were also observed in the analyses at species and family levels (Figure S5). The significant enrichment of the olive tree ASV, which was solely found in filtered samples of Sierra Nevada, might have been caused by the high abundance of olive tree pollen (ca. 20 μm), which would indeed be expected to be enriched by filtering. ASVs and genera from all other protist clades did not differ between filtered and bulk soil samples. At the clade level, filtration significantly enriched three shelled protist clades (Euglyphida, Arcellinida, Chrysophyceae) and Rotifera (Figure 2). Filtration also significantly reduced the relative abundance

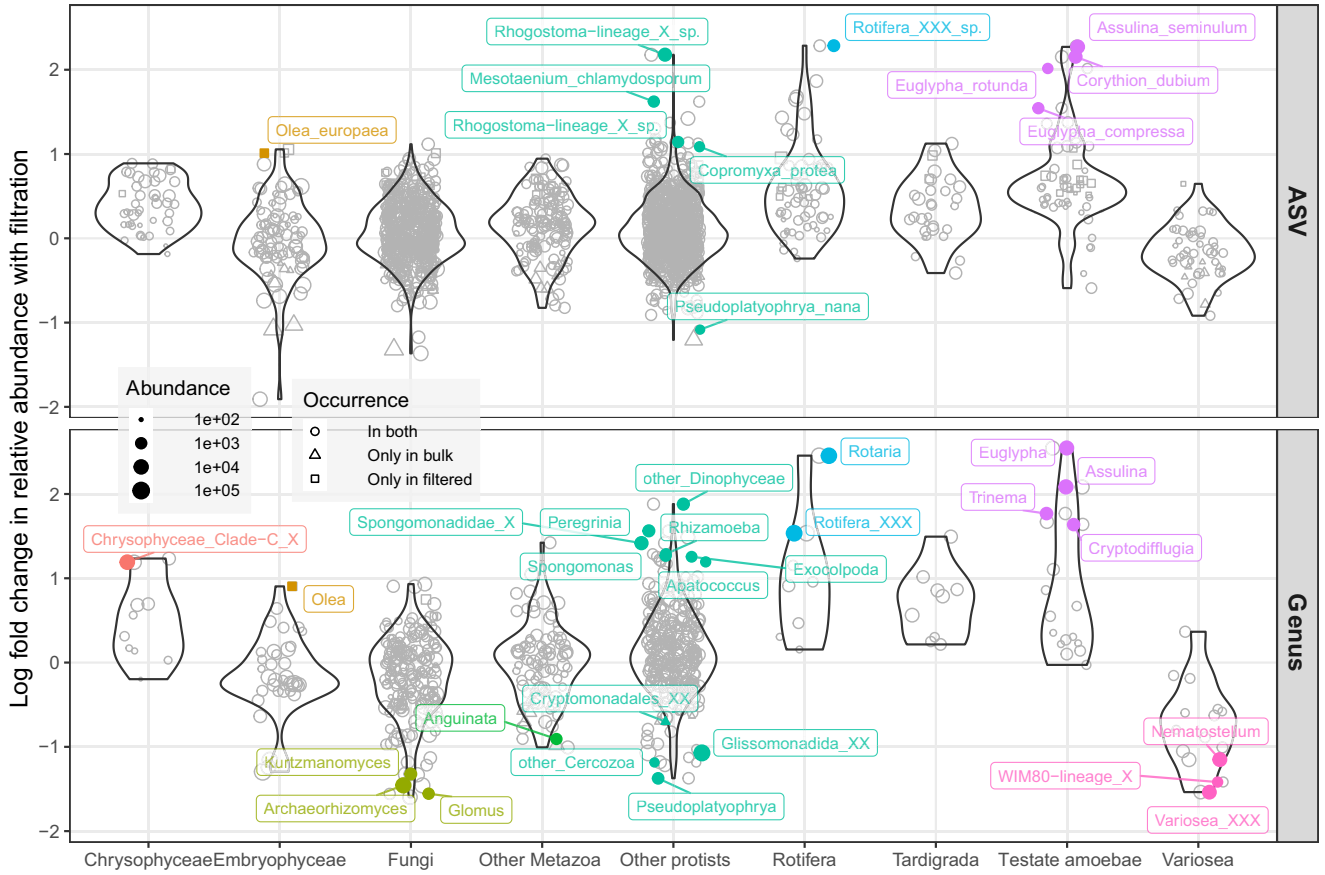

FIGURE 1 Differential abundance enrichment of ASVs and genera due to filtration. Log fold changes were computed with ANCOM-BC. ASV and genera are grouped by main clades or clades with systematic enrichment or depletion. Coloured and labelled points are for ASV and genera with statistical significant enrichment or depletion (positive or negative log-fold changes respectively; $p < .05$). Point size (i.e. 'abundance' legend) is proportional to the total read counts in the most abundant fraction (i.e. in filtered samples for positive log fold change and conversely). Only taxa with more than 100 reads in the most abundant fraction are displayed. Testate amoebae groups Arcellinida, Euglyphida, Pseudodifflugiidae and Amphitraemidae.

of two naked protist clades, Variosea and Sarcomonadea, the land plants (Embryophyceae) and fungi. The same trends were observed when using normalised read counts over all habitats and regions (i.e. without taking into account the block design), with significantly more Arcellinida, Euglyphida, Chrysophyceae and Rotifera reads and significantly fewer Variosea reads in the filtered samples (Figure S6). Relative abundance enrichments were systematic and significant in most regions and habitats for shelled protists and Rotifera when tested separately, as well as depletions of Variosea (Figure S7). Shifts in relative abundances showed variable directions between regions and habitat for Sarcomonadea, land plants and fungi. Finally, the coefficients of variation in relative abundance, as computed from the replicates in each combination of region and habitat, were significantly lower in filtered than in bulk samples for Ciliophora (Figure S8).

3.2 | Filtration effect on alpha diversity

Overall, there were more protist ASVs in bulk (5568) than in filtered samples (5143). Significant differences in protist alpha diversity between bulk soil and filtered samples were only detected in the Japan forest and alpine samples and La Réunion alpine samples, with a higher richness in the filtered samples (Mann–Whitney test, p-value $< .05$; Figure 3; Figure S9). Analyses conducted for each region and habitat separately showed strong and significant increases in uncorrected ASV richness with filtration for Arcellinida, Chrysophyceae and Euglyphida in alpine samples of Japan and alpine and forest samples of Sierra Nevada, with up to 10 times increase in ASV richness (Mann–Whitney test, p-value $< .05$; Figure S7). Variosea showed significant reduction with filtration of ASV richness only in alpine soils of Switzerland and forest soil of La Réunion, with a maximum reduction by a factor of 3. Sarcomonadea did not show any significant changes in ASV richness despite significant reduction in relative abundances (Figure 2; Figure S7).

Comparing protist alpha diversity between regions or habitats provided almost the same outcome when using bulk soil or filtered samples only. In bulk soil samples, the richness was significantly lower in Japan and La Réunion alpine soils in Swiss alpine soils (Figure 3). No significant differences in alpha diversity were observed between regions for filtered samples, nor between alpine

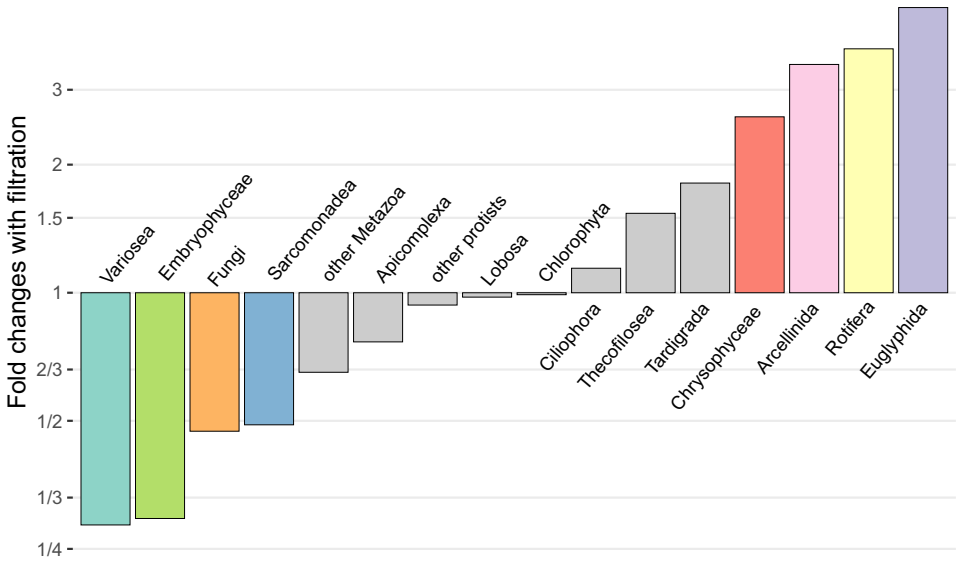

FIGURE 2 Differential abundance enrichment of whole protists and other micro-eukaryotes clades due to filtration in soil samples from Hokkaido, Japan, La Réunion, the Spanish Sierra Nevada and Swiss Alps. Fold changes were computed with ANCOM-BC. Coloured bars are for clades with statistically significant enrichment or depletion (fold changes above or below 1 respectively; $p < .05$).

and forest samples within a region for the same type of samples. At the phylum level, few significant differences were observed between habitats: higher richness of Apicomplexa in forest than in alpine filtered samples of Japan, of Choanoflagellida and Conosa in forest than in alpine filtered samples of Sierra Nevada, and of Chlorophyta in alpine than forest bulk soil samples of Japan (Figure S10).

The increases in relative abundances were well matched by a significant increase in uncorrected ASV richness in Arcellinida, Euglyphida, Chrysophyceae, Rotifera and Tardigrada (Mann–Whitney test, p-value $< .05$; Figure 3). Likewise, Variosea displayed lower uncorrected ASV richness in filtered samples. Interestingly, the coefficient of variance in uncorrected ASV richness was lower in filtered samples for Ciliophora, Embryophyceae, Euglyphida and Thecofilosea (Figure S5).

3.3 | Filtration effect on beta diversity

Protist community compositions varied primarily between regions and habitats, while the filtration effect was more marginal (Figure 4a). Permutational analyses of variances confirmed the NMDS patterns, with regions and habitats jointly explaining 41% of the variance, while filtration explained a lower (2%) but still significant proportion of soil protist community composition differences (Table S2). In the constrained RDA analyses conducted on the two datasets separately using only quantitative factors as explaining variables, three variables were significant: elevation and pH in both models and bioavailable phosphorus for the bulk soil samples only (Figure 5, Table S3). A similar dominant significant predictive power of regions and habitats was observed for the other clades (animal, fungi, plants) and the total eukaryotic community (Table S2). The average dissimilarity in community compositions of protists was significantly reduced in the filtered samples (Mann–Whitney test, p-value $< .05$; Figure 4b). The best model selection of RDA for individual protist clades was similar for most clades when analysing bulk soil and filtered samples separately (Table S4). When analysing all testate amoebae together (Arcellinida, Euglyphida, Pseudodifflugiidae and Amphitraemidae), the best models were different, with pH as sole explaining factor for bulk soil samples, and C/N ratio, elevation and northness of the slope orientation as explaining factor for filtered samples.

4 | DISCUSSION

Obtaining an unbiased coverage of all protist taxa in studies of terrestrial communities has long been a central methodological question (Fiore-Donno et al., 2018; Jacquiod et al., 2016; Lentendu et al., 2014). With the development of eDNA and metabarcoding, the debate has focused on the best primer pair covering the majority of the protist clades, with, so far, no unique and universal solution (Vaulot et al., 2022). One neglected leverage to reach this goal is the preparation of the soil samples before DNA extraction. The only standard approach is currently to sieve soil at 2 mm in order to remove coarse plant debris (International Organization for Standardization, 2020). Here, we show that an additional filtration-sedimentation significantly improves the recovery of shelled protist clades without modifying much the overall diversity estimates of the full protist community. This method has the additional advantage of significantly reducing the relative abundance of land plants and fungi ASVs in filtered samples, thus increasing the coverage of the full protist diversity. Our results show that studying rotifers and, to a lesser extent, tardigrades with eDNA will also profit from this filtration-sedimentation method.

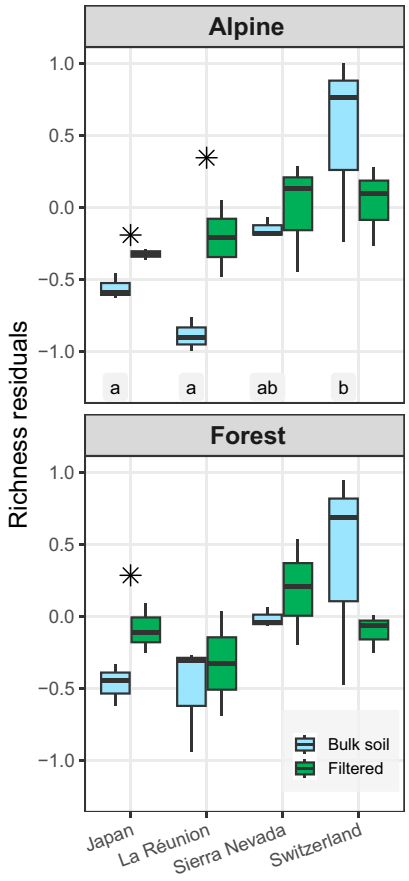

FIGURE 3 Protist richness variations in response to filtration. Sequencing depth bias is removed by using only the residual variance of the linear models between the square root transformed read count per sample and the richness (Figure S3). A star denotes significant differences in alpha diversity between sample types as computed for each region, habitat and diversity index separately (Mann–Whitney test, $p < .05$). Different letters below boxplots stand for significant differences in alpha diversity between regions as computed for each habitat and sample type separately (Tukey Honest Significant Difference, $p < .05$).

4.1 | Enrichment in shelled protists

The observed enrichment of the two main testate amoebae clades (Arcellinida and Euglyphida) and Chrysophyceae can be directly linked to their size and the presence of a shell, at least in one of their life stages. Most soil testate amoebae have a shell length comprised between 20 and 150 µm (Meisterfeld, 2009). Furthermore, most are elongated in shape and thus much narrower than 150 µm, allowing them to easily pass the 150 µm mesh filter and their shell will allow them to sediment quickly. The enriched Clade-C family of Chrysophyceae is a part of the Ochromonadales, which includes many undescribed *Spumella*-like environmental taxa as well as the well-described *Spumella* species, and has an average size ranging from 2 to 12 µm (Grossmann et al., 2016). There were 11 *Spumella* ASVs in the filtered samples, but only five in the bulk samples, but this difference was not-significant. However, as Ochromonadales species are non-scaled (Kristiansen & Skaloud, 2017), their enrichment may be due to the sedimentation of their siliceous stomatocysts. This will need further microscopic and molecular analyses to be confirmed. Land plants and fungi were likely depleted due to filtration, as fungal hyphae, plant debris, fine roots and their fungal endophytes may be retained by the filter. The significant reduction of the whole Glomeromycetes class (i.e. obligatory root endosymbionts) supports this interpretation. Variosea was the most impacted protist clade by filtration. Within this group, the Schizoplasmodiida was most abundant in bulk soils and was depleted by 50% in filtered samples. Schizoplasmodiids are mainly found on fresh and decaying surface litter and on tree bark, so they may have been reduced by filtration due to the removal of the plant substrate (Spiegel et al., 2017). Among Sarcomonadea, only one (Glissomonadida_X) out of 28 genera was depleted in filtered samples. The most abundant ASV in this genus has an exact match (Megablast, *E*-value = 0) with a newly described species, *Saccharomycomorpha psychra* (accession MH791032), likely parasitising mosses and lichens (Feng et al., 2021), so that thalli retention by the filter may also explain their reduction.

4.2 | Equivalent diversity patterns with filtration

The reduced overall diversity in filtered samples did not imply much difference in diversity patterns. In forest soils from Japan, protists in filtered samples were significantly more diverse than their bulk soil counterparts. This discrepancy with bulk soils appeared to be mainly due to the significantly lower diversity of Chlorophyta in bulk soil forest samples compared to filtered forest samples. As this clade was not found to be impacted by the shift in relative abundances over all samples, there might be a more general process of enrichment in protist cells and intracellular DNA with filtration. However, filtration-sedimentation reduced the coefficient of variances of relative abundance and alpha diversity, which might contribute to strengthening the significance of statistical tests. This is in line with the higher number of significant differences in alpha diversity between habitats detected at the phylum level in the filtered samples compared to bulk samples, with a general tendency of higher local diversity in the forest habitat.

The main drivers explaining differences in community compositions were identical between bulk soil and filtered samples. Filtration alone had only a minor, though significant, effect on the observed community composition, with a slight significant reduction of dissimilarity between all sample pairs compared to bulk soil sample pairs. These results are in line with the reduced alpha diversity, but do not impair the ecological interpretation, which shows in both cases a strong geographical signal, likely driven by pH (acidic soils on the two volcanic islands, acidic to neutral soils on the mainland), followed by a habitat effect between forest and alpine samples. This corresponds to the main expected drivers of terrestrial protist community at the global scale (Bates et al., 2013) and validates our selection of sites to test the filtration-sedimentation method to analyse terrestrial protist diversity and responses to environmental drivers.

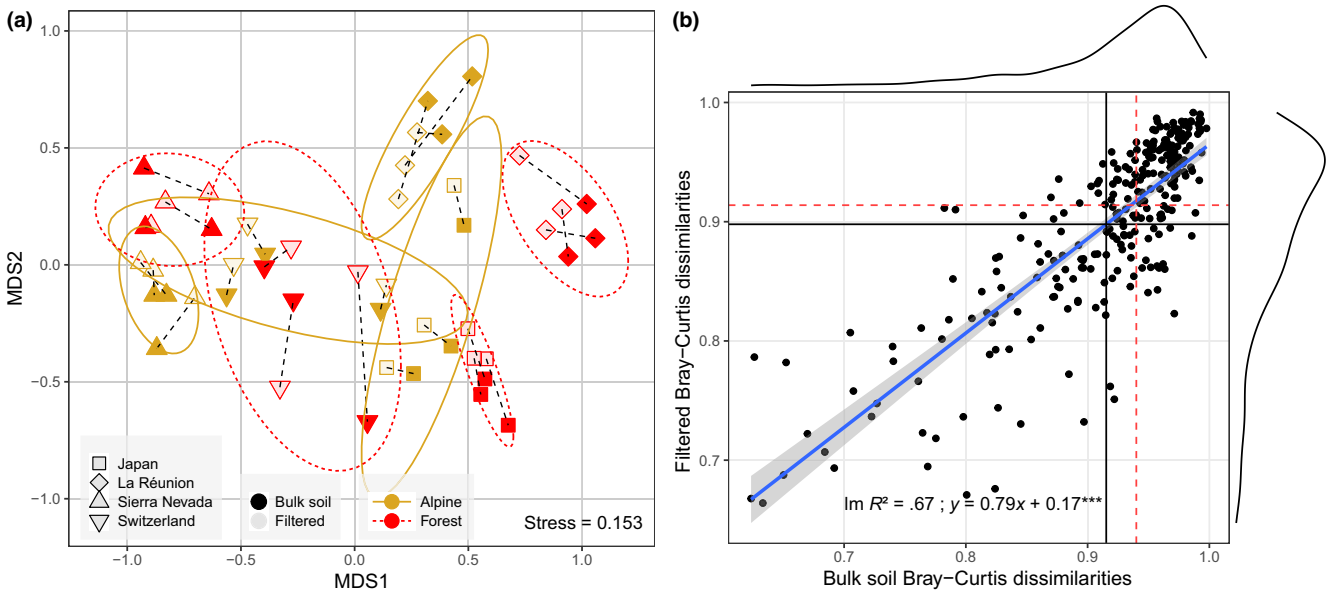

FIGURE 4 Effect of sample type on protist community composition. Dotted lines link bulk soil and filtered communities of the same sample in the Non-Metric Multidimensional Scaling plot (NMDS; a). Ellipses are 90% confidence intervals around samples from the same region and habitat. Yellow plain ellipses are for alpine habitat, red dotted ellipses are for forest habitat. The scatter plot presents the relationship between bulk soil and filtered Bray–Curtis dissimilarity values for the same pairs of samples (b). Density distributions of each dataset are presented in the margins. Filtered samples have a significant lower dissimilarity on average (paired Mann–Whitney test, p-value < .05). Blue line and surrounding grey area are the linear regression and its 95% confidence interval. Black plain and red dotted lines are mean and median values respectively.

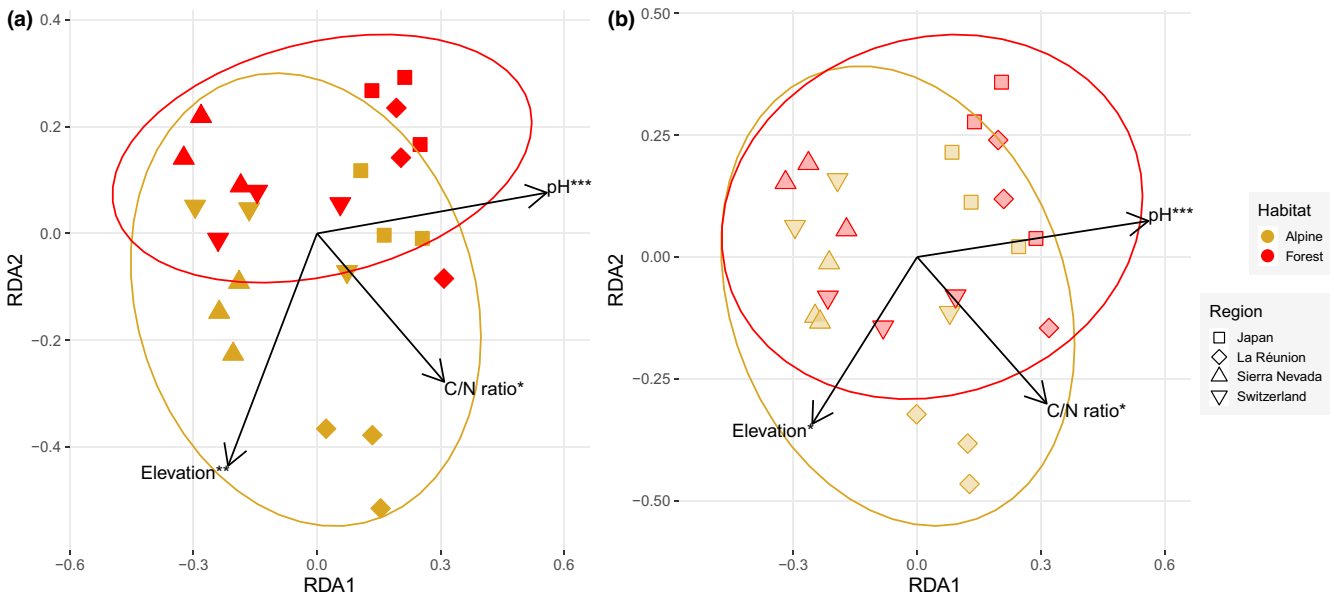

FIGURE 5 Effect of sample type on protist RDA. Constrained redundancy analyses were used to find the best model of soil parameters explaining the change in protist community compositions for bulk soil (a) or filtered samples (b), using all topographic and soil abiotic parameters to select best models based on AIC. Only the significant parameters are drawn.

There were strong variations in individual clades alpha diversity and relative abundance responses to filtration depending on region and habitat. While an overall effect of only 2% on the protist community composition is due to the filtration method, analyses conducted on more homogeneous soils (e.g. from same habitat in the same region) would have allowed to detect more drastic changes in community composition. Also, when beta diversity is analysed at the clade level, it was already possible to detect clear differences in the main drivers of the testate amoebae composition between bulk soil and filtered samples. This was in line with the drastic increase in

testate amoebae ASV richness which was higher by up to one order of magnitude in some regions and habitats. This suggests that the true diversity patterns of additional protist clades can be revealed by a simple soil preparation and does not necessarily require specific primers to target them (Fiore-Donno et al., 2018; Lentendu et al., 2014). The absence of any significant loss in sensitivity in the protist diversity patterns when the filtration-sedimentation method is used in addition to the standard bulk soil 2 mm sieving preparation advocates for the sole analysis of filtered samples.

4.3 | Methodological consideration

Compared to existing methods relying on extracellular DNA in order to process higher quantities of soil for DNA extraction (Taberlet et al., 2012; Zinger et al., 2016), our method has the advantage to remove more filamentous organisms and fine roots (e.g. fungi and land plants), while catching DNA from cells, in particular of testate amoebae and Chrysophyceae. The advantage of extracellular DNA extraction is to use more soil, which is also the case here, though the concentration factor remains unknown. Indeed, only a fraction of the 10 g of soil makes it through the strainer and the 150 µm filter, and the sedimentation allows to concentrate the heavy particles and cells. However, only 1 mL of the pellet is used which may still not be enough to capture the rarest taxa. For these, a method combining filtration and using a larger volume of filtered sample (thus starting with a much larger sample) could be useful. However, we feel that for most studies our approach will be a good compromise.

Only three species were used to control for tag-jump, which limits the number of ASVs ($n = 14$) from which the multiple linear models used to correct abundances were built. Using a mock community of multiple strictly aquatic species could improve such model-based correction by predicting more accurately the thresholds below which the read count of an ASV in a sample can only be due to tag-jump. Nevertheless, the method used here is clearly much preferable than using a fix threshold which would remove all truly rare ASVs (i.e. those with low read counts). Indeed, rare ASV have almost no chance to produce tag-jump as the amount of tag-jumping of an ASV was strictly correlated to the total amount of read of this ASV over all samples. A recent methodological advance allows for drastic reduction of tag-jumping (Carøe & Bohmann, 2020) and should be adopted in future research to avoid such additional data curation and validation steps.

5 | CONCLUSION

The filtration-sedimentation method presented in this study to prepare soil samples before eDNA extraction improves the detection of terrestrial protist communities by allowing better coverage of low abundant shelled unicellular eukaryotes. This method is, however, not suited for studying protist exclusively associated with leaf-litter and root endophytes due to the removal of most plant material. These taxa may, however, be enriched in the >150 µm fraction, but this should be further determined. The reduced variance in diversity estimates between replicates with the filtration method will, however, allow better statistical support when testing ecological hypotheses. Owing to the fact that testate amoebae and Chryophyceae play key functions in soil microbial food webs and nutrient cycles (Beisser et al., 2017; Wilkinson & Mitchell, 2010), we now recommend to use the filtration-sedimentation method as a standard preparation procedure for eDNA metabarcoding of whole protist community in soils.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

Raw sequences data and associated metadata are deposited on SRA (Bioproject PRJEB56206, Table S1). Details of accessions for the samples used in this study are listed in Table S1. All codes used to analyse the data are provided in the supplements (Data S1). The analysed sequences data (ASV sequences, community matrix and taxonomic assignments), the soil abiotic data and the samples metadata are provided as a R binary file in the supplements (Data S2).

BENEFIT-SHARING STATEMENT

A research collaboration was developed with researchers from the countries providing the genetic resources, all of them are included as co-authors. Benefits from this research accrue from the sharing of our data and results on public databases as described above.

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SUPPORTING INFORMATION

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