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Moderate changes in nutrient input alter tropical microbial and protist communities and belowground linkages

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We investigated the response of soil microbial communities in tropical ecosystems to increased nutrient deposition, such as predicted by anthropogenic change scenarios. Moderate amounts of nitrogen and phosphorus and their combination were added along an altitudinal transect. We expected microorganisms and microbial grazers (testate amoebae) to significantly respond to nutrient additions with the effect increasing with increasing altitude and with duration of nutrient additions. Further, we expected nutrients to alter grazer–prey interrelationships. Indeed, nutrient additions strongly altered microbial biomass (MB) and community structure as well as the community structure of testate amoebae. The response of microorganisms varied with both altitude and duration of nutrient addition. The results indicate that microorganisms are generally limited by N, but saprotrophic fungi also by P. Also, arbuscular mycorrhizal fungi benefited from N and/or P addition. Parallel to MB, testate amoebae benefited from the addition of N but were detrimentally affected by P, with the addition of P negating the positive effect of N. Our data suggests that testate amoeba communities are predominantly structured by abiotic factors and by antagonistic interactions with other microorganisms, in particular mycorrhizal fungi, rather than by the availability of prey. Overall, the results suggest that the decomposer system of tropical montane rainforests significantly responds to even moderate changes in nutrient inputs with the potential to cause major ramifications of the whole ecosystem including litter decomposition and plant growth.

Introduction

With the growing human activity, such as farming, livestock breeding, burning of fossil fuels and biomass, nutrients are increasingly distributed over wide geographical areas, thereby, also entering natural ecosystems. Emissions of nitrogen increased at least fourfold over the last century and are predicted to further increase (Mahowald *et al.*, 2005; Phoenix *et al.*, 2006; Galloway *et al.*, 2008; Reay *et al.*, 2008). Atmospheric nutrient input may detrimentally affect ecosystems at the regional scale; however, they are also transported over long distances, thereby entering ecosystems assumed to be devoid of human impacts, such as tropical rainforests (Fabian *et al.*, 2005). By modifying the nutrient limitation of primary productivity and altering decomposition processes, these inputs

likely feedback to climate change, as tropical forests account for about one-third of the annual global carbon dioxide exchange between the atmosphere and terrestrial ecosystems (Mahowald *et al.*, 2005; Galloway *et al.*, 2008; Pett-Ridge, 2009). Further, nutrient inputs are expected to threaten biodiversity; Sala *et al.* (2000) considered N deposition to be the third most important driver of biodiversity loss after land use and climate change. Deposition of N and P is generally expected to significantly alter species composition, diversity and productivity of virtually any ecosystem; however, the magnitude and the direction of change depend on the nutrient status of ecosystems and vary between biomes with the reasons for the variable responses still being little understood (Phoenix *et al.*, 2006; Pett-Ridge, 2009; Homeier *et al.*, 2012).

Tropical rainforests, especially montane rainforests, are thought to be limited by N or N and P, and this applies to both primary producers and decomposers (Vitousek *et al.*, 1993; Tanner *et al.*, 1998; Krashevska *et al.*, 2010). Potentially related to low nutrient supply, tropical montane rainforests are among the most species-rich ecosystems of the

world and this applies in particular to Andean rainforests (Myers et al., 2000; Beck et al., 2008), and nutrient inputs may threaten this diversity. However, knowledge on how these highly diverse ecosystems respond to the ongoing increase in nutrient deposition is lacking.

A number of experiments have been performed to investigate effects of increased input of nutrients on primary producers, including tree growth and litter production; however, most of these studies were performed using high doses of nutrients (for example, 125–225 kg N and 50–75 kg P ha^{-1} per year; Tanner et al., 1992; Wright et al., 2011). Only few studies investigated the response of tropical montane rainforest ecosystems to moderate nutrient additions (for example, 50 kg N and 10 kg P ha^{-1} per year; Homeier et al., 2012; Martinson et al., 2012). Knowledge on the response of the belowground system to low or moderate input of nutrients into tropical rainforests is entirely lacking. This is unfortunate as decomposers and trophic interactions below the ground affect plant performance and diversity, and have an important role for ecosystem processes (Scheu et al., 1999, 2005; Wardle 2002; Kottke et al., 2008). For soil processes, such as decomposition and nutrient turnover, microorganisms and microfauna are most important, and therefore understanding their response to increased nutrient input is essential. As small organisms, protists quickly respond to abiotic and biotic changes, as well as anthropogenic changes, for example, fertilizer and pesticide application, thereby functioning as indicators of recent as well as past ecosystem changes (Foissner, 1994; Mitchell et al., 2008).

Testate amoebae are among the most important and abundant protists in acidic forest ecosystems including most tropical rainforests (Krashevska et al., 2007). Despite their importance in trophic and antagonistic interactions with microorganisms, and their potential as bioindicators of environmental change, they are still poorly studied soil organisms (Krashevska et al., 2010, 2012).

We investigated the response of microorganisms and testate amoebae of montane rainforests of the Andes of southern Ecuador to moderate nutrient addition such as those predicted by anthropogenic change scenarios (Galloway et al., 2008; Homeier et al., 2012). Rainforests along an altitudinal transect were investigated to get insight into variations in the effects of nutrient inputs with altitude. We hypothesized that (1) the response of microorganisms to moderate nutrient additions increases with increasing altitude, parallel to the increased nutrient shortage due to high nutrient leaching by high precipitation. Further, we posit that the response of microorganisms increases with duration of nutrient additions, that is, from 12 to 36 months after start of the experiment. On the basis of previous experiments with high nutrient input (Krashevska et al., 2010), we hypothesized that (2) N but not P addition

will increase the density of testate amoebae, whereas both N and P will decrease their diversity. By investigating both, the response of testate amoebae and microorganisms to increased nutrient input, we assumed to be able to identify whether responses of testate amoebae are driven by changes in prey availability, that is, we tested the hypothesis that (3) the response of testate amoebae is due to nutrient-mediated changes in prey availability.

Materials and methods

Study sites
The study sites are located in southern Ecuador at the northern border of the Podocarpus National Park on the eastern slopes of the Andes. Three study sites along an altitudinal transect were selected at 1000, 2000 and 3000 m above sea level. Study sites at 1000 m comprise old-growth premontane rainforest, at 2000 m old-growth lower montane rainforest and at 3000 m upper montane rainforest. Soil types at 1000, 2000 and 3000 m were dystric cambisol, stagnic cambisol and stagnic histosol. The thickness of organic layers increases with altitude from about 5 to 15 to 25 cm at 1000, 2000 and 3000 m, respectively. More details of the study sites are summarized in Beck et al. (2008), Moser et al. (2007) and Martinson et al. (2012); details on testate amoebae and their interrelationships with abiotic and biotic factors are given in (Krashevska et al., 2007, 2008, 2012).

Experimental design and sampling
A full-factorial nutrient manipulation experiment was performed on plots of 20 × 20 m resulting in four treatments: control, +N, +P and +N+P. Minimum distance between two plots was 10 m. Each treatment was replicated four times, and the plots were arranged in a randomized complete block design with four blocks at each altitude.

N and P were added at an annual rate of 50 kg N ha^{-1} as urea and 10 kg P ha^{-1} as monosodium phosphate (Homeier et al., 2012). Fertilizers were dispersed homogeneously over the plots with two applications per year starting in 2008. First samples were taken after 12 months in May 2009, that is, after three fertilization campaigns; second samples were taken after 36 months and seven fertilization campaigns in May 2011. Samples from the litter/fermentation layer were taken from each plot to a depth of 5 cm using a corer (Ø 5 cm) resulting in 96 samples in total.

Environmental factors
Litter/fermentation layer material was dried at 65 °C for 72 h, milled and analyzed for total C and N concentrations using an elemental analyzer (Carlo Erba, Milan, Italy). Further, pH(CaCl$_2$) was measured

using a digital pH meter. Water content was determined gravimetrically.

Microorganisms

Microbial respiration and biomass (MB) were determined by measuring O_2 consumption using an automated respirometer system (Scheu, 1992; for details see Krashevska et al., 2012). For measuring phospholipid fatty acids (PLFAs) and neutral lipid fatty acids, 2 g wet weight litter was extracted following the procedure of Frostegård et al. (1993), for details see Krashevska et al. (2008). Individual PLFAs were calculated as percentage of total PLFAs (nmol per g dry litter material). The concentration of the fungal-specific fatty acid 18:2ω6,9 was used as a relative fungal marker (Frostegård and Bååth, 1996; Ruess et al., 2002; Haubert et al., 2004). The sum of 16:1ω7, cy17:0 and cy19:0 was used as a relative marker for Gram-negative, and the sum of i15:0, a15:0, i16:0 and i17:0 as a relative marker for Gram-positive bacteria (Zelles et al., 1995; Zelles, 1997; Schoug et al., 2008). The sum of 20:2 and 20:4ω6 was used as a relative animal marker (Salomonová et al., 2003; Chamberlain et al., 2005). The neutral lipid fatty acid 16:1ω5c was used as an indicator for arbuscular mycorrhizal fungi (AMF) (Lekberg et al., 2012).

Testate amoebae

Testate amoebae were extracted by washing samples over 500 μm mesh and then back sieving the filtrate over 20 μm mesh. Testate amoebae were separated into living cells and empty shells after staining with aniline blue (Wanner and Elmer, 2009). Encysted testate amoebae were not abundant (less than 5% of total) and were added to living cells. The mean shell size of live specimens was 90 ± 44 μm and ranged between 17 and 169 μm. Determination of species was based on morphological characters (morphospecies); details on identification and taxonomic references are given in Krashevska et al. (2007). Full names of taxa are listed in alphabetical order in Supplementary Appendix S1.

Statistical analysis

To analyze differences in the assemblages of live testate amoebae among experimental treatments, their density, species richness and small (< 90 μm)-to-large cell (> 90 μm) abundance ratio were calculated and analyzed. These data and data on microbial parameters (microbial basal respiration (BR), MB, PLFAs and neutral lipid fatty acid) as well as data on abiotic environmental factors (C-to-N ratio, pH and water content of litter) were analyzed by four-factorial repeated measures analysis of variance (rm-ANOVA), with the fixed factors N (with and without), P (with and without) and altitude (1000, 2000 and 3000 m), and time as repeated factor

(sampling dates after 12 and 36 months). Before statistical analyses, data on microbial BR, MB and litter C-to-N ratio were $\log(x + 1)$ transformed; PLFA data were arcsine square-root transformed. Tukey's HSD test ($\alpha < 0.05$) was used to identify significant differences between means. Data on living cells of testate amoebae were analyzed by discriminant function analysis to identify treatment effects on community composition. Squared Mahalanobis distances between group centroids (control, $+N$, $+P$ and $+N+P$) and the reliability of sample classification were determined. Two significant canonical roots were derived and graphically presented in two-dimensional space illustrating significant differences between control, $+N$, $+P$ and $+N+P$ treatments at 1000, 2000 and 3000 m at the two sampling dates. Only species present in at least two replicates were included in discriminant function analysis, resulting in 114 taxa. For identifying which of these species were responsible for significant differences between treatments, protected ANOVAs were carried out (Scheiner and Gurevitch, 2001). ANOVA were performed using SAS 9.13 (SAS Institute Inc., Cary, NC, USA) and discriminant function analysis using STATISTICA 7.0 for Windows (StatSoft, Tulsa, OK, USA).

Relationships between live specimens of testate amoebae and environmental factors were analyzed using redundancy analysis as implemented in CANOCO (Ter Braak and Smilauer, 1998). Redundancy analysis was chosen as the length of gradient was < 2 (Lepš and Šmilauer, 2003). The analysis allowed testate amoebae taxa (dependent variables) to be related to a set of environmental factors (independent variables) by direct ordination. Environmental factors included only variables significantly affected by N, P, altitude and time. We focused on the effect of the addition of N and P and excluded species that were only affected by altitude, time or altitude × time interaction; a total of 59 taxa remained and were included in redundancy analysis. Monte Carlo tests (999 permutations) were performed to evaluate the significance of individual axes (Ter Braak, 1996). The approach aimed at identifying individual environmental factors driving testate amoebae community composition and affected by nutrient additions, thereby contributing to disentangle the pathways through which testate amoebae community composition was changed due to nutrient additions.

Results

Environmental factors

Water content in the litter layer was on average $290 \pm 117\%$ of dry weight and increased significantly with altitude (Supplementary Figure S1a; Table 1). It decreased significantly from the first to the second sampling date at 1000 and 2000 m, but increased at 3000 m. The addition of nutrients also

Table 1 Effect of the addition of moderate amounts of nutrients on environmental factors (water content, pH and C-to-N ratio of litter; microbial BR, MB, PLFA 18:2ω6,9 (FM) and NLFA 16:1ω5c (AMF)), and species richness, density of living cells testate amoebae and small-to-large shell abundance ratio of living testate amoebae (small-to-large ratio)

	Between-subject effects						Within-subject effects				
	Altitude	N	P	Altitude × P	N × P	Altitude × N × P	Time	Time × Altitude	Time × N	Time × P	Time × N × P
Degrees of freedom	2,36	1,36	1,36	2,36	1,36	2,36	1,36	2,36	1,36	1,36	1,36
Litter water content	98.6**	1.5	6.7*	0.2	4.8*	1.3	0.9	15.2**	1.4	0.5	2.9
Litter pH	94.8**	1.7	11.6**	2.4	0.9	1.7	79.6**	17.7**	0.1	2.3	8.3*
Litter C-to-N ratio	131.4**	17.2**	0.2	0.4	0.5	5.9*	8.1*	0.8	1.8	0.3	0
BR	13.9**	0.7	0.6	5.2*	0.2	1.7	3.1	0.2	0.4	4.2*	7.6*
MB	5.8*	1.7	0.2	2.0	0.5	1.6	6.7*	2.3	0.7	3.7	4.3*
FM	76.9**	2.5	0.1	0.1	1.3	4.3*	1.6	0.4	0.0	0.0	0.1
AMF	101.3**	0.1	0.1	0.5	9.5**	1.5	103.7**	23.3**	0.2	0.7	0.1
Species richness	2.0	1.1	19.6**	0.7	0.2	1.0	36.4**	1.1	2.5	1.4	0
Density	18.0**	10.9**	20.6**	1.9	4.2*	5.0*	3.2	9.4**	4.1*	3.1	1.5
Small-to-large ratio	8.4**	0.0	5.1*	2.0	1.0	0.6	15.5**	1.7	0.3	0.0	17.3**

Abbreviations: AMF, arbuscular mycorrhizal fungi; BR, basal respiration; NLFA, neutral lipid fatty acids; MB, microbial biomass; PLFA, phospholipid fatty acids.
*$P<0.05$, **$P<0.005$.
Four-factorial repeated measures ANOVA table of F-values for between-subject effects with the fixed factors N, P and altitude (1000, 2000 and 3000 m), and within-subject effects with the repeated factor time (sampling dates after 12 and 36 months).

affected the water content of the litter; it was significantly reduced by the addition of P, but the effect varied with the addition of N. Water content was reduced by the addition of N but only if P was also added.

Litter pH was on average 4.5 ± 0.7 and decreased significantly with altitude (Supplementary Figure S1b; Table 1). Further, it increased significantly from the first to the second sampling date at 1000 and 3000 m, but changed little at 2000 m. Overall, pH significantly increased by the addition of P. However, at the first sampling date, pH was little affected by the addition of P and N, whereas at the second sampling date it increased in the +P and +N+P treatments.

Litter C-to-N ratio was on average 30.4 ± 8.2. Generally, litter C-to-N ratio decreased significantly from the first to the second sampling date (Supplementary Figure S1c; Table 1). Further, it increased significantly with altitude. Litter C-to-N ratio also varied with the addition of nutrients. It decreased significantly by the addition of N. Further, it decreased significantly by the addition of P but only if N was also added at 1000 and 2000 m but not at 3000 m.

Microorganisms
Both BR and MB increased significantly with altitude (Figures 1a and b; Table 1). MB increased significantly by 24% from the first to the second sampling date. The addition of P significantly affected BR and MB, but the effect varied with the addition of N and sampling date. At the first sampling, BR and MB were increased by the addition of P, but particularly in combination with N. In contrast, they were reduced by P at the second sampling date and were highest in the N only treatment. Further, P addition increased BR at 2000
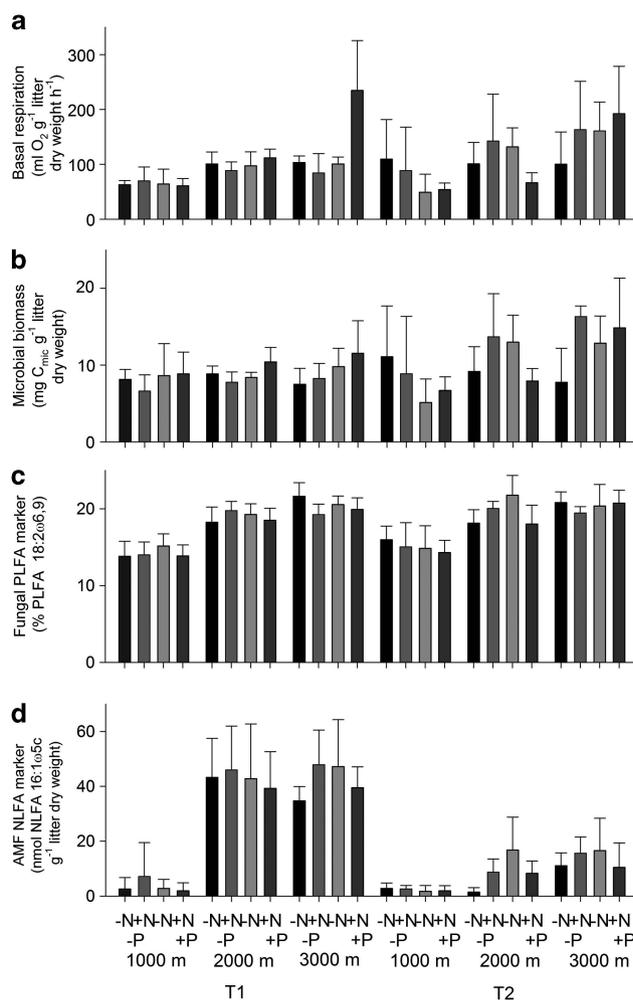
Figure 1 Effects of the addition of N and P on basal respiration (**a**), microbial biomass (**b**), fungal PLFA marker (**c**) and AMF NLFA marker (**d**) along an altitudinal transect (1000, 2000 and 3000 m) at two sampling dates (T1, after 12 and T2, after 36 months) in tropical montane rainforests of southern Ecuador, means with s.d. ($n = 4$).

and especially at 3000 m, whereas the opposite was true for 1000 m.

The total amount of PLFAs averaged 670 ± 453 nmol per g litter dry weight and was not affected by the addition of nutrients. Also, the addition of nutrients did not affect Gram-negative and Gram-positive bacteria, as well as animal marker fatty acids. In contrast, the fungal marker fatty acid $18:2\omega6,9$, which significantly increased with altitude, was reduced at 1000 and 2000 m in the combined $+N+P$ treatment as compared with the $+N$ and $+P$ treatments (Figure 1c; Table 1). At 3000 m, the fungal marker was lowest in the $+N$ treatment. AMF significantly increased with altitude and decreased significantly from the first to the second sampling date at 2000 and 3000 m (Figure 1d; Table 1). AMF significantly increased in the $+N$ and $+P$ treatments, but decreased in the $+N+P$ treatment.

Testate amoebae

A total of 203 taxa of testate amoebae were identified of which 171 taxa were found as living individuals (see Supplementary Appendix S1). Species richness decreased significantly from the first to the second sampling on average by 17%. Further, the addition of P reduced testate amoebae species richness by 12% (Figure 2a; Table 1).

Generally, the density of testate amoebae increased with altitude (Figure 2b; Table 1). Further, the density at 1000 m decreased significantly from the first to the second sampling, whereas the opposite was true for 2000 and 3000 m. Similar to testate amoebae species richness, the addition of P reduced the density of testate amoebae by 26%. In contrast, the addition of N increased the density by 22%. The positive effect of N increased significantly from the first to the second sampling. Notably, the combined addition of P and N negated the positive effect of the addition of N only, with the density in the $+N+P$ treatment being reduced on average by 34% as compared to the $+N$ treatment; however, the reduction was more pronounced at 1000 m than at 2000 and 3000 m.

Overall, the ratio between the number of small ($<90\,\mu m$) and large shells ($>90\,\mu m$) of testate amoebae was 1.19 ± 0.69. The ratio increased significantly from the first to the second sampling and decreased significantly with altitude (Figure 2c; Table 1). Further, it increased significantly by the addition of P. However, the effect of P depended on the addition of N and changed with sampling date. At the first sampling, the ratio was decreased in the $+N$ treatment and increased in the $+N+P$ treatment, whereas at the second sampling the opposite was true.

Discriminant function analysis not only separated testate amoeba communities of different altitude (axis 1), but also the communities of control and fertilized treatments (axis 2; Wilks' $\lambda = 0.249$,
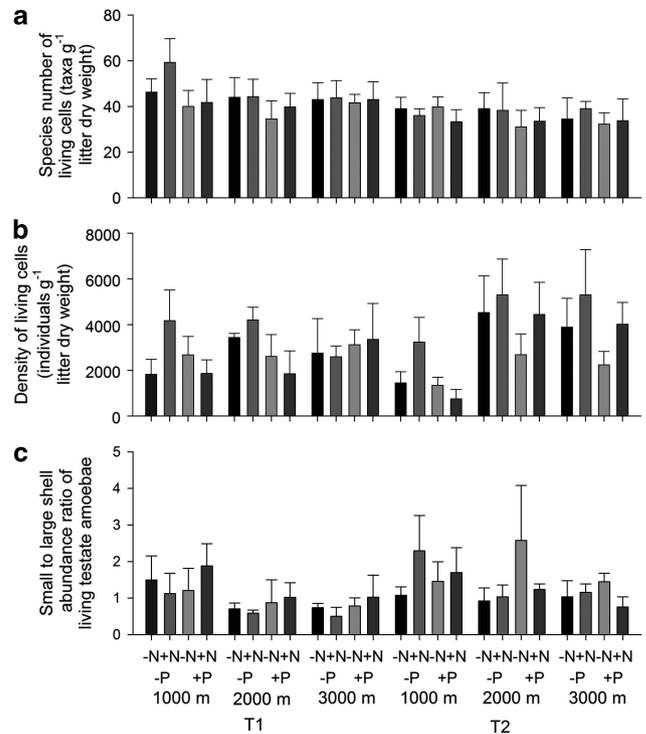
Figure 2 Effects of the addition of N and P on species number (**a**), density (**b**) and small-to-large shell abundance ratio of living testate amoebae (**c**) along an altitudinal transect (1000, 2000 and 3000 m) at two sampling dates (T1, after 12 and T2, after 36 months) in tropical montane rainforests of southern Ecuador, means with s.d. ($n = 4$).

$F_{46,142} = 16.4$, $P < 0.0001$; Figure 3; Supplementary Table S1). Generally, communities of testate amoebae at 3000 m differed markedly from those at 2000 and 1000 m irrespective of sampling date and fertilization. Nutrient addition did not affect testate amoeba communities at 3000 m at the first sampling, whereas at the second sampling nutrients caused a shift in the composition of testate amoebae, that is, community structure of testate amoebae in the $+P$ treatment differed significantly from that in the control. Also, the community structure of the $+P$ and $+N$ treatments differed significantly from that of the $+N+P$ treatment. Further, the community structure in $+P$ and $+N+P$ treatments at the first sampling differed significantly from that at the second sampling. At 2000 m, fertilization did not affect community structure of testate amoebae at the first sampling, whereas at the second sampling community composition in $+P$ and $+N+P$ treatments differed significantly from that in the control. At 1000 m, the addition of N caused a shift in community composition already at first sampling, but only in the $+N$ treatment, whereas at the second sampling nutrients did not affect testate amoeba communities. Protected ANOVAs suggested that 92 taxa of living testate amoebae significantly responded to at least one of the studied factors (see Supplementary Appendix S2).
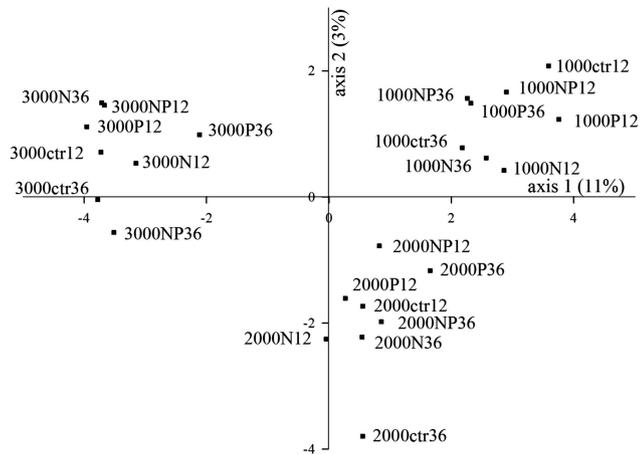
Figure 3 Discriminant function analysis of living cells of testate amoebae along an altitudinal transect (axis 1; 1000, 2000 and 3000 m) in control and experimental treatments (axis 2; ctr for control, N, P and NP) at two sampling dates (T1, after 12 and T2, after 36 months). Axis 1 and 2 explained 11% and 3% of the variation in species data, respectively.

In the forward selection procedure of redundancy analysis, four of the eight quantitative explanatory variables were significantly related to the community structure of testate amoebae ($P < 0.05$). Together, these variables explained more than 20% of the variation in species data, with the trace being significant (0.206; $F = 2.8$, $P = 0.001$). Litter C-to-N ratio accounted for most of the variation in species data, that is, 11% of total ($F = 11.9$, $P = 0.001$). The second environmental variable with significant explanatory power was water content, accounting for an additional 3% of the variation ($F = 2.5$, $P = 0.001$). The third was AMF, accounting for 2% of the variation ($F = 2.1$, $P = 0.001$) and the fourth was MB accounting for 1% of the variation ($F = 1.5$, $P = 0.002$).

Discussion

We investigated the response of tropical montane rainforests' soil communities to increased nutrient deposition resembling that predicted by anthropogenic change scenarios (Fabian *et al.*, 2005; Mahowald *et al.*, 2005; Homeier *et al.*, 2012). We focused on the response of microorganisms and testate amoebae as major predators of bacteria to moderate additions of N and P. Generally, in agreement with hypothesis (1), microorganisms, that is, MB, fungal and AMF fatty acid markers, significantly responded to moderate nutrient additions. However, in contrast to our expectation, the effect of nutrients on MB did not increase with increasing altitude, and long-term effects of nutrient addition were more complex than expected. After 12 months, irrespective of altitude, MB was the highest in treatments with P, especially if also N had been added. However, if only N was added, MB declined

significantly. This suggests that microorganisms were primarily limited by P, with the effect of P overriding the detrimental effect of N. Similarly, in other tropical rainforests, microorganisms have been shown to be limited by P and detrimentally affected by N (Cleveland *et al.*, 2002; Treseder, 2008; Homeier *et al.*, 2012). However, after addition of nutrients for 36 months, MB was the highest in the +N treatment and lowest in the +N+P treatment, opposing the response of microorganisms after 12 months. The data suggest that with duration of nutrient addition microorganisms benefited from increased N supply. Potentially, these changes are related to a shift in plant limitation from N to P due to prolonged N addition and associated accumulation of N (Matson *et al.*, 1999; Homeier *et al.*, 2012).

Plant–microbial interactions are complex, for example, microorganisms immobilize P for microbial growth, thereby reducing plant P availability, but plants may increase P capture via investing carbon into mycorrhizal symbionts (Marschner *et al.*, 2011). Indeed, in the studied forests, most tree species are associated with AMF (Kottke *et al.*, 2004), known to increase plant capture of P but presumably also N (Ames *et al.*, 1983). Therefore, in the studied rainforests, competition for nutrients likely occurs mainly between mycorrhizal fungi and saprotrophic litter microorganisms, rather than between plant roots and microorganisms. Potentially, the combined addition of N and P increased the competitive strength of saprotrophic as compared with mycorrhizal fungi. Supporting this scenario, the biomass of AMF was at a minimum in the combined +N+P treatment. However, MB was also lowest in the combined +N+P treatments at the second sampling, contrasting the response at the first sampling. Potentially, after the initial increase in biomass, the continuous additional supply of nutrients increased competitive interactions among microorganisms, resulting in increased production of toxins and reduced MB (Becker *et al.*, 2012). Hence, the results support our suggestion that plant limitation shifted from N at the first to P at the second sampling, resulting from reduced competitive interactions between AMF and saprotrophic microorganisms for N. The response of microorganisms to both N and P further indicates that both nutrients were in short supply, potentially co-limiting microorganisms as suggested earlier (Krashevska *et al.*, 2010).

In contrast to MB, the fungal marker PLFA 18:2ω6,9 was not affected by the duration of the nutrient deposition, but the effect of nutrients increased with increasing altitude. N addition decreased the C-to-N ratio of litter material, and the C-to-N ratio correlated negatively with the fungal marker PLFA 18:2ω6,9. Fungi are generally considered to dominate in low nutrient litter material; increased nutrient supply therefore likely reduced their competitive strength against bacteria relying on more homogenously distributed nutrients

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and higher nutrient concentrations (Bardgett et al., 1996; Frostegård and Bååth, 1996; Hodge et al., 2000; Güsewell and Gessner, 2009).

In agreement with hypothesis (2), testate amoebae density and diversity significantly responded to moderate nutrient additions. Addition of N generally increased the density of testate amoebae, indicating that N primarily limits testate amoebae communities irrespective of altitude. Notably, the effect of N increased from the first to the second sampling. Unexpectedly, however, the addition of P detrimentally affected testate amoebae, and the detrimental effect prevailed even if N was also given, thereby, the addition of P negated the beneficial effect of N with this being most pronounced at 1000 m. These results correspond to earlier findings that the addition of P detrimentally affects testate amoebae at the studied montane rainforests; however, much higher amounts of P had been added in the previous study (Krashevska et al., 2010). Contrary to these results, increased N input into terrestrial ecosystems typically boosts diversity loss in terrestrial ecosystems (Sala et al., 2000). Notably, in our study, the addition of P detrimentally affected both the diversity and density of testate amoebae suggesting that the addition of P fostered antagonistic interactions. Further, the uniform response of testate amoebae contrasted the variable response of MB, suggesting that the effect of P was not closely linked to the biomass of microorganisms, that is, the availability of prey, contrasting our hypothesis 3. Generally, testate amoebae quickly responded to variations in environmental factors and nutrient additions as documented by the rapid and strong changes in the density, diversity and community structure of testate amoebae to fertilization. Community structure of testate amoebae changed significantly with the duration of nutrient addition; the delayed response at 2000 and 3000 m suggests that nutrients needed to accumulate to annihilate nutrient limitation at higher altitude. Of the studied environmental factors, litter C-to-N ratio, water content and pH were most important. However, these abiotic factors were also affected by nutrient additions irrespective of duration of the experiment and altitude. Generally, testate amoebae density correlated closely with litter C-to-N ratio, water content and pH, with N addition significantly decreasing litter C-to-N ratio (Fanin et al., 2012) and P addition significantly increasing litter pH (DeForest et al., 2012). Further, litter water content significantly decreased by the combined addition of N and P, and this may have been responsible for the decline in density of testate amoeba species relying on high water availability. Only two biotic factors, AMF and MB, accounted for variations in the community structure of testate amoebae, but their explanatory power was low. This was unexpected as testate amoebae predominantly feed on microorganisms; however, MB and also microbial PLFAs have been found previously to only poorly correlate with the community structure of testate amoebae (Krashevska et al., 2010). The results therefore confirm that general parameters of the microbial community, such as MB, and fungal and bacterial PLFA markers, poorly reflect the diet of testate amoebae. Further, as suggested previously, rather than serving as food, microorganisms may also antagonistically affect testate amoebae and this may apply in particular to mycorrhiza (Krashevska et al., 2010; Vohnik et al., 2011). Changes in cell size distribution in testate amoebae with the relative abundance of large cells declining from the first to the second sampling suggest that changes in community composition are related to cell size with larger species being more sensitive to nutrient additions and potentially more sensitive to antagonistic interactions.

In conclusion, testate amoebae of tropical montane rainforests significantly responded to moderate nutrient additions as those predicted by future global change scenarios. Both diversity and density of testate amoebae benefited from the addition of N, whereas the addition of P detrimentally affected their diversity and density. Nutrient-mediated changes in MB and microbial community structure (as indicated by PLFA analysis) contributed only little to these changes. Rather, the response of testate amoebae appeared to be mainly due to nutrient-mediated changes in litter C-to-N ratio, water content and pH. This supports earlier conclusions that testate amoebae communities are structured predominantly by abiotic factors rather than by the availability of food, but a more detailed analysis of microbial communities are needed to test these suggestions. The results suggest that testate amoebae communities of tropical montane rainforests are structured by both positive and negative interactions via both biotic and abiotic factors, but more information on ecological niches of testate amoebae species is needed for understanding these interactions. As testate amoebae form a major component of the decomposer food web responsible for litter decomposition and nutrient mineralization, these changes are likely to propagate into plant growth, primary productivity and carbon dioxide exchange between the atmosphere, plants and the decomposer system.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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