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ORIGINAL ARTICLE

Response of protists to nitrogen addition, arbuscular mycorrhizal fungi manipulation, and mesofauna reduction in a tropical montane rainforest in southern Ecuador

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Abstract

The tropical Andes are a species-rich and nitrogen-limited system, susceptible to increased nitrogen (N) inputs from the atmosphere. However, our understanding of the impacts of increased N input on belowground systems, in particular on protists and their role in nutrient cycling, remains limited. We explored how increased N affects protists in tropical montane rainforests in Ecuador using high-throughput sequencing (HTS) of environmental DNA from two litter layers. In addition, we manipulated the amount of arbuscular mycorrhizal fungi (AMF) and mesofauna, both playing a significant role in N cycling and interacting in complex ways with protist communities. We found that N strongly affected protist community composition in both layers, while mesofauna reduction had a stronger effect on the lower layer. Changes in concentration of the AMF marker lipid had little effect on protists. In both layers, the addition of N increased phagotrophs and animal parasites and decreased plant parasites, while mixotrophs decreased in the upper layer but increased in the lower layer. In the upper layer with higher AMF concentration, mixotrophs decreased, while in the lower layer, photoautotrophs increased and plant parasites decreased. With reduced mesofauna, phagotrophs increased and animal parasites decreased in both layers, while plant parasites increased only in the upper layer. The findings indicate that to understand the intricate response of protist communities to environmental changes, it is critical to thoroughly analyze these communities across litter and soil layers, and to include HTS.

KEYWORDS
environmental changes, environmental DNA, high-throughput sequencing, nutrient cycling, protist community, trophic groups

INTRODUCTION

THE tropical Andes are a biodiversity "hotspot" with a high number of endemic animal and microbial taxa (Bax & Francesconi, 2019; Myers et al., 2000) involved in complex interactions with plants (de la Cruz-Amo et al., 2020; Hagedorn et al., 2019). These species-rich but nutrient-poor systems are likely to be sensitive to changes in nutrient inputs, such as nitrogen (N) and phosphorus (P), especially at high elevation (Camenzind et al., 2018; Dalling et al., 2016; Graefe et al., 2010). Changes in nutrient input occur even in pristine tropical forests; since

the 1960s, the global atmospheric amount of N oxides has increased mainly due to increased combustion of fossil fuels (Galloway et al., 2008). Atmospheric N input into distant nonagricultural ecosystems has increased and will increase further in the future (Mahowald et al., 2005). Even minor input of nutrients affects not only plants, but also alters soil microbial communities and threatens biodiversity in particular in tropical biodiversity hotspots (Homeier et al., 2012; Krashevska et al., 2014). Cycling of nutrients largely is mediated via decomposition processes, performed by interactions of a diverse community of soil- and litter-dwelling organisms (Bardgett, 2005; Scheu et al., 2005).

One of the most important players in the complex decomposer system below the ground are protists, single-celled organisms comprising the majority of all eukaryotic life forms (Adl et al., 2019). Protists may reach high densities and diversity in soils (Foissner, 1999; Geisen & Bonkowski, 2017), and include virtually all trophic levels in ecosystems (Geisen et al., 2018). Therefore, they are ideal model organisms for studying interactions in the belowground system and factors impacting these interactions. For example, phagotrophs are grazers and predators of microorganisms and smaller soil animals (Adam et al., 2017; Clarholm, 2005; Geisen, Rosengarten, et al., 2015; Mitchell, 2015). Photoautotrophs are primary producers and contribute to the organic input into soils (Bamforth, 2008; Metting, 1981; Seppey et al., 2017). Mixotrophs use photosynthesis for nutrition, but simultaneously employ a phagotrophic lifestyle or switch between both modes (Jassey et al., 2015; Unrein et al., 2014). Furthermore, parasites of plants and animals constitute a large faction in the belowground system, rivaling the diversity of arthropods (Foissner, 1987; Geisen et al., 2018; Kinne & Lauckner, 1980; Mahé et al., 2017; Strona, 2015). While not the primary focus of this study, it is worth noting that a substantial portion, perhaps the majority, of autotrophic protists exhibit varying degrees of osmotrophic capabilities (Geisen et al., 2018). While it is known that N affects protist communities (Pan et al., 2020; Wang et al., 2014), it is not clear which trophic groups are most vulnerable to increased atmospheric N input. Although the importance of protists in the belowground system has been stressed (Bonkowski, 2004; Bonkowski & Clarholm, 2012; Geisen & Quist, 2021), experiments investigating interactions between protists and other players in soil, such as arbuscular mycorrhizal fungi (AMF; Henkes et al., 2018; Koller et al., 2013) and mesofauna (Erktan et al., 2020), are limited. This is due, at least in part, to the fact that extraction and cultivation of the majority of protist taxa is difficult or impossible (Geisen, Tveit, et al., 2015). However, high-throughput sequencing (HTS) of environmental DNA offers the possibility to analyze whole protist communities (Heger et al., 2018; Mahé et al., 2017; Oliverio et al., 2020; Schulz et al., 2019). Most of the existing HTS data is based on samples from the top layers of the mineral soil, though protists also colonize the litter layer, which is of eminent importance for element cycling. In the tropical montane rainforest of southern Ecuador, our study region, testate amoebae are the only group of protists, which have been investigated so far (Krashevska et al., 2007, 2010, 2014). These mostly microbial grazers showed high abundance and diversity at 2000 m a.s.l., with both being higher in the upper litter than the deeper fermentation layer (Krashevska et al., 2007). Testate amoebae benefit from the addition of high and moderate amounts of N, but likely compete with saprotrophic fungi and AMF for nutrients (Krashevska et al., 2010, 2014). However, it is unknown how other groups of protists respond to increased input of N, and variations in AMF or mesofauna abundance, both potential competitors for resources of protists (Camenzind & Rillig, 2013; Treseder & Cross, 2006).

In this study, we used HTS of environmental DNA targeting the 18S rRNA gene to investigate protist community structure in an experimental setup. We manipulated N input and AMF and mesofauna abundance to investigate their impact on the diversity and community composition of protists, as well as on protist functioning in tropical montane rainforests. We hypothesized that diversity, community composition, and relative abundance of trophic groups of protists will be differentially affected (1) by the addition of N, (2) the reduction of AMF, and (3) the reduction of mesofauna, with the effect of N input and AMF reduction being strongest on autotrophic protists essentially relying on mineral N and plant C inputs, whereas the effect of reduced mesofauna is expected to be strongest in phagotrophic protists relying on organic matter resources and microorganisms as food as most detritivore soil mesofauna species. Furthermore, we expected mesofauna to act as hosts for parasitic protists, thereby favoring their diversity. Generally, we assumed these effects to be more pronounced on the functional than on the taxonomic level.

MATERIALS AND METHODS

Study site

The study site is located near the Estación Científica San Francisco at 2000 m a.s.l. (3°59′S, 79°05′W) in the northern part of the Podocarpus National Park on the eastern slope of the Andes in southern Ecuador. The forest is classified as lower montane rainforest (Homeier et al., 2008) and part of the private reserve Reserva Biologica San Francisco. It is in close to pristine condition and well studied in regard to vegetation and above- and belowground arthropods, with extraordinary richness in tree species as well as other plant and animal species (Beck et al., 2008; Homeier et al., 2010). The climate is warm (mean annual temperature of ~15°C) and humid (mean annual precipitation

~2200 mm). The soil is Cambisol with a thick organic layer, but nutrient poor (Wilcke et al., 2002). The organic layer has a thickness of 11 cm, with clear separation of litter (L) and fragmentation/humus (F/H) layers (Krashevska et al., 2007).

Experimental setup

The experiment was set up as described in Sánchez-Galindo et al. (2019). Briefly, microcosms consisted of plastic tubes of 15 cm diameter and 20 cm length. Two rectangular holes (10 × 15 cm) were cut opposite to each other into the tube and covered with 45 µm nylon mesh. Furthermore, the tubes were equipped with two sheets of 45 µm nylon mesh at 2 and 7 cm from the bottom to allow drainage of water, but prevent ingrowth of roots and mycorrhizal hyphae. The microcosms were closed with a lid of 4 mm nylon mesh to allow colonization by mesofauna (Figure 1A).

The effect of three factors—N addition, rotation (reduction of AMF; two levels), and defaunation (reduction of mesofauna; two levels)—on protist communities in the L and F/H layers were investigated in the framework of the nutrient manipulation experiment (NUMEX; Homeier et al., 2012). Briefly, NUMEX is a fertilization experiment set up in 2007 in a complete randomized block design with four blocks. For our experiment, four soil samples of approximately 3000 cm^3 were taken randomly with a stainless steel corer of 14.5 cm inner diameter from two subplots (2 × 2 m) of the N addition and control plots of the four blocks of the NUMEX experiment at 2000 m a.s.l. The N addition plots received 50 kg N ha^{-1} $year^{-1}$ applied as urea twice per year. The samples comprising the L and F/H layers were placed into the microcosm tubes fitting on top of the upper 45 µm mesh sheet. For defaunation, half of the cores were frozen at −20°C for 1 week. Microcosms were placed into the holes from which the samples were excavated. To manipulate AMF abundance, half of the microcosms were rotated 45° every 2 days preventing ingrowth of AMF hyphae (Figure 1B). Microcosms were installed in June 2015 and harvested after 5 months in November 2015. At harvest, the soil cores were taken out of the tubes and vertically split into half and then separated into L and F/H layers, resulting in 128 samples in total (2× fertilization, 2× rotation, 2× defaunation, 4× blocks, 2× layers, 2× subsamples). Half of each sample was frozen at −20°C on the day of sampling and transported to Germany for analysis of fatty acids, soil properties, and extraction of environmental DNA. The other half of each sample was used for extraction and determination of soil mesofauna (Sánchez-Galindo et al., 2019).

DNA extraction and amplification

DNA was extracted from 200 mg of lyophilized sample material using the MoBio PowerSoil isolation kit (Dianova) following the manufacturer's instructions. The variable region V4 of the 18S rRNA gene was amplified using the general eukaryotic primers TA-Reuk454FWD1 (5′-CCAGCASCYGCGGTAATTCC-3′) and TA-Reuk REV3 (5′-ACTTTCGTTCTTGATYRA-3′; Stoeck et al., 2010) paired with the MiSeq-adapters forward overhang (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′) and reverse overhang (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′). For amplification, the Phusion High-Fidelity DNA Polymerase kit (Thermo Fisher Scientific) was used. The PCR reaction mixture contained 10 µL of fivefold Phusion GC buffer, 1 µL of the forward and reverse primers (1 µM), 1 µL $MgCl_2$ (50 mM), 1 µL dNTPs (50 mM), 2.5 µL DMSO, 0.5 µL Phusion polymerase (1 U), and 1 µL template DNA. The following thermocycling scheme was used for amplification: initial denaturation at 98°C for 1 min, 35 cycles of denaturation at 98°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 1 min, followed by a final extension period at 72°C for 5 min. Amplicon length was ~400 bp. All amplicon PCRs were performed three times and pooled in equal amounts for sequencing. PCR products were purified using the QIAquick Purification Kit (Qiagen) following the manufacturer's protocol. Göttingen Genomic Laboratory determined the sequences of the 18S rRNA amplicons using the dual index paired-end approach (2× 300 bp) with v3 chemistry for the Illumina MiSeq platform.

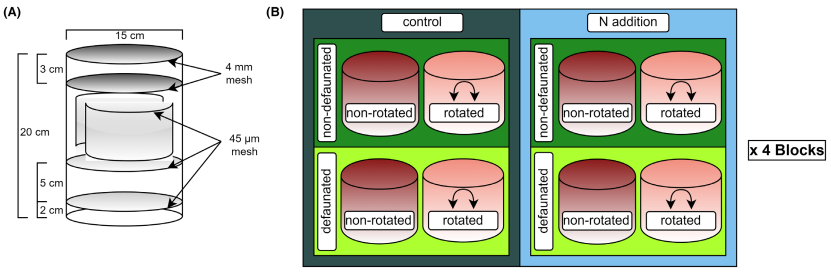

FIGURE 1 Scheme of the microcosms (A) and study design (B).

Bioinformatic analysis

Paired-end sequencing data from the Illumina MiSeq were quality filtered with fastp (version 0.19.4; Chen et al., 2018) using default settings with the addition of an increased per base Phred score of 20, base pair corrections by overlap (−c), as well as 5′- and 3′-end read trimming with a sliding window of 4, a mean quality of 20 and minimum sequence size of 50 bp. After quality control, the paired-end reads were merged using PEAR (version 0.9.11; Zhang et al., 2014) and primers clipped using cutadapt (version 1.16; Martin, 2011) with default settings. Sequences were then processed using VSEARCH (version 2.8.4; Rognes et al., 2016). This included sorting and size filtering of the paired reads to ≥250 bp (–sortbylength –minseqlength 250), dereplication (–derep_fulllength). Dereplicated amplicon sequence variants (ASVs) were denoised with UNOISE3 using default settings (–cluster_unoise –minsize 8) and chimeras were removed (−uchime3_denovo). An additional reference-based chimera removal was performed (−uchime_ref) against the SILVA SSU NR database (version 132). Quality-filtered and merged reads were mapped to ASVs (−usearch_global–id 0.97). The taxonomy was assigned using BLAST 2.7.1+ (Altschul et al., 1990) against the PR2 database (version 4.10) resulting in a total of 10,368 ASVs. The taxonomy was checked manually and curated following Ruggiero et al. (2015), Cavalier-Smith (2017), and Adam et al. (2017).

Data analysis

All statistical analysis, data handling, and graphical processing were done in R 3.6.3 (R Core Team, 2020) using the packages ampvis2 (Andersen et al., 2018), car (Fox & Weisberg, 2019), compositions (van den Boogaart & Tolosana-Delgado, 2008), ggpubr (Kassambara, 2020), gridExtra (Auguie, 2017), kableExtra (Zhu, 2019), MOTE (Buchanan et al., 2019), nlme (Pinheiro et al., 2017), tidyverse (Wickham & Henry, 2017), vegan (Oksanen et al., 2017), and zCompositions (Palarea-Albaladejo & Martín-Fernández, 2015).

Metagenomic data are compositional by nature (Pawlowsky-Glahn & Egozcue, 2006). To account for that, we used the centered log-ratio transformation (clr; Aitchison, 1982) in contrast to the popular rarefaction approach, which drops information (McMurdie & Holmes, 2014). In order to apply this transformation, we imputed zeros in the read counts using the count zero multiplicative replacement. The clr-transformed data were used for all further analyses, except for the assessment of alpha diversity, that is, richness and evenness. Due to the clr transformation we could use Euclidean distances for all PERMANOVAs (Pawlowsky-Glahn & Egozcue, 2006).

Differences of the protist communities, based on the clr-transformed read counts, between the L and F/H layers were assessed using principal component analysis (PCA) and tested with a PERMANOVA with 10,000 replicates. Additionally, the major protist groups Euglenozoa, Excavata, Sarcomastigota, Stramenopiles, Alveolata, Rhizaria, Hacrobia, and Archaeplastida, based on their clr-transformed read counts, were tested for differences between layers with a PERMANOVA with 10,000 replicates. In addition to report p values, we calculated the effect size ω^2 of all applicable analyses (Lakens, 2013; Olejnik & Algina, 2003).

We used exploratory data analysis to test for successful treatment procedures. The treatment was considered successful if in the rotation treatments the concentration of the AMF marker NLFA 16:1ω5 in rotated microcosms was reduced, if in the defaunation treatments the abundance of mesofauna was reduced, and if in the N addition treatments the concentration of N was increased. This was tested by individual ANOVAs for each of the three treatments. The rotation treatment did not significantly affect the AMF marker concentration, but the concentration of the AMF marker varied across the samples and showed a bimodal distribution; in addition to using the rotation treatment as factor, we therefore used the AMF marker concentration as covariate in all analyses (see 'Efficacy of experimental setup' in the Results and Discussion for details).

Alpha diversity was analyzed by comparing the observed ASV richness based on untransformed read counts, usually underestimating real richness (Calle, 2019). Therefore, we used the Chao1 index, which adjusts for missed species, in addition, resulting in elevated numbers compared to the observed richness (Calle, 2019). Evenness within samples was assessed by using the Shannon index as well as the inverse Simpson index. All four diversity measures were used in individual ANOVAs as independent variables to test for significant treatment effects.

Community composition was assessed by PCA of the clr-transformed read counts, looking into each treatment individually. To test for treatment effects on the community, the scores of PCA axes 1 and 2 were used as variables in a MANOVA for each layer.

To gain further insight into the functional variation of the protist communities, protists were assigned to five trophic groups: phagotrophs, photoautotrophs, mixotrophs, and animal and plant parasites, based on recent studies (Adam et al., 2017; Adl et al., 2019; Schulz et al., 2019). We used the most inclusive taxonomic ranks, for example, class, of the respective protists for assignment whenever possible. Higher taxonomic ranks, for example, family or genus, were used to prevent assignment of protists to multiple trophic groups (Table S1).

PERMANOVAs (with 10,000 replicates) using the clr-transformed read counts for all five trophic groups for each layer revealed significant effects of the

experimental treatments N addition and defaunation in both layers and of the AMF marker concentration in the F/H layer on the clr-transformed read counts (Table S2; Figure S1). Therefore, the effects of the experimental treatments, variation in the concentration of the AMF marker concentration, and possible interactions on each trophic group were analyzed individually with further PERMANOVAs with the same number of replicates. Finally, we compared the ranked clr-transformed read counts of the respective trophic groups to assess effects of the experimental treatments on the most abundant protists.

Sequence data deposition

The raw 18S rRNA gene sequences were deposited at the European Nucleotide Archive under PRJEB23549 (ERP105307).

RESULTS

Data quality and general community overview

Using general eukaryotic primers to amplify the variable V4 region of the 18S rRNA gene (Stoeck et al., 2010), we obtained 5,021,942 curated sequence reads from 128 samples, which could be ascribed to 10,368 ASVs. After filtering out reads of Fungi, Metazoa, and Streptophyta (37.0%) as well as all unidentified reads (18.8%), the remaining reads of protists targetable with this PCR-based approach accounted for 44.2% of the total reads comprising 2,503,427 reads, ascribed to 4369 ASVs (Table 1). Rhizaria and Alveolata of the SAR supergroup dominated the protist community, followed by Sarcomastigota, Stramenopiles, Archaeplastida, Hacrobia, Excavata, and Euglenozoa in both ASV richness and read counts (Table 1). The clr-transformed read counts of all protists in the L and F/H layers differed significantly ($F_{1,126}=14.61$, $p<0.001$, $\omega^2=0.10$; Figure 2A). The major protist groups in the L and F/H layers also differed significantly ($F_{1,126}=12.44$, $p<0.001$, $\omega^2=0.08$; Figure 2B). Alveolata, Sarcomastigota, and Archaeplastida were more abundant in the L layer (Table S3), while Rhizaria, Excavata, and Hacrobia were more abundant in the F/H layer (Table S4).

Efficacy of experimental treatments

Data exploration of the three experimental treatments—N addition, rotation, and defaunation—revealed that only N addition and defaunation were successful (Figure 3A,C). N addition increased N concentration in both the L and F/H layers ($F_{1,62}=13.53$, $p<0.001$, $\omega^2=0.16$ and $F_{1,62}=8.08$, $p=0.006$, $\omega^2=0.10$, respectively). Defaunation reduced mesofauna abundance in both the L and F/H layers ($F_{1,62}=27.17$, $p<0.001$, $\omega^2=0.28$ and $F_{1,62}=17.86$, $p<0.001$, $\omega^2=0.21$, respectively). However, there was no effect of rotation in either layer as measured by the concentration of the AMF marker (Figure 3B). Rather, the AMF marker concentration showed a roughly bimodal distribution in both layers, suggesting an uneven distribution of AMF, not influenced by the rotation of the microcosms. Therefore, we retained the rotation treatment as an independent variable, but included the concentration of the AMF marker as covariate in the following statistical analyses to test for possible effects on protists.

Variation in community composition between layers

In the L layer, neither ASV richness nor any of the diversity indices varied significantly within treatments (Figure S1 and Table S2). In the F/H layer, ASV richness increased by N addition ($F_{1,56}=19.90$, $p\leq0.001$, $\omega^2=0.23$) and defaunation ($F_{1,56}=4.17$, $p=0.046$, $\omega^2=0.05$). The Chao1 index also increased by N addition ($F_{1,56}=21.67$, $p\leq0.001$, $\omega^2=0.24$) and defaunation ($F_{1,56}=4.68$, $p=0.035$, $\omega^2=0.05$). In the L layer, the protist community was significantly affected by N addition and defaunation, while in the F/H layer the AMF marker concentration also affected the protist community, whereas rotation was neither significant in the L nor in the F/H layers (Figure 4). In both layers, N addition explained most of the variation in the relative community abundance data (78% and 72% for the L and F/H layers, respectively) and had overall a large effect size ($\omega^2=0.76$ and $\omega^2=0.70$, respectively). Defaunation explained 14% and 61% of the variation in the respective layers, but had a rather low effect size ($\omega^2=0.10$) in the L layer compared to the F/H layer ($\omega^2=0.58$). The concentration of the AMF marker showed a significant effect on the F/H layer, explaining 11% of the variation, but with a small effect size ($\omega^2=0.08$), and was nonsignificant in the L layer.

Distribution of trophic groups between layers

Three of the five trophic groups of protists differed in their clr-transformed relative abundances between the L and F/H layers ($F_{1,127}=23.77$, $p<0.001$, $\omega^2=0.15$; Figure 5). Phagotrophs showed a higher proportional abundance in the F/H than the L layer (79% and 63% of total, respectively). The top 10 ASVs in this layer could be ascribed exclusively to Glissomonadida, while in the L layer few Cercomonadida ASVs were present in the middle and upper ranks. The majority of Glissomonadida belonged to Allapsidae and Sandonidae, that is, bacterivores.

Photoautotrophic protists showed a higher relative abundance in the L than the F/H layer (5% and 2% of

TABLE 1 Reads and amplicon sequence variants (ASVs) of all eukaryotic (all eukaryotes) and protist phyla (protists) amplified by PCR of 128 samples from southern Ecuador.

			All eukaryotes		Protists	
Phylum	**# of reads**	**# of ASVs**	**Read %**	**ASV %**	**Read %**	**ASV %**
Unikonts						
Euglenozoa						
Euglenozoa	1031	13	0.02	0.13	0.04	0.30
Excavata						
Loukozoa	48	1	<0.01	0.01	<0.01	0.02
Metamonada	78	2	<0.01	0.02	<0.01	0.05
Percolozoa	2112	13	0.04	0.13	0.08	0.30
Sarcomastigota						
Amoebozoa	212,636	530	4.23	5.11	8.49	12.13
Choanozoa	24,166	129	0.48	1.24	0.97	2.95
Sulcozoa	1786	10	0.04	0.10	0.07	0.23
Opisthokonta						
Metazoa	1,176,044	1837	23.42	17.72		
Fungi	727,698	2004	14.49	19.33		
Bikonts						
Harosa						
Incertae Sedis – Harosa	368	9	0.01	0.09	0.01	0.21
Stramenopiles						
Bigyra	69,013	456	1.37	4.40	2.76	10.44
Ochrophyta	4719	38	0.09	0.37	0.19	0.87
Alveolata						
Pseudofungi	3508	23	0.07	0.22	0.14	0.53
Ciliophora	119,160	354	2.37	3.41	4.76	8.10
Miozoa	760,123	723	15.14	6.97	30.36	16.55
Rhizaria						
Cercozoa	1,242,675	1625	24.74	15.67	49.64	37.19
Hacrobia						
Cryptista	281	5	0.01	0.05	0.01	0.11
Haptophyta	927	14	0.02	0.14	0.04	0.32
Heliozoa	4130	33	0.08	0.32	0.16	0.76
Picozoa	5504	56	0.11	0.54	0.22	1.28
Archaeplastida						
Viridiplantae						
Chlorophyta	46,632	306	0.93	2.95	1.86	7.00
Streptophyta	116,976	214	2.33	2.06		
Biliphyta						
Rhodophyta	4530	29	0.09	0.28	0.18	0.66
Unidentified						
No blast hit	497,797	1944	9.91	18.75		

Note: For protists unidentified reads, Fungi, Metazoa, and Streptophyta were filtered out. Percentages based on total numbers of combined samples of unfiltered eukaryote data and filtered protist data.

total, respectively). The top 10 ASVs in the L layer were ascribed to green algae (Chlorophyta) and comprised eight Trebouxiophyceae, one Chlorophyceae, and one Ulvophyceae. The following algae could be further identified to genus level: *Elliptochloris*, *Coccomyxa*, *Pseudococcomyxa*, *Viridirella*, *Printzina*, *Lobosphaeropsis*, and *Leptosira*. Compared to the L layer, in the F/H layer more Chlorophyceae ASVs (*Chlorosarcinopsis*, *Coccomyxa*) as well as a brown algae ASV (Phaeophyceae) were present in the middle ranks.

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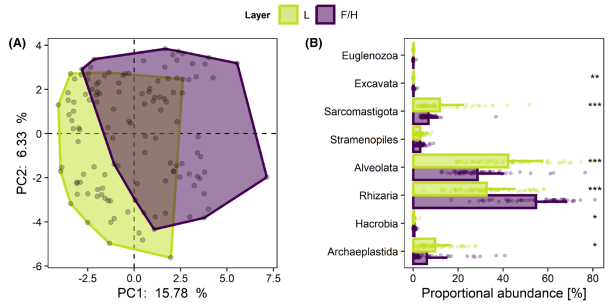

FIGURE 2 Differences between the litter (L) and fragmentation/humus (F/H) layers of (A) read abundances of all protists visualized as principal component analysis (PCA) and (B) proportional abundances of major protist groups. Asterisks mark significant differences in proportional abundances between layers (*t*-test; $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$).

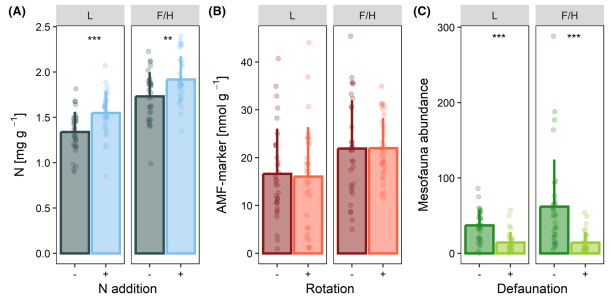

FIGURE 3 Effects of (A) N addition, (B) rotation, and (C) defaunation measured as N concentration, concentration of the arbuscular mycorrhizal fungi (AMF) marker NLFA 16:1ω5 and mesofauna abundance, respectively; − control, + treatment. Asterisks mark significant differences within layers (*t*-test; $**p \leq 0.01$; $***p \leq 0.001$).

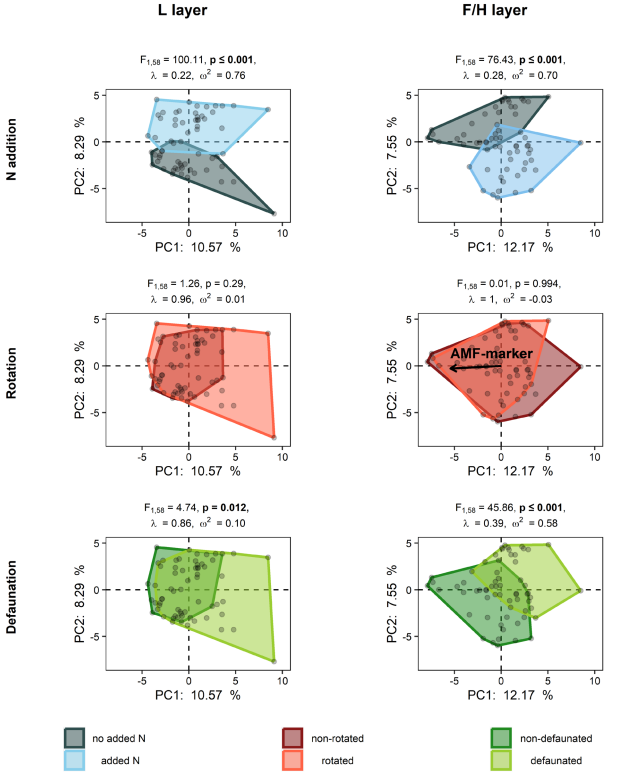

FIGURE 4 Principle component analysis (PCA) on the effects of N addition (blue), rotation (green), and defaunation (red) on protist communities in the litter (L) layer (left) and fragmentation/humus (F/H) layer (right). The significant effect ($\lambda = 0.89$, $F_{2,58} = 3.67$, $p = 0.032$, $\omega^2 = 0.08$) of the covariate arbuscular mycorrhizal fungi (AMF) marker concentration is indicated as black arrow. Dark color indicates control, light color indicates treatment.

Given that brown algae predominantly inhabit marine environments, with only seven species known from freshwater habitats, this identification needs to be confirmed.

Animal parasites showed a higher relative abundance in the L than the F/H layer (31% and 18% of total, respectively). The top 10 animal parasitic ASVs belonged exclusively to Gregarines. In the L layer, *Gregarina* and *Ascogregarina* could be further identified, and in the F/H layer additionally *Paraschneideria*.

Neither the relative abundance of mixotrophs nor plant parasites differed between layers (both 2%). The mixotrophs comprised Ochrophyta (Stramenopiles), Haptophyta and Cryptista (Hacrobia), and Miozoa (Alveolata). The plant parasites comprised mostly Plasmodiophorida (Endomyxea) and few Peronosporales (Oomycota).

Treatment effects on trophic groups

N addition

In the L layer, N addition affected phagotrophs, mixotrophs, animal parasites, and plant parasites (Table 2). The relative abundance of phagotrophs and animal parasites increased by 3.0% and 0.1%, the relative abundance of mixotrophs decreased by 4.2% (Figure 6A–C), whereas that of plant parasites generally decreased by 61.1%. However, the strong decrease of the relative abundance of plant parasite varied with defaunation (N addition × defaunation interaction; $F_{1,58} = 5.27$, $p = 0.024$) and was mainly due to the very high relative abundance of plant parasites in the defaunated treatment without N addition (Figure 6D). In the F/H layer, N addition affected phagotrophs, mixotrophs, and plant parasites. The relative abundance of phagotrophs and mixotrophs increased by 8.2% and 40.0%, whereas that of plant parasites decreased by 46.7% (Figure 6E–G).

Based on the ranked clr-transformed read counts, the top 10 phagotrophic ASVs in the L layer without N addition could be ascribed to ASVs of the families Glissomonadida and Cercomonadida, both Cercozoa, but were mixed through the ranks. Additionally, a Colpodellida ASV (Miozoa) occurred in the middle and lower ranks. In the L layer with N addition, Glissomonadida also dominated the upper ranks, while the Cercomonadida filled the lower ranks. A Spongomonadida ASV occurred in the lower ranks as well, while the Colpodellida disappeared.

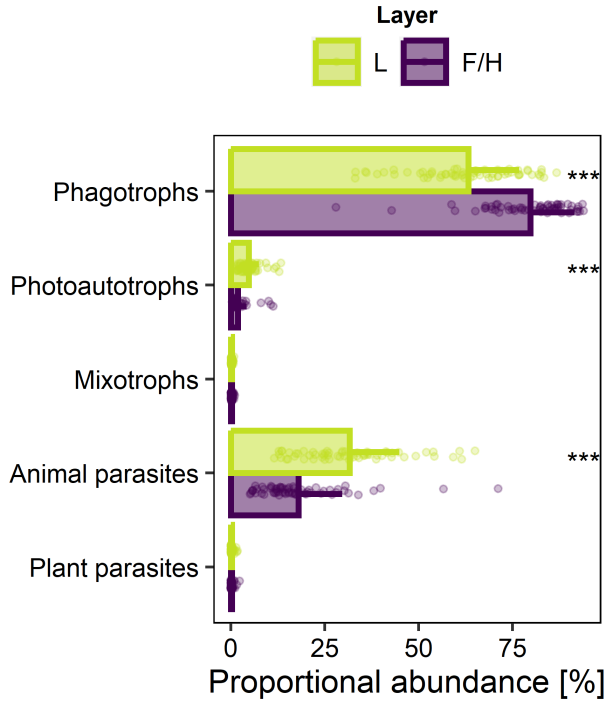

FIGURE 5 Distribution of trophic groups of protists in the litter (L) and fragmentation/humus (F/H) layers based on clr-transformed read counts, shown as proportional abundances. Asterisks mark significant differences between layers (*t*-test; ***$p \leq 0.001$).

In the F/H layer, Glissomonadida of the families Allapsidae, Cercomonadidae, and Sandonidae dominated the phagotrophs in microcosms with and without N addition. Although their relative abundances differed between treatments, the order of their ranks did not.

The top 10 mixotrophic ASVs in the L layer without N addition could be ascribed to members of three superphyla, that is, Alveolata, Hacrobia and Stramenopiles. In the L layer without N addition, members of Chrysophyceae Clade C dominated the top and middle ranks, with *Rufusiella* (Dinophyceae) second and *Isochrysis* (Prymnesiophyceae) third, while a further *Rufusiella* ASV and Prymnesiphyceae (Clade C1 and *Algiosphaera*) filled the lower ranks. In the L layer with N addition, no Hacrobia were in the top 10 ranks. ASVs ascribed to members of Chrysophyceae Clade C, and *Paraphysomonas* Clade F dominated virtually all upper and middle ranks. A *Rufusiella* ASV ranked first, while a different *Rufusiella* together with other Dinophyceae filled the lower ranks.

Of the mixotrophs in the F/H layer without N addition, a member of Clade C of the Chrysophyceae ranked highest, followed by Cryptomonodales (Cryptophyceae) and *Rufusiella* (Dinophyaceae). The middle ranks comprised *Ochromonas* (Chrysophyseae) and Cryptomonodales ASVs (Cryptophyceae), while

TABLE 2 Effects of N addition, rotation, and defaunation as independent factors as well as the concentration of the arbuscular mycorrhizal fungi (AMF) marker as covariate on the abundance of trophic groups of protists in the litter (L) and fragmentation/humus (F/H) layers (PERMANOVAs; 10,000 replicates).

		L layer			F/H layer		
Trophic group	**Treatment**	$F_{1,58}$	p	ω^2	$F_{1,58}$	p	ω^2
Phagotrophs	N addition	10.58	**0.002**	0.13	22.27	**<0.001**	0.25
	Rotation	0.04	0.843	−0.02	2.11	0.149	0.02
	Defaunation	6.92	**0.011**	0.08	35.23	**<0.001**	0.35
	AMF marker	0.02	0.900	−0.02	2.93	0.097	0.03
	Defaunation × N addition	0.27	0.595	−0.01	<0.01	0.998	−0.02
Photoautotrophs	N addition	1.58	0.209	0.01	1.10	0.300	0.01
	Rotation	<0.01	0.995	−0.02	0.75	0.388	0.01
	Defaunation	0.67	0.413	−0.01	0.80	0.379	0.01
	AMF marker	0.18	0.670	−0.01	15.87	**<0.001**	0.19
	Defaunation × N addition	1.39	0.242	0.01	3.78	0.058	0.04
Mixotrophs	N addition	4.43	**0.037**	0.05	16.66	**<0.001**	0.20
	Rotation	0.42	0.527	−0.01	0.72	0.393	0.01
	Defaunation	3.92	0.056	0.04	1.53	0.225	0.01
	AMF marker	5.2	**0.026**	0.06	0.95	0.336	0.01
	Defaunation × N addition	0.61	0.431	−0.01	0.70	0.397	0.01
Animal parasites	N addition	5.55	**0.022**	0.07	0.19	0.664	−0.01
	Rotation	0.48	0.498	−0.01	0.69	0.417	0.01
	Defaunation	13.61	**<0.001**	0.16	9.25	**0.003**	0.11
	AMF marker	2.06	0.153	0.02	0.26	0.618	−0.01
	Defaunation × N addition	1.47	0.238	0.01	0.02	0.889	−0.02
Plant parasites	N addition	12.36	**<0.001**	0.15	10.94	**0.001**	0.13
	Rotation	0.84	0.366	0.01	<0.01	0.954	−0.02
	Defaunation	7.87	**0.005**	0.1	1.66	0.208	0.01
	AMF marker	0.28	0.599	−0.01	10.20	**0.002**	0.13
	Defaunation × N addition	5.27	**0.024**	0.06	0.59	0.445	−0.01

Note: The only interaction between defaunation and N addition was included as interactions with rotation (as well as rotation as main factor) were generally not significant.

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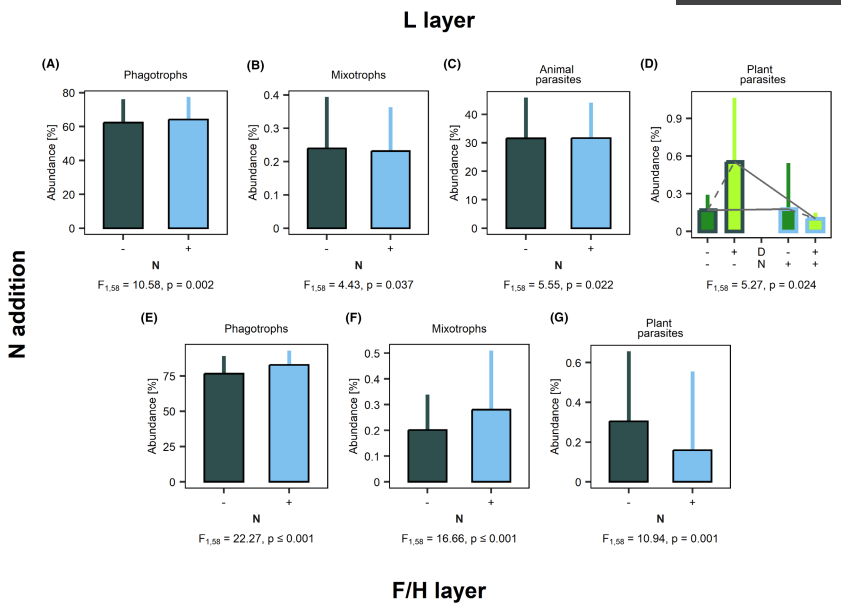

FIGURE 6 Effects of N addition (control = −, N addition = +) on the proportional abundance of trophic groups of protists in the litter (L; top) and fragmentation/humus (F/H; bottom) layers. Panel D shows the interactive effects of N addition (N; control = −, N addition = +) and defaunation (D; nondefaunated = −, defaunated = +) on the relative abundance of plant parasitic protists in the L layer.

the lower ranks comprised members of the Prymnesiophyceae (*Isochrysis*, *Algiosphare*, and an ASV of Clade C1). With N addition, Cryptophyceae were reduced in diversity and filled the lower ranks. By contrast, Chrysophyceae increased in diversity with more members of Clade C dominating not only the top ranks, but also the middle and lower ranks comprised the genus *Paraphysomonas* Clade F. The Dinophyceae filled the upper ranks as well.

The top 10 animal parasitic ASVs in the L layer without N addition exclusively comprised Gregarines. In the L layer with N addition, an ASV ascribed to the phylum Perkinsozoa entered the lower ranks but could not be further identified.

The top 10 plant parasitic ASVs in both layers could be ascribed to mostly Phytomyxea and few Oomycota. Phytomyxea comprised *Spongospora*, *Woronia*, and *Plasmodiophora* with other not further identified Plasmodiophorida. The Oomycota comprised the two Peronosporales *Phytium* and *Phytophthora*.

In the L layer without N addition, *Spongospora* dominated the top ranks, followed by *Plasmodiophora* and further unidentified Plasmodiophorida ASVs, with *Pythium*, *Woronia*, and *Phytophthora* in the lower ranks. With fertilization, *Woronia* dominated the top rank in the L layer. Their relative abundance increased, while that of the other Phytomyxea where reduced, but ranked as in the nonfertilized ingrowth cores. The relative abundance of Oomycota increased as well with N addition.

In the plant parasites of the F/H layer, Plasmodiophora dominated the top 10 ranks as in the L layer. However, with N addition *Woronia* appeared in the middle and lower ranks. Only one ASV of Oomycota and *Phytophthora* (Peronosporales) occurred in the middle ranks without N addition and in the lower ranks with N addition.

Rotation and AMF marker concentration

Although the rotation did not affect the relative abundance of protists, the concentration of the AMF marker did (Table 2). In the L layer, with increasing AMF marker concentration, the relative abundance of mixotrophs decreased, whereas in the F/H layer, the relative abundance of photoautotrophs increased and the relative abundance of plant parasites decreased (Figure 7A–C).

Defaunation

In the L layer, with decreasing mesofauna abundance, that is, in the defaunated microcosms, the relative abundance of phagotrophs and plant parasites increased by 21.9% and 94.1%, while the relative abundance of animal parasites decreased by 32.0% (Figure 8A,B; Table 2). However, the increase in plant parasites varied with N addition (N addition × defaunation interaction; $F_{1,58} = 5.27$, $p = 0.024$; Figure 6D). In the F/H layer, defaunation increased the relative abundance of phagotrophs by 18.5%, while animal parasites decreased by 49.8% (Figure 8C,D).

Based on the ranked clr-transformed read counts, the top 10 phagotrophic ASVs in the L layer without defaunation, that is, with mesofauna, could be ascribed virtually exclusively to members of the Cercozoa.

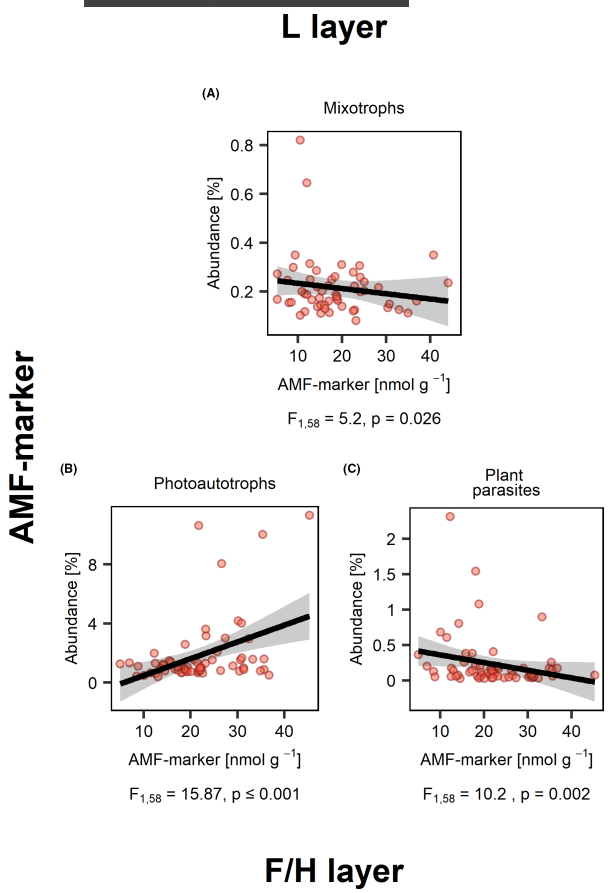

FIGURE 7 Significant correlations of the concentration of the arbuscular mycorrhizal fungi (AMF) marker with the relative abundance of trophic groups of protists in the litter (L; top) and fragmentation/humus (F/H; bottom) layers.

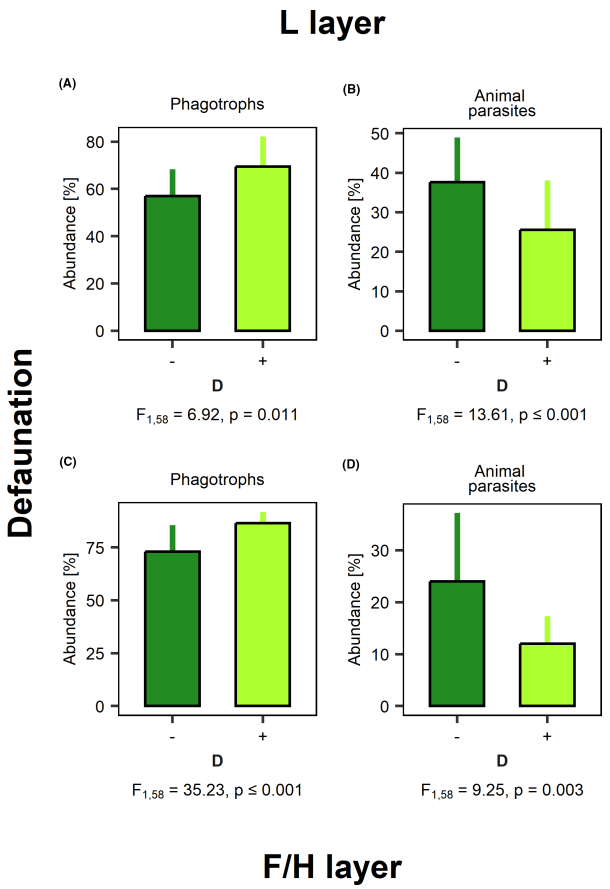

FIGURE 8 Significant effects of defaunation (control = −, defaunated = +), that is, reduction of mesofauna, on the relative abundance of trophic groups of protists in the litter (L; top) and fragmentation/humus (F/H; bottom) layers.

Several Glissomonadida of the family Allapsidae filled the top ranks, while not further identifiable Glissomonadida, but also ASVs of the genus *Cercomonas* (Cercomonadida) as well as two Colpodellida (Miozoa) filled the middle and lower ranks. With defaunation, that is, reduced mesofauna, another Glissomonadida family, Sandonidae, including the genus *Sandona*, occupied the top ranks. Allapsidae and Cercomonadidae mixed in the middle and lower ranks, while contrasting microcosms without defaunation both Colpodellida were not in the top ranks.

The top 10 phagotrophic ASVs in the F/H layer without defaunation could be ascribed to a mix of Glissomonadida (Sandonidae, Allapsidae, as well as not further identifiable Glissomonadida), with the addition of an ASV ascribed to Variosea (Amoebozoa) and an apicomplexan Colpodellida (Miozoa). With defaunation, Sandonidae dominated not only the top ranks but also appeared in the lower ranks. Allapsidae filled the middle ranks, while the ASVs ascribed to Variosea and Colpodellida disappeared from the top ranks.

The top 10 ASVs of animal parasites in the L layer could be ascribed exclusively to Gregarines. Some ASVs could be further identified to the genera *Gregarina* and *Ascogregarina*. In the nondefaunated microcosms, that is, with mesofauna, the *Gregarina* dominated the upper ranks, while *Ascogregarina* appeared in the lower ranks. With defaunation, the animal parasites kept their order of ranks, but the relative abundance of the individual ASVs changed.

The animal parasites in the F/H layer kept their order of ranks, but changed in their relative abundance, as in the L layer. However, an ASV could be ascribed to the genus *Paraschneideria* and was positioned in the lower ranks, while *Ascogregarina* was positioned in the middle ranks.

The top 10 plant parasitic ASVs in the L layer without defaunation could be ascribed to a mixture of Phytomyxea (Cercozoa) and Oomycota (Pseudofungi), but exclusively to Phytomyxea in microcosms with defaunation. All Phytomyxea could further be identified as Plasmodiophorida, including the genera *Spongospora* and *Woronia*. The Oomycota could further be identified as *Pythium* and *Phytophthora* of the Peronosporales. All families were well mixed in the nondefaunated microcosms, while in the defaunated microcosms no Oomycota were present in the top ranks. Instead, additional ASVs of the genus *Woronia* appeared in the upper and middle

ranks. However, a single *Spongospora* dominated clearly the top ranks.

DISCUSSION

We investigated the response of protist communities to increased N deposition, a limiting macroelement in montane ecosystems (Camenzind et al., 2018), similar to that forecasted by anthropogenic change scenarios (Fabian et al., 2005; Homeier et al., 2012). We also investigated the role of concentrations of AMF, common fungi associated with plants in tropical montane rainforests (Treseder & Cross, 2006), on protist communities. Furthermore, we investigated effects of the reduction of mesofauna, important soil arthropods (Maraun et al., 2008), on protist communities. To the best of our knowledge, this study is the first employing HTS for investigating protist communities in tropical montane rainforests. In general, 44.2% of the total number of 5,021,942 reads could be ascribed to 4369 protist ASVs, resembling figures from studies on other tropical regions (Araujo et al., 2018; Mahé et al., 2017; Oliverio et al., 2020), but being higher than those from studies of temperate regions (Venter et al., 2017). Most studies on protists focus on the mineral soil ignoring organic layers despite the fact that the density and diversity of microorganisms and animals in organic layers typically exceeds that in the mineral soil (Krashevska et al., 2008; Maraun et al., 2008). To account for the vertical distribution of protist communities, we analyzed protist communities of the upper L and underlying F/H layers.

Community composition

Of the three treatments, N addition had the strongest impact, that is, large effect size and high explained variance, on the protist communities in both layers. The amount of N added (50 kg N ha^{-1} $year^{-1}$ applied twice per year) resembled predicted atmospheric anthropogenic input at our study region highlighting the sensitivity of microorganisms to nutrient inputs at this nitrogen-limited ecosystem (Camenzind et al., 2018; Homeier et al., 2012). Both ASV richness and Chao1 index increased with N addition, reflecting an overall increase in protist diversity, but this was restricted to the F/H layer. The lack of effect on the L layer might have been due to the accumulation of the added N in the F/H layer 7 years after the start of the N addition in the NUMEX experiment. The Shannon index and the inverse Simpson index was high in both layers irrespective of the addition of N, indicating species-rich protist communities that differ in abundance but not in species diversity.

Although the rotation of the microcosms did not reduce the concentration of the AMF marker NLFA 16:1ω5 in the microcosms, we detected a weak but significant effect of the concentration of the AMF marker on the protist communities in the F/H layer, where it explained 11% of the variance in the relative abundance data. This is in line with recent studies showing direct or indirect effects of AMF on protists (Bukovská et al., 2018; Henkes et al., 2018; Koller et al., 2013; Krashevska et al., 2014). AMF mostly suppress protists and other microorganisms, however, in a context-dependant way. To facilitate N capture, AMF depend on the microbial loop in which protists as grazers of bacteria play a crucial role (Bonkowski, 2004; Jansa et al., 2019). Presumably, AMF suppress only selected groups of protists excluding, for example, bacterial grazers, to improve N capture. This has been demonstrated experimentally for *Acanthamoeba* sp. and *Ischnamoeba* sp. (Bukovská et al., 2018.

The reduction of mesofauna abundance in the defaunation treatment affected protist communities in both layers, but the effect on the F/H layer was less pronounced than that of N addition. Generally, the effect was weak in the L layer with a small effect size and only 14% of explained variance in the relative abundance data, it was much stronger in the F/H layer with a large effect size and 61% of explained variance. In the F/H layer, the reduced mesofauna abundance was associated with an increased ASV richness and Chao1 index of protist communities, while the Shannon index and the inverse Simpson index were not affected. This mirrored the effect of N addition, that is, increased abundance of protists but no effect on the community composition. Lower abundance in the nondefaunated treatments with higher numbers of mesofauna might be due to not only mesofauna preying on protists, but also an indirect effect via reduction of mesofauna as consumers of nematodes (Crotty et al., 2012; Ruiter et al., 1995; Rusek, 1998). Alphei et al. (1996) showed that nematodes may increase the abundance of flagellates, but decrease the abundance of amoebae. In our study, the mesofauna mainly comprised Collembola and Acari (Sánchez-Galindo et al., 2019), including groups preying on nematodes, such as Uropodina (Koehler, 1997), but also Oribatida (Heidemann et al., 2011). The reduction in mesofauna abundance likely increased nematode abundance and this may have beneficially affected protists. While defaunation reduced mesofauna abundance in both layers, the reduction was stronger in the F/H layer than the L layer (Sánchez-Galindo et al., 2019), suggesting a more pronounced influence of mesofauna on protists in the deeper F/H layer compared to the upper L layer.

Trophic groups

To explore possible effects of the treatments on protists with respect to their function, we assigned ASVs to trophic groups including phagotrophs, photoautotrophs, mixotrophs, and animal and plant parasites. As the relative abundance based on sequence reads of the trophic

groups of protists differed between the L and F/H layers for three of the five groups, that is, phagotrophs, photoautotrophs, and animal parasites, treatment effects are discussed separately for each layer.

N addition

In both layers, N addition affected phagotrophs, mixotrophs, and plant parasites, while in the L layer animal parasites were also affected. The relative abundance of phagotrophs and animal parasites increased by N addition, while the relative abundance of plant parasites decreased. The relative abundance of mixotrophs decreased slightly in the L layer, but increased in the F/H layer by N addition.

The addition of N likely benefitted bacteria, increasing the food resource of phagotrophic protists and thereby their relative abundance. This has been shown in a greenhouse experiment (Xiong et al., 2018) as well as in agricultural fields (Schulz et al., 2019). While the addition of N may benefit phagotrophic protists in general, Zhao et al. (2019) showed that only certain taxa may indeed benefit, while others may be detrimentally affected. Typically, in N addition experiments higher amounts of N have been applied than in our study, highlighting the sensitivity of microbial communities of montane rainforests to changes in nutrient inputs (Homeier et al., 2012). Interestingly, the relative abundance of phagotrophs was higher in the F/H than in the L layer, which contrasts bacteria as indicated by phospholipid fatty acid markers (Sánchez-Galindo et al., 2019). As the most abundant ASVs in both layers were ascribed to bacterivorous groups, such as glissomonads Allapsidae and Sandonidae (Howe et al., 2011), this suggests that phagotrophs actually exert a stronger top-down pressure on bacteria in the F/H than in the L layer. However, not all phagotrophs benefitted from the addition of N, which might be related to the fact that they not only include bacterivores, but also predators. While an ASV of the Colpodellida, predatory flagellates preying on other protists (Simpson & Patterson, 1996), was among the most abundant phagotrophs in the L layer without N addition, it disappeared from the predominant phagotrophs in the treatments with N addition. Although speculative, this suggests that the addition of N may have improved food resources of Colpodellida, presumably mainly algae, and thereby enhanced defense against predators. Alternatively, the N addition might have directly detrimentally affected Colpodellida.

Mixotrophs can switch trophic strategies depending on the availability of nutrients, that is, bacteria and other protists as well as the availability of light (Liu et al., 2016). As discussed earlier, N addition likely increased the abundance of bacteria also serving as prey for mixotrophs. However, the addition of N only increased the relative abundance of mixotrophs in the F/H layer, whereas it was reduced in the L layer. Due to the lack of light, in the F/H layer, mixotrophs are likely to predominantly live as phagotrophs. Therefore, similar arguments for the beneficial effects of N addition as for phagotrophs may apply, but again, their response likely varies among taxa. Different species of *Ochromonas* thrive in dark environments if bacterial prey is present (Sanders et al., 2001), while other species such as *Isochrysis* employs phagotrophy to survive in the absence of light, but cannot grow properly relying on bacteria alone (Anderson et al., 2018). Some Chrysophyceae such as *Paraphysomonas* grow better if, in addition to prey, nutrients are added (Sin et al., 1998). This suggests that the addition of N not only increased bacterial prey and thereby indirectly influenced mixotrophic protists, but also beneficially affected the growth of individual mixotrophs directly by increasing nutrient availability.

Animal parasites in the L layer increased slightly in relative abundance with N addition. Virtually all dominant ASVs were gregarines, one member of Perkinsozoa occurred in the treatment with N addition. Gregarines are parasites of virtually all major arthropod lineages including mites and collembolans (Chen, 1999; Clopton, 2009), while Perkinsozoa infect not only marine mollusks, but also amphibians in freshwater environments (Gleason et al., 2014; Mangot et al., 2011). As the animal parasites could not be identified further than phylum or class level, interpretation of their response is difficult.

Plant parasites in both layers decreased in relative abundance with N addition, with a stronger effect on the L than the F/H layer. They comprised mostly Plasmodiophorida (Cercozoa, Endomyxea) and low numbers of Peronosporales (Pseudofungi, Oomycota). Plasmodiophorida have a wide variety of hosts, such as vascular plants, green and brown algae, diatoms, but also other plant parasites, for example, Oomycota (Bulman & Braselton, 2014). Peronosporales are a diverse group of mostly plant parasites with narrow to wide host spectra (Beakes et al., 2014; Thines, 2014). Both groups are well-known from arable systems, where they play a significant role as pathogens (Adam et al., 2017). In such systems fertilization with N can reduce their abundance (Zhao et al., 2019). This is likely due to positive effects of N on their hosts improving defense (Mur et al., 2016). Mittelstraß et al. (2006) showed that low rather than high amounts of N fertilizer improved plant resistance to *Phytophthora* (Peronosporales), resembling the effect of low N addition in our study. Overall, the effect of the N addition was strongest in plant parasites among all the trophic groups of protists, underscoring the impact of indirect effects on microbial communities in low nutrient ecosystem. In the litter layer, N addition and defaunation had an interactive effect on the plant parasites. Potentially, mesofauna increased the availability of N in litter (Kandeler et al., 1999; Scheu et al., 2005),

which may have beneficially affected host plants resulting in more effective defense against plant parasites. Conversely, the nitrogen released by the mesofauna was missing in the defaunated microcosms and the plant parasites could more easily infest their hosts, resulting in increased relative abundance. Notably, N addition overpowered the positive effects of defaunation on plant parasites and reduced their relative abundance below the level in control treatments.

Rotation

By rotating the microcosms every second day, we expected to reduce colonization of the microcosms by AMF hyphae. Contrasting this assumption, rotation did not reduce AMF. Presumably, this is due to different growth of AMF in tropical forests compared to temperate ecosystems where AMF form extensive exploitative mycelia extending away from roots to capture nutrients in soil (Phillips et al., 2013). In montane rainforests, AMF may predominantly stay closely attached to roots (Camenzind & Rillig, 2013), which in lieu develop an extensive network in the litter layer (Kottke et al., 2004). Despite the failure to manipulate AMF via rotation of the microcosms, the amount of AMF, as measured by the concentration of the AMF marker NLFA 16:1ω5 in the microcosms, varied widely allowing to explore associated variations in protist communities by using the concentration of the AMF marker as covariate in our statistical models. Doing that, we assumed that AMF hyphae were still active within the microcosms potentially in a saprotrophic stage (Azcón-Aguilar & Barea, 1995). The significant correlation with the overall abundance of protists in the F/H layer as well as with mixotrophs in the L layer and photoautotrophs and plant parasites in the F/H layer suggests that protist community composition in fact varies with AMF abundance. We expected that reduced AMF abundance beneficially affect protists in general. Contrasting this assumption, the effect on the relative abundance of individual trophic groups of protists differed and varied between layers. In the L layer, the relative abundance of mixotrophs decreased with increased concentration of the AMF marker and the same was true for plant parasites in the F/H layer. By contrast, the abundance of photoautotrophs decreased with reduced concentration of the AMF marker. AMF are important for P and N acquisition of plants, which is mediated by interactions of AMF with bacteria and protists (Bonkowski et al., 2019; Jansa et al., 2019). It has been shown that AMF can suppress not only specific bacteria, but also protists such as *Acanthamoeba* (Bukovská et al., 2018; Henkes et al., 2018). However, *Acanthamoeba* as bacterivorous phagotrophs were not affected by changes in the concentration of the AMF marker in our experiment, but the negative effect of AMF on mixotrophs in the L layer may have been linked to the phagotrophic phase of these protists. Overall, the results based on correlations between protists and concentrations of the AMF marker fatty acid need to be interpreted with caution as not only hyphae but also spores of AMF contain high amounts of the marker fatty acid (Olsson, 1999) complicating their interpretation.

Defaunation

Reduction in mesofauna affected phagotrophs and animal parasites in both layers, as well as plant parasites in the L layer. The relative abundance of phagotrophs and plant parasites increased in defaunated microcosms, while the relative abundance of animal parasites declined. This suggests that phagotrophic protists thrive in the absence of mesofauna, such as Oribatida and Collembola, potentially functioning as competitors for food in particular by feeding on bacteria (Ngosong et al., 2009). Although defaunation resulted in a more pronounced reduction in mesofauna in the F/H than in the L layer, the effect on phagotrophs was somewhat less pronounced. This may have been due to the fact that there were more predatory protists, for example, Colpodellidae, in the F/H than in the L layer, which likely did not benefit from the reduction in bacterivorous mesofauna.

The reduction in the relative abundance of animal parasites likely was linked to the reduction in host organisms. The higher relative abundance of animal parasites in the L than the F/H layer suggests that the majority of hosts did not belong to mesofauna, which are more abundant in the F/H than the L layer (Sánchez-Galindo et al., 2019). For interpreting the response of animal parasites, more detailed information on their taxonomic affiliation is needed.

Unexpectedly, the relative abundance of plant parasites increased with the reduction of mesofauna abundance in the L layer. Presumably, mesofauna also consumed hosts of plant parasitic protists such as algae, thereby reducing algal parasites. However, mesofauna may also directly feed on plant parasitic protists.

CONCLUSIONS

We showed that the protist communities in tropical montane rainforests of southern Ecuador are not only taxonomically but also trophically complex and sensitively respond to moderate increase in N input as well as variations in mesofauna abundance and concentrations of the AMF marker fatty acid. N addition strongly affected virtually all trophic groups of protists, highlighting the susceptibility of microbial food webs to human disturbances. Reduced mesofauna abundance resulted in increased relative abundance of phagotrophs, presumably competing with mites and collembolans for bacterial food, as well as reduced relative abundance

of animal parasites. Although our treatment of reducing AMF abundance by rotating the microcosms was not successful, significant correlations between the AMF marker fatty acid and photoautotrophic, mixotrophic, and plant parasitic protists suggest that at a wide range of protist trophic groups closely interact with AMF. While the experimental treatments typically affected trophic groups of protists in the L and F/H layers in a similar way, some trophic groups differentially responded in the two layers suggesting that protist communities need to be studied across layers to fully understand their role in ecosystem functioning as well as their response to environmental changes.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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SUPPORTING INFORMATION

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

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