Response of root associated fungal communities to increased atmospheric deposition of nitrogen and phosphorus in tropical montane forests

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This work was carried out between March 2016 and October 2021, under the supervision of **Prof. Dr. Matthias C. Rillig**, **Prof. Dr. Britta Tietjen** and **Dr. Tessa Camenzind**, at the Institut für Biologie of Freie Universität Berlin. The doctoral work proceeded within the framework of the doctoral studies program **Biodiversity, Evolution & Ecology** at Dahlem Research School of Freie Universität Berlin.

1st reviewer: Prof. Dr. Matthias C. Rillig
2nd reviewer: Prof. Dr. Britta Tietjen

Date of defense: 14th December 2021

Declaration:
I, Juan Fernando Dueñas Serrano, hereby confirm that my doctoral thesis has been preared independently and without impermissible help. I further confirm that my doctoral thesis is not based on work written as part of any of my previous degrees.

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Foreword

This dissertation is cumulative, and encompasses three manuscripts that are either published or in preparation (Chapter 2–4). The publication of Chapter 2 was done in compliance with my supervisor, Prof. Dr. Matthias C. Rillig. Two additional chapters provide a general introduction (Chapter 1) and a general discussion (Chapter 5). All the reference cited in this work have been merged in a common reference section at the end of the dissertation.

I. **Chapter 2: Dueñas JF, Camenzind T, Roy J, Hempel S, Homeier J, Suárez JP, Rillig MC**. **2020**. Moderate phosphorus additions consistently affect community composition of arbuscular mycorrhizal fungi in tropical montane forests in southern Ecuador. *New Phytologist* **227**: 1505–1518. doi: 10.1111/nph.16641

II. **Chapter 3: Dueñas JF, Hempel S, Homeier J, Suárez JP, Rillig MC, Camenzind T**. **2021**. Non-mycorrhizal root associated fungi of a tropical montane forest are relatively robust to the long-term addition of moderate rates of nitrogen and phosphorus. In preparation.

III. **Chapter 4: Dueñas JF, Rillig MC**. Finding a suitable base for soil microbial diversity: should we account for differences in bulk density in comparative microbial community studies? In preparation.

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Summary/Zusammenfassung

Summary

Primary tropical montane forests along the eastern Andes contribute significantly to the storage of carbon and to the designation of the tropical Andes as a biodiversity hotspot. Despite their remoteness, these unique ecosystems are increasingly experiencing the effects of different global change factors. Of particular concern, given the adaptation of eastern Andean forests to infertile soils, is the increased deposition rate of nitrogen and phosphorus resulting from the intensification of human activities in neighboring regions. Tropical montane forest trees rely on arbuscular mycorrhizal fungi (AMF) to obtain nutrients from the scarcely available pool found in eastern Andean soils. In addition to AMF, it is believed that tropical trees interact with a broad range of fungal taxa that range from facultative saprobes to specialized plant pathogens. Given root associated fungi stand at the interphase of soil and plants and are directly involved in the flow of nutrients, studying their response to an increased availability of N and P can give insight into the response of the ecosystem to this disturbance.

The work presented in this dissertation is a compendium of three scientific contributions. Two of them document the response of root associated fungal communities to increased availability of nitrogen and phosphorus and one is a methodological viewpoint that critically assesses the suitability of current sampling strategies to study soil microbial communities. The empirical studies collected mixed root samples from a fertilization experiment seven years after the manipulation started. This experiment simulated the atmospheric deposition rates expected for the eastern Andes, by fertilizing the forest floor at a rate of 50 kg N ha^{-1} yr^{-1} and 10 kg P ha^{-1} yr^{-1}. To increase the external validity of the manipulation, the experiment was deployed in forests occurring along an elevation gradient (1000–3000 m above sea level) that represents the typical transition from pre-montane to upper montane forests observed in the eastern Andes. High-throughput sequencing was employed to characterize AM and non-AM fungal communities recovered from DNA extracted from mixed root samples.

Based on previous studies, it was hypothesized that chronic fertilization will decrease AMF diversity and community composition given plants will invest less C in the symbiosis. As the ambient N and P availabilities shift with elevation and it is likely that AMF clades have distinct nutritional niches, it was further hypothesized that site and clade specific responses will be observed. Regarding additional clades of root associated fungi, it was expected that fertilization will reduce diversity and alter community composition. It was assumed that the pool of soil fungi available to interact with roots decreases, since it is unlikely all fungal taxa are able to regulate their elemental stoichiometry to maintain homeostasis with the altered soil stoichiometry elicited by fertilization.

AMF alpha diversity decreased with elevation and there was a strong turnover of operational taxonomic units (OTUs) across sites, which indicates AMF taxa have narrow environmental niches. Non-AM fungal communities were taxonomically rich, mostly including phylotypes within Ascomycota, Basidiomycota and Mortierellomycota. Guild structure was also diverse, and mostly included fungal saprobes and plant pathogens. Fertilization consistently altered AMF community composition along the elevation gradient, but only reduced Glomeraceae richness. Compositional changes were mainly driven by increases in P supply while richness reductions were observed only after combined N and P additions. Taxonomic richness of non-AM fungal communities was not affected by fertilization, neither at the kingdom nor at the phylum level. In contrast, community composition shifted, particularly among Ascomycota and after the addition of P. These findings suggest that, unlike AMF, non-AM fungal communities are less sensitive to shifts in soil nutrient availability.

Overall the findings reported in this dissertation expand our understanding about the response of root associated fungi to increased nutrient availability in tropical systems. Sensitivity of AMF communities to P fertilization is in line with previous literature and causes concern in light of the current trends of atmospheric deposition. We confirmed that tropical montane trees interact with diverse fungal communities, and these appear to be robust to the addition of nutrients. Despite the fact that we characterized root associated fungal communities at a resolution that has never been achieved before in this region, we are still lacking the most basic understanding of the functional roles and trophic modes of most members of these communities. Hence, we hope the patterns revealed in these studies inspire further exploration of tropical montane fungi.

Zusammenfassung

Die tropischen Primär-Bergwälder entlang der östlichen Anden tragen erheblich zur Kohlenstoffspeicherung und zur Einstufung der tropischen Anden als Hotspot der biologischen Vielfalt bei. Trotz ihrer Abgeschiedenheit sind diese einzigartigen Ökosysteme zunehmend den Auswirkungen verschiedener Faktoren des globalen Wandels ausgesetzt. Besonders besorgniserregend ist angesichts der Anpassung der östlichen Andenwälder an unfruchtbare Böden die erhöhte Deposition von Stickstoff (N) und Phosphor (P), die sich aus der Intensivierung menschlicher Aktivitäten in benachbarten Regionen ergibt. Tropische Bergwaldbäume sind auf arbuskuläre Mykorrhizapilze (AMF) angewiesen, um Nährstoffe aus dem kaum verfügbaren Pool der Böden zu erhalten. Neben den AMF stehen tropische Bäume vermutlich in Wechselwirkung mit einem breiten Spektrum von Pilztaxa, die von fakultativen Saprophyten bis hin zu spezialisierten Pflanzenpathogenen reichen. Da wurzelassoziierte Pilze an der Schnittstelle zwischen Boden und Pflanzen stehen und direkt am Nährstofftransfer aus dem Boden in die Pflanzen beteiligt sind, kann die Untersuchung ihrer Reaktion auf eine erhöhte Verfügbarkeit von N und P Aufschluss über die Reaktion des Ökosystems auf diese Störung geben.

Die Dissertation ist eine Zusammenstellung aus drei wissenschaftlichen Beiträgen. Zwei Beiträge dokumentieren die Reaktion von wurzelassoziierten Pilzgemeinschaften auf eine erhöhte Verfügbarkeit von N und P. Der 3. Beitrag ist ein methodischer Standpunkt, der die Eignung aktueller Probenahmestrategien zur Untersuchung mikrobieller Gemeinschaften im Boden kritisch bewertet. In den empirischen Studien wurden gemischte Wurzelproben aus einem Düngeexperiment sieben Jahre nach Beginn der Behandlung entnommen. Dieses Experiment simulierte die für die östlichen Anden erwarteten atmosphärischen Depositionsraten, indem der Waldboden mit einer Rate von 50 kg N ha^{-1} yr^{-1} und 10 kg P ha^{-1} yr^{-1} gedüngt wurde. Das Experiment wurde in Wäldern durchgeführt, die entlang eines Höhengradienten (1000-3000 m über dem Meeresspiegel) liegen, der den typischen Übergang von prämontanen zu hochmontanen Wäldern in den östlichen Anden darstellt. In der aus den Wurzelmischproben extrahierten DNA wurden mithilfe von Hochdurchsatz-Sequenzierung AM- und Nicht-AM-Pilzgemeinschaften charakterisiert.

Auf der Grundlage früherer Studien wurde die Hypothese aufgestellt, dass eine chronische Düngung die -Diversität und die Zusammensetzung der AMF-Gemeinschaft verringert, da die Pflanzen weniger Kohlenstoff (C) in die Symbiose investieren. Da sich die N- und P-Verfügbarkeit in der Umgebung mit der Höhenstufe der Wälder verschiebt und AMF-Stämme wahrscheinlich unterschiedliche Nahrungsnischen haben, wurde außerdem angenommen, dass standort- und stämmespezifische Reaktionen zu beobachten sind. Hinsichtlich weiterer nicht-AMF wurzelassoziierter Pilze wurde ebenfalls erwartet, dass die Düngung die Diversität verringert und die Zusammensetzung der Gemeinschaft verändert. Es wurde angenommen, dass die Vielfalt der Bodenpilze, die für die Interaktion mit den Wurzeln zur Verfügung steht, abnimmt, da nicht alle Pilztaxa in der Lage sind, ihre interne Elementhomöostase bei der infolge der Düngung stark veränderten Bodenstöchiometrie aufrechtzuerhalten.

Die AMF-Alpha-Diversität nahm mit zunehmender Höhenstufe der Wälder ab, und es gab eine starke Fluktuation der operational taxonomic units (OTUs) zwischen den Standorten, was darauf hindeutet, dass AMF-Taxa enge ökologische Nischen haben. Nicht-AM-Pilzgemeinschaften waren taxonomisch reichhaltig und umfassten hauptsächlich Phylotypen innerhalb der Ascomycota, Basidiomycota und Mortierellomycota. Die Gildenstruktur war ebenfalls vielfältig und umfasste vor allem pilzliche Saproben und Pflanzenpathogene. Die Düngung veränderte die Zusammensetzung der AMF-Gemeinschaft entlang des Höhengradienten, reduzierte aber nur die Vielfalt an Glomeraceae. Veränderungen in der Zusammensetzung wurden hauptsächlich durch eine erhöhte P-Zufuhr verursacht, während eine Verringerung der Vielfalt nur nach kombinierten N- und P-Zugaben beobachtet wurde. Die taxonomische Vielfalt der Pilzgemeinschaften, die nicht zu den AM gehören, wurde durch die Düngung nicht beeinflusst, weder auf der Ebene des Pflanzenreiches noch auf der Ebene der Stämme. Diese Ergebnisse deuten darauf hin, dass Pilzgemeinschaften, die nicht zu den AMF gehören, im Gegensatz zu den AMF weniger empfindlich auf Veränderungen der Nährstoffverfügbarkeit im Boden reagieren.

Insgesamt erweitern die in dieser Dissertation erzielten Ergebnisse unser Wissen über die Reaktion von wurzelassoziierten Pilzen auf eine erhöhte Nährstoffverfügbarkeit in tropischen Wäldern. Die Empfindlichkeit der AMF-Gemeinschaften gegenüber der P-

Düngung steht im Einklang mit der bisherigen Literatur und ist angesichts der aktuellen Trends der atmosphärischen Deposition besorgniserregend. Wir haben bestätigt, dass tropische Bergwaldbäume mit verschiedenen Pilzgemeinschaften interagieren, wobei diese anscheinend gegenüber der Zugabe von Nährstoffen unempfindlich sind. Obwohl wir die wurzelassoziierten Pilzgemeinschaften mit einer Genauigkeit charakterisiert haben, die in dieser Region noch nie erreicht wurde, fehlt uns noch immer ein grundlegendes Verständnis der funktionellen Rollen und trophischen Formen der meisten Mitglieder dieser Lebensgemeinschaften. Wir hoffen daher, dass die in diesen Studien aufgedeckten Muster zur weiteren Erforschung der tropischen Bergwaldpilze anregen.

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"*This portion of the surface of the globe affords in the smallest space the greatest possible variety of impressions from the contemplation of nature*"

Alexander von Humboldt

1. Chapter 1 General Introduction

1.1. Tropical montane forests on the eastern Andes

Tropical forests are renowned for their biodiversity and their contribution to maintain the stability of earth's climate (Kreft & Jetz, 2007; Hubau *et al.*, 2020). They are primary forests (according to the definition of the Food and Agriculture Organization, FAO, 2020) surrounding the Equator between the tropics of Capricorn (23.44° N) and Cancer (23.44° S). Tropical forests occurring in Central and South America belong to the Neotropical biogeographical province (Antonelli *et al.*, 2015). While the largest portion of primary forests in the Neotropics lies in the Amazon basin, tropical forests further occur along the foothills of major and minor mountain ranges across the region. The elevation shift within a short area imposes a series of unique environmental permutations that give rise to distinct ecosystems known collectively as tropical montane forests (Homeier *et al.*, 2010; Dalling *et al.*, 2016).

In tropical South America, large stretches of primary montane forests still occur along the humid eastern slopes of the Andes. Eastern Andean forests largely contribute to the declaration of the Andes as a biodiversity hotspot and are important carbon sinks (Myers *et al.*, 2000; Spracklen & Righelato, 2014; Duque *et al.*, 2021). Yet despite their remote location and ecological significance, these forests are facing multiple threats. In addition to high deforestation rates (Tapia-Armijos *et al.*, 2015), eastern Andean forest are increasingly experiencing the effects of global change (e.g. warming and atmospheric pollution, Cusack *et al.*, 2016). As there is generally little information on how tropical ecosystems will respond to global change, it is crucial to generate an understanding of how these unique ecosystems are being affected by global change factors.

The field work presented in this dissertation has taken place in southern Ecuador, in a set of sites representing the transition from tropical pre-montane to upper montane forests along the eastern slopes of the Andes (Figure 1.1). The information presented

in the following sections places a special focus on the literature of forests located in this specific region. For the remainder of this work, the denomination of “tropical montane forests” and “eastern Andean forests” will be used interchangeably.

1.2. Root associated fungal communities

The kingdom Fungi constitutes a diverse, widely distributed and abundant eukaryote clade (Spatafora *et al.*, 2017; Bar-On *et al.*, 2018; Naranjo-Ortiz & Gabaldón, 2019). Fungi, together with bacteria, are prominent soil inhabitants and constitute the foundation of the soil trophic chain (Fierer, 2017). As fungi are one of the primary decomposers found in soils, particularly in the organic layer of forests (Baldrian, 2017), their activities are essential to support plant and animal life (van der Heijden *et al.*, 2008). Thus it is not an overstatement to say that fungi are crucial for the maintenance of element cycles in terrestrial ecosystems.

By virtue of their broad metabolic capacities (Treseder & Lennon, 2015), fungi are capable of exploiting a series of substrates, enabling them to live independently as free living saprobes or in association with other organisms as mutualists, parasites or commensals (Rodriguez *et al.*, 2009; Hardoim *et al.*, 2015). The degree of association between fungus and host ranges from obligate to facultative (Hardoim *et al.*, 2015).

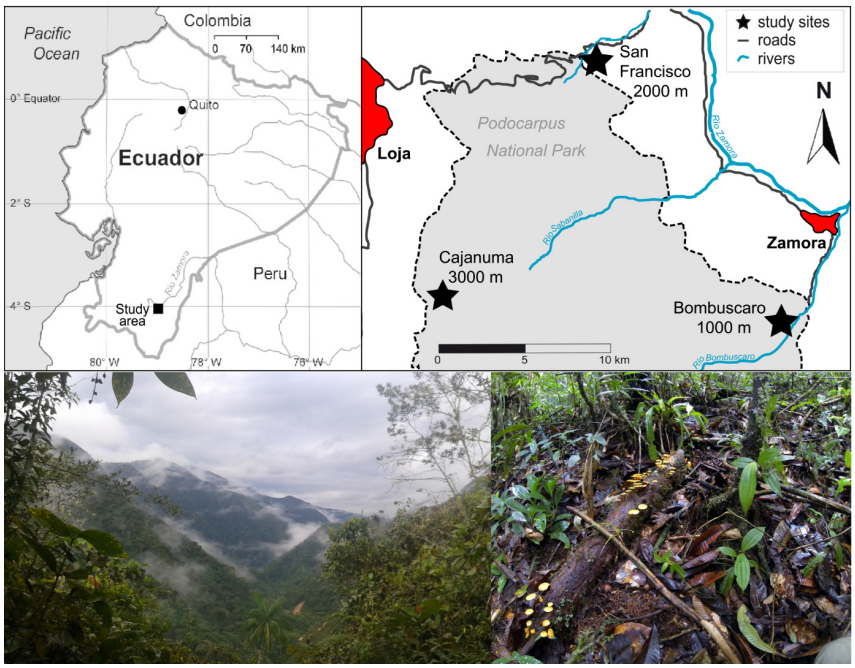

Figure 1.1 Overall impression of the study area

The above panels provide an overall impression of the study area. The black square on the map in the upper left shows the location of the study site in continental Ecuador. Black stars

in the upper right map indicate the location and elevation of each experimental site surveyed as part of this dissertation. The picture in the lower left depicts a typical vista of eastern Andean forests and the constant incidence of clouds. The lower right picture shows fungal fruiting bodies emerging from a decomposing log which lies over the organic matter-rich forest floor in San Francisco River valley; one of the most intensively studied sites included in this dissertation. The map is taken from Homeier *et al*. (2013) and reproduced with permission of the author and publisher. Pictures were taken by the author of this dissertation.

1.3. Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi (AMF) belong to the phylum Glomeromycota, an early diverging monophyletic clade within the fungal kingdom (Naranjo-Ortiz & Gabaldón, 2019). AMF species have lost their capacity to derive Carbon (C) independently, thus requiring to form associations with living plants in order to obtain C and lipids (Powell & Rillig, 2018). AMF are well known for their capacity to form nutritional mutualisms with plants, particularly for their capacity to mineralize and exchange Phosphorus (P) with their hosts. In addition to the nutritional benefit, AMF are thought to improve plant fitness by protecting hosts from the attack of parasites (Veresoglou & Rillig, 2012). AMF are further considered important C conduits to the soil, thus contributing to the stimulation of soil processes such as the cycling of Nitrogen (N, Veresoglou *et al.*, 2012) but also to the fixation of C belowground (Rillig, 2004). Because AMF have broad host ranges, i.e. AMF species can form simultaneous partnerships with different plant species, their presence is considered to promote plant diversity (van der Heijden *et al.*, 2015; Bachelot *et al.*, 2017).

AMF are the dominant mycorrhizal form in Neotropical forests (Kottke *et al.*, 2004; Smith & Read, 2008; Steidinger *et al.*, 2019) and are in general the most frequent mycorrhizal association found in the plant kingdom (van der Heijden *et al.*, 2015). While most of our understanding of AMF in tropical systems is derived from studies conducted in temperate ecosystems, there have been several ecological studies of these symbionts taking place in tropical montane forests in recent years (Homeier *et al.*, 2012; Haug *et al.*, 2013, 2019; Camenzind & Rillig, 2013; Camenzind *et al.*, 2014, 2016b,a; Cárate-Tandalla *et al.*, 2018). The present dissertation intends to contribute to this growing body of literature.

1.4. Root associated fungi beyond AMF

In contrast to the extensive knowledge about AMF ecology, studies focusing on additional clades of root associated fungi (RAF) are rare (Baldrian, 2017), especially in the tropics (Luo *et al.*, 2014; Sato *et al.*, 2015; Schroeder *et al.*, 2018, 2019). One

of the reasons for this paucity might be that plants engage in associations with a wide range of fungi whose nutritional modes are unknown or manifest only in very specific conditions (Aguilar-Trigueros *et al.*, 2014). Unlike AMF, other clades of RAF do not share a common morphology, trophic mode or ancestry. Rather, RAF are a heterogeneous group that include other mycorrhizal types, mutualistic endophytes, parasites and facultative saprotrophs (Rodriguez *et al.*, 2009; Hardoim *et al.*, 2015). Root association is thus an ecological parameter that describes members of the fungal kingdom that can be found on the surface or on the inside of plant roots, but whose function or nutrition mode can only be hinted at by their taxonomic identity and prior information. Uncertainty in such designations is large and arises because many fungal taxa display a wide range of lifestyles and trophic modes (Aguilar-Trigueros *et al.*, 2014; Griffin & Carson, 2018).

While the categorization of RAF is admittedly imprecise, it still represents a useful framework to organize the study of fungi at the community level, and to reveal general patterns that can later be confirmed by focused experimentation. For instance, some of the most devastating plant pathogens are found in the fungal kingdom (Spatafora *et al.*, 2017; Naranjo-Ortiz & Gabaldón, 2019). Pathogenic fungi are a highly diversified guild in the tropics and are believed to perform an important control function, thus allowing the coexistence of diverse plant species (Wright, 2002; Bachelot *et al.*, 2015). Hence characterizing plant pathogen communities is an important first step to determine whether parasite pools are shifting in response to environmental change, and whether this represents a cause of concern as it can potentially affect seed germination and seedling recruitment (Mangan *et al.*, 2010; Sarmiento *et al.*, 2017). In addition to the characterization of plant pathogens, assessment of root associated communities can uncover the presence of alternative mycorrhizal modes or mutualistic endophytes, thus allowing to discover additional mechanisms by which tropical plants cope with nutrient limitation or other environmental constraints. Finally, considering fungal saprobe abundance and richness are high in the organic layers of tropical montane forests (Cusack *et al.*, 2011; Whitaker *et al.*, 2014; Peay *et al.*, 2017) and assuming a considerable portion of root associated fungi are inactive saprobes recruited from soil, monitoring root associated saprobe communities can offer insight on how soil fungal saprobes respond to shifts in the environment.

1.5. The study area - floristic and climatic aspects of eastern Andean forests

Montane tropical forests on the eastern Andes host a large number of plant species, many of which are endemics (Myers *et al.*, 2000; Homeier *et al.*, 2010). An active geology (Hoorn *et al.*, 2010), past climatic events or the onset of steep geomorphological gradients within a short area are some of the historical contingencies explaining the extraordinary biodiversity and endemism observed in this region today (Antonelli & Sanmartín, 2011; Rangel *et al.*, 2018). Although the region as a whole is highly diverse, the environmental variation imposed by elevation and topography represents an insurmountable barrier to many species. As a consequence, increments in elevation mark a decrease in spermatophyte richness as well as an extensive replacement of species (Homeier *et al.*, 2010; Bañares-de-Dios *et al.*, 2020).

Mean annual temperature decreases with elevation at an approximate rate of 4° C for every 1000 m gained in elevation (Girardin *et al.*, 2010; Baez *et al.*, 2015). This roughly places eastern Andean forests occurring between 1000 m above sea level and the tree line within the lower and upper montane thermal belt categories of Körner *et al*. (2011). Eastern Andean forests are further characterized by high precipitation rates (> 2000 mm yr^{-1}) and the permanent incidence of ground level clouds (Bendix *et al.*, 2006; Rollenbeck & Bendix, 2011). Rain seasonality is complex, but is mainly governed by the transport of water and air masses from the Amazon to the Andes through the trade winds. In the eastern Andean forests of southern Ecuador, precipitation maxima can be observed between the months of May and August while a period of reduced precipitation can occur during the austral spring months when dominant easterlies give way to westerlies (September-December, Rollenbeck & Bendix, 2011). Cooler temperatures, high precipitation and cloud immersion are correlated with a series of structural and physiological changes in vegetation relative to lowland tropical forests (Oliveira *et al.*, 2014; Dalling *et al.*, 2016). That is, forest growth, aboveground productivity, leaf area and stand stature generally decrease with elevation (Moser *et al.*, 2007; Girardin *et al.*, 2010; Unger *et al.*, 2012; Baez *et al.*, 2015). By contrast, the belowground portion of these forests shows the reverse trend, with root biomass increasing along with elevation (Leuschner *et al.*, 2007; Moser *et al.*, 2011; Girardin *et al.*, 2013).

1.6. Soil in the eastern Andean forests

Eastern Andean forests soils are shallow, acidic and generally poor in exchangeable nutrients. Lower and upper montane forests soils are often classified as Cambisols (Wilcke *et al.*, 2002; Unger *et al.*, 2012), soils at the beginning of soil formation, whereas soils towards higher elevations have been characterized as Histosols or Podzols (Moser *et al.*, 2011). Relative to the deeper and highly weathered soils found in eastern Amazonia, soils near the Andes tend to contain higher nutrient pools (Quesada *et al.*, 2010). This pattern is often explained as the result of a prolonged denudation of nutrients from Andean soils towards the western Amazon lowlands. In addition to the loss of nutrients towards western Amazonian soils, tectonic activity, high precipitation rates and steep slopes trigger frequent landslides, thereby transporting soil to the valley bottoms and re-setting the process of soil formation on the exposed slopes (Fisher *et al.*, 2013; Dalling *et al.*, 2016).

A defining feature of tropical montane forest soils is the accumulation of thick layers of slowly decomposing biomass (hereafter referred to as organic layers). These layers are densely rooted (Leuschner *et al.*, 2007; Soethe *et al.*, 2008; Moser *et al.*, 2011), thus playing a crucial role in nutrition of these ecosystems. Organic layer thickness generally increases with elevation (Fisher *et al*., 2013; Soethe *et al*., 2008; Unger *et al*., 2012; Wilcke *et al*., 2008), but varies within elevation belts according to the slope position (Wolf *et al.*, 2011). That is, forests on gentle slopes and at elevations higher than 1500 m above sea level tend to accumulate thick organic layers (10 – 60 cm). Leaves, seeds, flowers, twigs and wood can still be recognized in the first few centimeters of these layers, while organic matter is fractionated and devoid of the features that link it to its origin as depth increases. By contrast, the organic layer thickness in valleys or at lower elevations is gradually reduced, preserving only recently shed plant material.

Macronutrient stocks (N, P, K, Ca, Mg and S) in the organic layers are substantial, tending to increase with depth of the organic horizon (Wilcke *et al.*, 2002, 2008; Unger *et al.*, 2010). Similar to other nutrients, C stocks in the densely rooted organic layers is considerable. However, as these nutrients are bound to organic matter, their availability in the ecosystem depends largely on the mineralizing capacity of soil organisms. This capacity is generally reduced as elevation increases, indicated by the high C:N and C:P ratios of plant litter (Wilcke *et al.*, 2008; Soethe *et al.*, 2008; Unger

et al., 2010). Low bulk density of the organic layer (Unger *et al.*, 2010), lower temperature and hypoxia due to frequent water stagnation (Wilcke *et al.*, 2008; Soethe *et al.*, 2008) are often presented as probable causes for this phenomenon. So a paradox arises: lower mineralization rates lead to a reduction in the supply of macronutrients in exchangeable form whereas the nutrient stocks are maintained across elevations by virtue of an increase in the organic layer thickness. Low availability of nutrients affect forest productivity and growth, as indicated by a significant decrease in foliar N:P ratios at higher elevations (Wilcke *et al.*, 2008; Soethe *et al.*, 2008; Fisher *et al.*, 2013). This suggests that while pre-montane and lower montane plant communities suffer primarily from P-limitation (Homeier *et al.*, 2012; Cárate-Tandalla *et al.*, 2018), N and P co-limitation becomes more frequent at upper montane sites (Moser *et al.*, 2011; Fisher *et al.*, 2013).

1.7. Increased rates of atmospheric nutrient deposition and its relevance to Andean forests

Increased atmospheric N deposition is a global phenomenon triggered by a strong increment of air pollution with reactive inorganic N compounds (Galloway *et al.*, 2008). The most frequent of these compounds are nitrogen oxides (NO_x) and volatilized ammonia (NH_3), with combustion of fossil fuels and agricultural activities the main known sources. It is also believed that an undetermined portion of these emissions result from wildfires (Galloway *et al.*, 2008). Increased N deposition drastically augments the N load to ecosystems, thus eventually leading to saturation and acidification (Aber *et al.*, 1998). Beyond the point of N saturation, it is hypothesized that ecosystems decrease their capacity to assimilate N, thus leading to N losses back to the atmosphere and further to water systems. In addition to an increment in the export of unassimilated N, ecosystems could gradually lose productivity and biodiversity as other soil nutrients become scarcer and new soil conditions favor species adapted to high N availability. While the environmental impacts of increased N deposition have long been known and have been researched in temperate systems (Aber *et al.*, 1998), not much is known about how tropical ecosystems will respond to increased N depositions (Matson *et al.*, 1999). This causes concern, given it is anticipated that N pollution will continue to increase during the first half of this century, mainly driven by emissions produced in developing nations (Galloway *et al.*, 2008).

This means that increased N deposition will reach remote areas of high ecological significance, such as tropical montane forests (Phoenix *et al.*, 2006).

In contrast to N pollution, human-driven increments in the availability of atmospheric P are not large (Mahowald *et al.*, 2008; Peñuelas *et al.*, 2013). P does not have a stable gaseous phase, hence it reaches the atmosphere as solid aerosol particles (Mahowald *et al.*, 2008; Peñuelas *et al.*, 2013). Examples of P-aerosols are dust clouds originated in deserts, small particles of biological origin (e.g. polen and spores), or particles derived during the combustion of fuels or biomass (Mahowald *et al.*, 2008). Particles released during fires can be carried across large distances, ultimately to be 'recaptured' by the canopy of forests downwind (Bauters *et al.*, 2021). P losses to the atmosphere have attracted attention, as they signal an irreparable reduction in the fertility of the source ecosystems. However, the environmental consequences of P transport and its subsequent deposition should also be carefully considered. Low P availability is considered an important driver of plant diversity (Wassen *et al.*, 2005; Ceulemans *et al.*, 2014). Hence, it is reasonable to believe that an increment in P deposition could, in the medium to long term, affect the biodiversity of receiving ecosystems. On the other hand, increased P deposition could mitigate some of the deleterious impacts of increased N deposition (Chen *et al.*, 2016).

Consistent with the projections of Phoenix *et al*. (2006), just at the turn of the century, traces of inorganic N compounds were detected in fog and water samples collected in several stations located across the eastern Andes of southern Ecuador (Fabian *et al.*, 2005). Although the rate of N deposition inferred from these samples was low (1.5–4.4 kg N ha^{-1} yr^{-1}), it was clearly higher than the expectation for areas without a significant human influence (~ 0.5 kg N ha^{-1} yr^{-1}, Galloway *et al.*, 2008). As the eastern Andes ridge acts as a barrier that shields montane forests from the pollution of inter Andean cities, it was suggested that episodic increases in N deposition could be related to the burning of forests in the neighboring Amazon. Conversion of land to agriculture via forest fires is an extended practice throughout the tropics and certainly prevalent in the Amazon (Cochrane, 2003), thus lending credibility to this hypothesis. A four-year monitoring (1998–2002) of the incident rain over different micro catchments distributed across a lower montane forest in southern Ecuador confirmed initial results (Boy *et al.*, 2008). It was determined that eastern Andean forests were experiencing a significant increment of inorganic N (i.e. nitrate and ammonium)

deposition (9.5–10 kg N ha^{-1} yr^{-1}) and a non-significant increment in P deposition (0.64–1.1 kg P ha^{-1} yr^{-1}). Boy and colleagues (2008) further found a strong correlation between nutrient deposition increments and the incidence of fire events in the Amazon basin, thus ruling out other possible sources. Extensions of this initial monitoring effort over longer time periods further confirmed the increment of N and P loads on this system (Wilcke *et al.*, 2013, 2019). It was shown that in little more than a decade (1998–2013), eastern Andean forests have experienced a strong increase in atmospheric N and P deposition rates (~9 kg N ha^{-1} yr^{-1} and ~2.4 kg P ha^{-1} yr^{-1}). This means that the N deposition rate observed in this remote ecosystem is well within the range of critical N loads[1] determined for European forests (Phoenix *et al.*, 2006; Waldner *et al.*, 2015).

Early awareness of the increases in N and P deposition rates, motivated the establishment of a nutrient manipulation experiment (NUMEX) in the eastern Andean forests in southern Ecuador (Homeier *et al.*, 2012, 2013). NUMEX has been applying 50 kg N ha^{-1} yr^{-1} and 10 kg P ha^{-1} yr^{-1} in a full factorial randomized blocked design deployed at three different sites since 2008 (Figure 1.1). Such experiment offered an ideal platform to assess how increased rates of atmospheric N and P deposition affect different aspects of the ecology of eastern Andean forests.

1.8. Aims and main hypotheses of this dissertation

Given root associated fungi can exert strong feedbacks to the plant community and are sensitive to shifts in the soil nutrient condition, an assessment of fungal community responses to increases in the availability of nutrients is crucial. Prior studies within the NUMEX context have largely focused on measuring abundance shifts as a result of fertilization (Homeier *et al.*, 2012; Krashevska *et al*. 2013; Camenzind *et al.*, 2016a; Cárate-Tandalla *et al.*, 2018). The present set of contributions rather focuses on changes in diversity and community composition elicited by fertilization, which so far have been assessed two years after the experiment begun (Camenzind *et al*. 2014). Thus the main goal of this dissertation is to assess how root associated fungal communities in tropical montane forests respond to increases in N and P availability, seven years after the experiment started.

[1] The critical load concept determines a maximum level of exposure to N deposition, beyond which significant harmful effects on selected ecosystem properties are expected given current knowledge

Three main hypotheses were addressed:

1) AMF diversity will decrease and community composition will change, possibly favoring lineages adapted to higher nutrient availability, due to increases in N and P availability

2) the strength and direction of the response will be modulated by the time since the fertilization was initiated and by the acclimation of plant and AMF communities to differences in background nutrient availabilities observed at different elevations

3) non-AM fungal diversity will decrease and community composition will be altered given fertilization will favor taxa capable of adapting to the shifted elemental stoichiometry of both plant roots and the organic layer elicited by fertilization.

In addition to the main goal, this dissertation includes a methodological excursus that adresses the question of how current soil sample collection practices can affect our inference about microbial communities.

2. Chapter 2 Moderate phosphorus additions consistently affect community composition of arbuscular mycorrhizal fungi in tropical montane forests in southern Ecuador

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2.1. Introduction

Tropical Andean forests are centers of endemism and constitute the most biodiverse region of the world per unit area (Rahbek *et al.*, 2019b). Despite the large contribution of these forests to preserve Earth's biodiversity, many aspects of their ecology remain unresolved. Most notably, the role that soils –and soil dwelling organisms– play in shaping these ecosystems' response to global change drivers (Baez *et al.*, 2015; Hagedorn *et al.*, 2019). This is particularly relevant for tropical Andes, as montane forest soils store considerable amounts of carbon (C) (Girardin *et al.*, 2010; Moser *et al.*, 2011; Spracklen & Righelato, 2014), yet the drivers controlling C fluxes are shifting in this region. In the past two decades, the intensification of human activities in the neighboring Amazonian plains has fueled a moderate increment in the deposition rates of reactive nitrogen (N) (Wilcke *et al.*, 2013; Velescu *et al.*, 2016) and phosphorus (P) (Wilcke *et al.*, 2019) into the eastern Andes. Given that N and P are arguably the main soil elements regulating C cycling, and that their availability also affects soil microbes and the processes they drive (Camenzind *et al.*, 2018), understanding how tropical montane forests change in the face of ongoing soil eutrophication, requires a deeper understanding of how soil microbial communities respond to these disturbances.

Arbuscular mycorrhizal fungi (AMF) –a basal sub-phylum of mutualistic fungi (Glomeromycotina, Spatafora *et al.*, 2016)–form the most common type of mycorrhizal symbiosis worldwide (van der Heijden *et al.*, 2015), and are the dominant mutualists

in Andean tropical forests (Smith & Read, 2008; Kottke *et al.*, 2008). AMF are ecologically relevant because they increase the uptake of P in exchange for plant derived C (Smith & Smith, 2012), and to a lesser extent the uptake of inorganic N (Hodge & Storer, 2015; Ushio *et al.*, 2017). Because of their prominent role in the flow of nutrients, assessing AMF community responses to shifting nutrient pulses might serve to establish a link between AMF diversity and ecosystem function (Rillig, 2004).

Based on what is currently known about the nutritional attributes of the symbiosis, several predictions on how AMF diversity may respond to increased N and P availability can be attempted. From a resource economy perspective (Johnson, 2010), when atmospheric deposition increases P supply beyond limitation, the benefit of the symbiosis is reduced (Johnson *et al.*, 2015). This may intensify competition between AMF taxa for plant derived C and for soil nutrients, as well as between the host and AMF for inorganic N. In both cases, a reduction in AMF diversity can be expected. Conversely, in cases when P supply is the most limiting resource (i.e. N supply increases beyond limitation), the benefit of the symbiosis is enhanced. In this case AMF diversity levels might be maintained. The situation is considerably more nuanced when hosts are N and P co-limited. In this scenario, the nutritional benefit of the symbiosis will still be required, yet weak competition between AMF taxa for resources (Powell & Rillig, 2018) might lead to shifts in community composition. This last prediction is congruent with the co-adaptation model (Johnson, 2010). This model predicts that over time, ambient nutrient status selects sets of plants and fungi that are able to co-exist and maximize the exchange of resources (Johnson *et al.*, 2010).

Quite importantly, all these predictions assume that each AMF taxon occupies a defined nutritional niche (Treseder & Allen, 2002). This assumption is underpinned by the fact that AMF isolates differ in the benefits they provide to plants (Koch *et al.*, 2017), and by different clades (e.g. families) differing in susceptibility to fertilization regimes (van der Heyde *et al.*, 2017; Treseder *et al.*, 2018; Roy *et al*., 2019). Using classical abundance measures (e.g., root colonization, hyphal length), which are frequently used to assess fertilization effects, it is not possible to capture differences in responses of different AMF taxa to nutrient enrichment (Treseder, 2004). Information at such higher level of resolution can only be obtained by sequencing surveys. Yet the scarcity of surveys of this kind in tropical areas has been repeatedly noted in the literature (Cotton, 2018; Lilleskov *et al.*, 2019), particularly for the tropical

Andes (Soteras *et al.*, 2019). We are aware of only two deep sequencing studies conducted at AMF dominated neo-tropical forests within the context of nutrient manipulation experiments (Camenzind *et al.*, 2014; Sheldrake *et al.*, 2018). These studies showed AMF diversity decreases when N is added alone or in combination with P, while community structure is affected mainly by the addition of P. These responses, however, appear to be modulated by the fertilization regime, the duration and dosage of the application, and whether AMF communities were characterized from DNA isolated from roots or soil.

Given that virtually all aspects of AMF ecology are understudied in the tropics, it is evident that important gaps in our understanding still remain. First, studies conducted on tropical AMF communities in the context of increased nutrient supply are geographically narrow. Given whole ecosystem manipulations are resource intensive, these can only be maintained over relatively small areas (Fayle *et al.*, 2015). Hence the majority of such experiments in the tropics have been established in mesic lowland forests that grow over P-deficient soils (Matson *et al.*, 1999; Mirmanto *et al.*, 1999; Kaspari *et al.*, 2008; Cusack *et al.*, 2011). In tropical montane forests, however, plants obtain most of their nutrients from thick layers of organic detritus of very heterogeneous nutritional condition (Tanner *et al.*, 1998; Wilcke *et al.*, 2002). This heterogeneity is thought to originate from the interaction of parent material of different age and composition (Hoorn *et al.*, 2010) with climate (i.e. thermal isoclines, cloud immersion, seasonal precipitation patterns, Rahbek *et al.*, 2019a) and topography (Tanner *et al.*, 1992; Werner & Homeier, 2015). In addition to the geographic bias, there is a temporal one. Up until now, assessments of tropical AMF communities within nutrient addition experiments have not been reproduced, thereby ignoring the temporal dimension of the disturbance (Zhang *et al.*, 2018). Finally, the majority of tropical nutrient manipulation experiments have set rates of mineral fertilization with the goal of assessing plant growth limitations (Tanner *et al.*, 1992; Mirmanto *et al.*, 1999; Kaspari *et al.*, 2008). These, however, often exceed the actual rates of atmospheric nutrient deposition that these regions experience (Cusack *et al.*, 2010).

In this paper, we assess the responses of tropical forest AMF communities to increased nutrient deposition in a more realistic scenario. We do so by surveying a seven year-long fully factorial nitrogen (N) and phosphorus (P) addition experiment in southern Ecuador (Homeier *et al.*, 2012). This experiment is fully replicated at three

sites where P is the main limiting element for tree growth (Cárate-Tandalla et al. 2018), but its availability, as well as that of mineralized N, is modulated by local environmental conditions (Martinson *et al.*, 2013). One of these sites was surveyed after two years of simulated atmospheric deposition (Camenzind *et al.*, 2014), indicating important short-term reductions in AMF species richness. Here we focus on assessing the long-term response and increasing the external validity of our results by including all three sites within the experiment. We hypothesized that: 1) there will be a decrease in AMF molecular diversity after fertilization in sites with greater P availability, 2) nutrient applications will shift AMF community composition, but these shifts will be mediated by ambient availability of nutrients at different sites, and 3) assuming AMF lineages differ in terms of nutrient use and exchange capacities, clade responses to nutrient applications will be also different. To the best of our knowledge, this constitutes the most encompassing assessment of nutrient addition effects on naturally occurring AMF dominated forests.

2.2. Materials and Methods

Study site

Experimental work occurred on three sites along the south eastern Andes of Ecuador. Sites are located at an average distance of 19 km and at an average elevation difference of 1000 m of each other, starting at ~ 1000 m above sea level (m.a.s.l., Appendix A, Fig. S2.1). All sites are within protected areas and are covered by different forest types (Homeier *et al.*, 2013). The lowest site corresponds to pre-montane forest, the mid site to lower montane forests and the highest site to upper montane forest. Tree species turnover is complete between pre- and upper montane forests while fewer than five species are shared between lower montane and the other two forest types (Homeier *et al.*, 2013). Canopy openness and stand height are reduced, while fine root biomass sharply increases at the upper montane forest in relation to the other two forest classes (Moser *et al.*, 2011). From pre-montane to upper montane forest, understorey vegetation becomes denser with decreasing canopy openness. This stratum is mainly composed of tree recruits, herbaceous monocots, ferns, and a few woody shrub species (J. Homeier, pers. comm).

Climate at the three sites is permanently humid and strongly influenced by the dominant easterlies coming from the Amazon. Radar and ground station data indicate

high precipitation totals that increase towards the upper montane forest (2000–4500 mm y^{-1}, Homeier *et al.*, 2010; Rollenbeck & Bendix, 2011). Precipitation patterns are weakly seasonal with a maximum usually distributed from April to July. Minima occur towards the end of the year (Sep-Dec), when the dominant easterlies briefly give way to westerlies coming from the Pacific Ocean (Oñate-Valdivieso *et al.*, 2018). Temperature regimes also shift between sites. Direct measurements of average daily temperature show a decrease from ~ 19° to ~9° C between the pre- and upper montane sites (Moser *et al.*, 2007).

Soil physical and chemical characteristics also change between sites. Soils at the lower and upper montane sites are covered by 10–40 cm deep organic layers, have a propensity to water logging and a loamy mineral fraction (Wolf *et al.*, 2011; Werner & Homeier, 2015). At the pre-montane forest, soil texture becomes sandy, leading to a better drainage and the organic horizon depth is reduced close to 0 cm. Organic layers are generally acidic (pH range: 3–5) and suffer from chronic nutrient deficiencies. N and P availability tends to increase in the pre-montane forest relative to the lower and upper montane forests (Wolf *et al.*, 2011; Werner & Homeier, 2015). Despite of this, tree growth at all sites is predominantly limited by P availability (Graefe *et al.*, 2010; Cárate-Tandalla *et al*. 2018).

Additional details of each site edaphic and climatic characteristics can be found in Appendix A, Table S2.1.

Experimental design

A full factorial nutrient manipulation experiment started at each site in January 2008 (Homeier *et al.*, 2012, Appendix A, Fig. S2.1b). Since then, urea (50 kg of N ha^{-1} y^{-1}) and monosodium phosphate (10 kg P ha^{-1} y^{-1}) were applied manually every six months. These rates of application are moderate relative to the rates applied in similar experiments elsewhere (Liu *et al.*, 2015a; Sheldrake *et al.*, 2018) and correspond well to the annual rates of atmospheric deposition quantified at the lower montane forest site between years 2007–2012 (Velescu *et al.*, 2016; Wilcke et al. 2019). Experimental factors are applied in a randomized block arrangement. That is, on each site there are four blocks of four plots each (16 plots per site, 48 plots total). Each block consists of three plots with different nutrient application regimes (+N, +P and +N+P) and one unfertilized plot (Ctrl). Ctrl plots were always located above fertilized plots to avoid

fertilizer runoff. Fertilization regime was assigned randomly at the start of the experiment for the remaining plots. While randomization mitigates the effects of confounding sources of variability, blocking ensures greater homogeneity in environmental conditions between sets of plots. Plots are 400 m^2 and are at least 10 m apart to ensure independence of experimental units.

In August 2015, a 10 cm soil core (Ø 5 cm) was extracted from the organic layer of six sub-plots (4 m^2) within each treatment plot. Sub-plots were randomly established along two orthogonal transects. We sampled one core within each sub-plot. This yielded a total of 96 cores per site (Figure 2.1). In order to standardize our sampling procedure, approximately 20 fine root pieces of 1–2 cm length and < 2 mm diameter were separated from the organic layer of each soil core and subsequently preserved in 97% EtOH. Roots were favored over soil, because DNA extraction from the organic layer of these forests is cumbersome and hinders amplification. Samples were kept frozen upon their transport to the molecular ecology laboratories at the Institute for Biology of Freie Universität Berlin, where they were finally stored at -20° C.

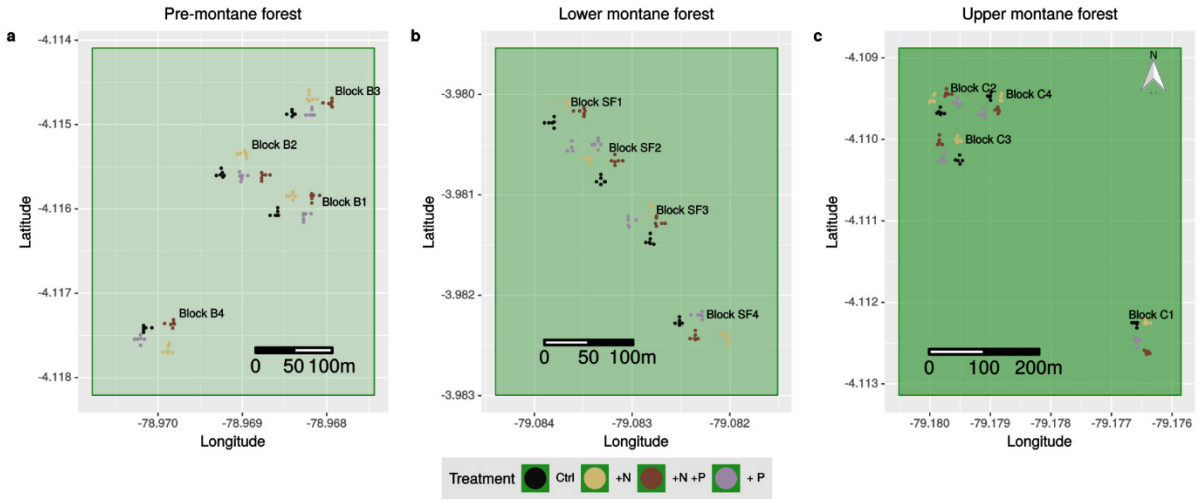

Figure 2.1 Spatial distribution of cores collected for this study

Each cluster of six points of the same color represent cores within a plot according to their fertilization regime. Text indicates the relative position of blocks at each site, each encompassing four plots (i.e. 24 cores). Core position was allocated randomly using two orthogonal transects within each plot. Map coordinates are in decimal degrees and polygons in various shades of green intend to remind the reader that each site is different in terms of soil and plant community composition, forest structure and climate.

DNA extraction, PCR amplification and sequencing

Roots from each of the samples were lyophilized overnight (Alpha 1-4 LDplus, Christ GmbH., Harz, Germany). Upon lyophilization, roots were pulverized by shaking a 2 mm metal bead along with roots in a 2 ml tube placed within a MM400 mill (2 min of 25 oscillations per second, Retsch GmbH., Hann, Germany). DNA was isolated from pulverized roots following the PowerSoil DNA isolation kit (MoBio Laboratories Inc., Carlsband, CA, USA) standard protocol. DNA extracts were stored at -20° C upon amplification. In order to minimize contamination, blank extracts were included, and all materials used were sterilized.

The genetic polymorphism within the nuclear rDNA operon was assessed adopting a nested PCR strategy. DNA extracts were amplified with a cocktail of Glomeromycotina specific primer sets developed by Krüger *et al.* (2009), in two consecutive PCR rounds. A third and final PCR round targeted a ~400 bp fragment spanning the D1 and D2 variable domains of LSU with the LR2rev–LR3 primer set[2] (Roy *et al.*, 2017). Amplicon libraries were sequenced in three separate reactions on an Illumina MiSeq platform using 2x250 paired-end chemistry at the Berlin Center for Genomics in Biodiversity Research (BeGenDiv).

Bioinformatic processing and taxonomic assignment

Paired-end reads were processed in USEARCH v.10 (Edgar, 2010). Reads from each site were processed separately for the merging, primer sequence removal and filtering steps. Reads that passed the filtering criteria were then combined in a single file for subsequent steps. MOTHUR (Schloss *et al.*, 2009) was employed to retain sequences

[2] DNA was amplified in 25 µl reactions containing a hot start polymerase with proofreading capacity. Approximately 20 ng of DNA extract (and 1 µl of product from the second reaction onwards), 0.2 mM of each dNTP, 0.3 µM of forward and reverse primers, 1x of KAPA-hifi buffer solution and 0.5 U of KAPA-hifi polymerase were added to each reaction. Final volume of reaction was completed with appropriate volumes of sterilized water. The first two reaction solutions were amplified by the following thermal cycling program: denaturation for 2 min at 95° C, followed by 30 cycles of 30 sec of denaturation at 94° C, 30 sec annealing at 60° C and 40 sec elongation at 72° C. A 2 min final elongation at 72° C closed the reaction. On the third PCR, initial denaturation ocurred for 3 min at 95° C, followed by 30 cycles of 20 sec denaturation at 98° C, 30 sec annealing at 47° C and 30 sec elongation at 72° C. Final elongation was increased to 3 min at 72° C. Negative controls were implemented across all phases of the amplification regime to detect cross-contamination. PCR products were purified using a gel purification kit (Macherey-Nagel, Düren, Germany). A 12 cycle amplification reaction ligated unique combinations of 8 nt tags on each extreme of the purified amplicons. Reaction conditions were the same as in the nested PCR. Labelled amplicons were further purified through the application of magnetic beads (GC Biotech, Alphen aan den Rijn, The Netherlands), followed by DNA quantitation with the Pico-Green assay kit in a fluorescence microplate reader (Thermo Fisher Scientific Inc., Germany). One individual library per elevation belt containing c. 25ng of each sample product was pooled.

with at least 375 bp and less than seven homopolymers. Sequences were clustered *de novo* into operational taxonomic units (OTUs) with UPARSE (Edgar, 2013), the minimum OTU cluster size was set to 8 and sequence similarity threshold to 97%. Chimera removal and clustering occurred simultaneously. Merged reads of each site were then mapped to OTUs to produce an OTU abundance table. Sequences representing these OTUs are deposited at the European Nucleotide Archive (ENA), under accession numbers LR656271–LR656682.

Phylotype taxonomic identity was assigned by aligning OTUs to Krüger *et al*. (2012) reference database using BLAST+ (Camacho *et al.*, 2009). Only the query sequences with alignment coverage ≥ 90 % were retained. Following Martinez-García *et al*. (2015), an OTU was assigned to species level when the best hit was ≥ 97% identical to a reference sequence, to genus when identity was between 90–96%, and to family when identity was between 80–90%.

Environmental factors

One composite sample of the organic layer was created by aggregating and homogenizing six sub-plot samples extracted from each plot (n = 48). Air dried samples were then transported to the plant ecology laboratories at the University of Göttingen, Germany. Soil pH was determined by suspension of the sample in a KCl solution; organic soil C and N with a C/N analyzer (Vario EL III, Elementar, Hanau, Germany) and plant-available P with the resin-bag method (Amer *et al.*, 1955). Finally, all trees with a diameter at breast height ≥ 10 cm were identified to species level in order to calculate tree species richness per plot.

Statistical analyses

All statistical analyses were performed in R (v. 3.4.3, R Core Team, 2017). Packages adespatial (Dray *et al.*, 2019), DESeq2 (Love *et al.*, 2014), dplyr (Wickham *et al.*, 2018), ggplot2 (Wickham, 2016), ggpubr (Kassambara, 2018), lme4 (Bates *et al.*, 2015), lmerTest (Kuznetsova *et al.*, 2017), mvabund (Wang *et al.*, 2012), phyloseq (McMurdie & Holmes, 2013), rgdal (Bivand *et al.*, 2019), sp (Bivand *et al.*, 2013) and vegan (Oksanen *et al.*, 2018) were employed. The commands used for the analyses can be found in Appendix A, Table S2.2.

Variability of environmental factors across sites and plots

To visualize how environmental factors varied across plots and sites, variability was collapsed using a principal component analysis (PCA). Variables were scaled and centered and the two most informative axes were plotted.

Normalization of sequencing data

As is typically observed in high throughput sequencing data, there was a high number of samples with few sequences and few samples with high number of counts (Appendix A, Fig. S2.2a). To account for the large differences in sequencing depth across samples, a variance stabilization transformation (VST) was applied (Love *et al.*, 2014). VST avoids rarefying to an arbitrary minimum sequencing depth while preserving the integrity of the data (McMurdie & Holmes, 2014; Sheldrake *et al.*, 2018). Applying VST normalized the density distribution of sequencing depth (Appendix A, Fig. S2.2b) while still allowing a sufficient coverage to characterize the diversity of AMF across samples (Appendix A, Fig. S2.3). Thus, the transformed table was used for all subsequent analyses.

AMF molecular diversity indices

Following Morris *et al*. (2014), per sample AMF molecular diversity (hereafter referred as 'alpha diversity') was quantified by two indices: Hill number 0 (H_0) and 2 (H_2). H_0 and H_2 are generalized forms of popular diversity indices that facilitate comparisons across studies given they express taxonomic diversity in standardized units (Hill, 1973). H_0 equals richness (S) and expresses the number of OTUs per sample while H_2 equals to the inverse of Simpson's dominance index and expresses the effective number of 'abundant' OTUs per sample (Chao *et al.*, 2014). To visualize how alpha diversity partitioned between different families within Glomeromycotina, H_0 and H_2 were also estimated by segregating OTU tables of the most represented families in our dataset (i.e. Acaulosporaceae, Glomeraceae and Gigasporaceae). To visualize AMF taxa turnover across sites, OTUs with relative abundance equal or greater to 1% were selected and their presence and relative abundance was plotted.

Effects of nutrient addition on AMF molecular diversity

The response of AMF alpha diversity to fertilization was inferred through linear mixed effects models (LMMs, Bates *et al.*, 2015). To meet model assumptions, H_0 and H_2 estimates were square root transformed and specified as response variables. N and

P were specified as fixed terms (i.e. sqrt(H_x) ~ N x P). To account for the random variability imposed by the experimental design, a nested random term was specified (i.e. 1|Site/Block/Plot). Given that including all components of the random term led to model over-fitting (blocks contributed to explain 0 % of residual variability in H_0 and H_2, Appendix A, Table S2.3), the random structure of the models was re-specified as 1|Site/Plot. The full OTU dataset and the per-family OTU data sub-sets were fitted to this model structure. The difference from control in mean H_x explained by the nutrient treatment regime, hereafter referred as the effect size, was used to infer the impact of nutrient addition on AMF alpha diversity. To visualize the magnitude and direction of these effect sizes and to provide a measure of uncertainty, 95 % confidence intervals around effect sizes were estimated by refitting the model 1000 times with parametric bootstraps of the original data (Morris, 2002). In addition to this, we ascertained the effect of nutrient application regimes with classical null hypothesis significance testing by performing *t* tests. The null hypothesis was that the difference from control was not different from 0. Given that the current implementation of mixed models in lme4 package does not estimate *P*-values, these were determined via lmerTest package (Kuznetsova *et al.*, 2017).

Effects of nutrient addition on AMF community composition

The effects of fertilization on AMF community composition were examined with multivariate generalized linear models (MGLM, Wang *et al.*, 2012). MGLMs can handle multivariate response variables in which the variance is not constant (Warton *et al.*, 2012), which is the case here (Appendix A, Fig. S2.4). Given the compositional nature of the data (Gloor *et al.*, 2017), phylotype proportions cannot be considered to represent the abundance of AMF taxa in the environment. Consequently, to assess if fertilization elicits a change in AMF community composition, we focused on OTU occurrence data. Because our goal was to assess if the effect of each fertilization factor differed among sites – and since MGLMs cannot handle random effects, a separate model for each site was specified. Spatial dependencies in OTU presence within each site were accounted for by Moran eigenvectors maps (MEMs, Dray *et al.*, 2006). MEMs were estimated according to the method developed by Bauman et al. (2018b). This is both an estimation and selection procedure that yields a set of MEMs that optimally describe the spatial structures observed in biotic communities (Bauman *et al.*, 2018a). Thus, the selected MEMs were specified as predictors in each of the

MGLMs (i.e. OTU occurrence ~ MEMs + N x P). The variance structure for all three models was specified as binomial. Finally, deviance tests were performed on each MGLM to measure the strength of nutrient addition effects on AMF community composition. If the sequential inclusion of explanatory terms significantly increased the fit of the data in relation to a reduced model, then such factor was considered to have a significant influence on OTU occurrence. In addition to this, distance based redundancy analysis plots based on Jaccard dissimilarity matrices were employed to visualize the effects of treatments on AMF community composition (RDA, Legendre & Anderson, 1999). One RDA per site was specified as a two-way model (N x P), including MEMs as conditional covariates.

Sensitivity analysis To test the robustness of our results and compare to previously observed short-term effects (Camenzind *et al*. 2014), the whole dataset was re-analyzed with traditionally applied statistical procedures (i.e. rarefying to a common minimum depth and PERMANOVA, Anderson, 2001; Oksanen *et al.*, 2018)[3].

2.3. Results

Taxonomic delineation and assignation

A total of 280 samples were amplified and generated 12,625,525 merged reads. Six samples with less than 10 reads each were discarded as they were considered defective and 503,495 unique sequences were retained after filtering. These sequences were clustered in 628 OTUs at 97% similarity, of which 65.6% (412 OTUs, 87.77% of reads) identified with known Glomeromycotina sequences. All Glomeromycotina OTUs were assigned to three orders and six families, but c. 75% of these reads could be assigned to a known genus.

[3] OTU abundance table was randomly re-sampled without replacement to a minimum depth of 850 reads, as this was the minimum read depth chosen by Camenzind et al. (2014). This procedure will be hereafter referred as rarefaction. Then richness and the inverse Simpson indices, as well as Jaccard dissimilarity matrices were estimated from the rarefied dataset. To test nutrient effects on alpha diversity, two way linear mixed models with the same structure described in the main method section of the text were fitted. Null hypothesis significance testing with *t*-test was employed to infer whether nutrient treatments elicited a meaningful effect on AMF alpha diversity estimates. The effect of fertilization on beta diversity was assessed by a two way permutational multivariate analysis of variance (Anderson, 2001) including MEMs as covariates. Results of these procedures are presented in Appendix A, Table S2.6–7, and briefly mentioned in the main text when differences are relevant.

Environmental variation and AMF community properties across sites

PCA of environmental factors indicated that environmental conditions in plots at the lower and upper montane forests were similar and differed from the conditions at the pre-montane forest site (Figure 2.2). In general, all experimental plots were characterized by low fertility and acidic soils. However, soils at the lower and upper montane plots had lower N and greater P availability than soils at the pre-montane site. In contrast, soils at the pre-montane site had higher pH and supported more diverse tree communities (Appendix A, Table S2.4). AMF OTU accumulation curves indicate that pre-montane plant communities hosted on average 126 more OTUs than lower and upper montane forests, which relative to each other reached a very similar number of OTUs (Appendix A, Fig. S2.5).

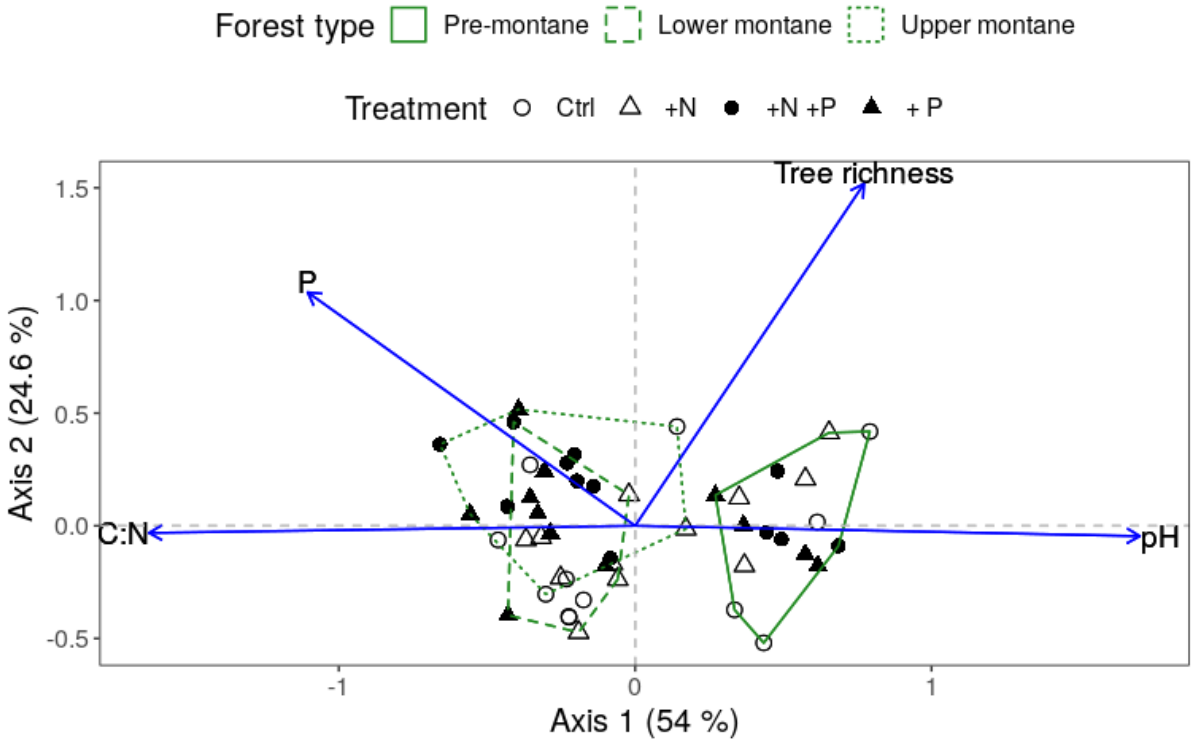

Figure 2.2 Site variability in environmental parameters

PCA of soil organic layer parameters and tree species richness. Two axes were sufficient to capture 79.3 % of within site variability in soil parameters (n = 48). Filled and open symbols represent homogenized soil samples according to fertilization regime and hulls enclose all samples within a site.

Alpha diversity and relative abundance of reads of the most represented families within Glomeromycotina traced this pattern. While in the pre-montane plots OTUs assigned

to Glomeraceae were more diverse and encompassed a greater proportion of reads than Acaulosporaceae, at both lower and upper montane sites OTUs assigned to Acaulosporaceae were more diverse and contributed with a greater proportion of reads than Glomeraceae (Fig. 2.3).

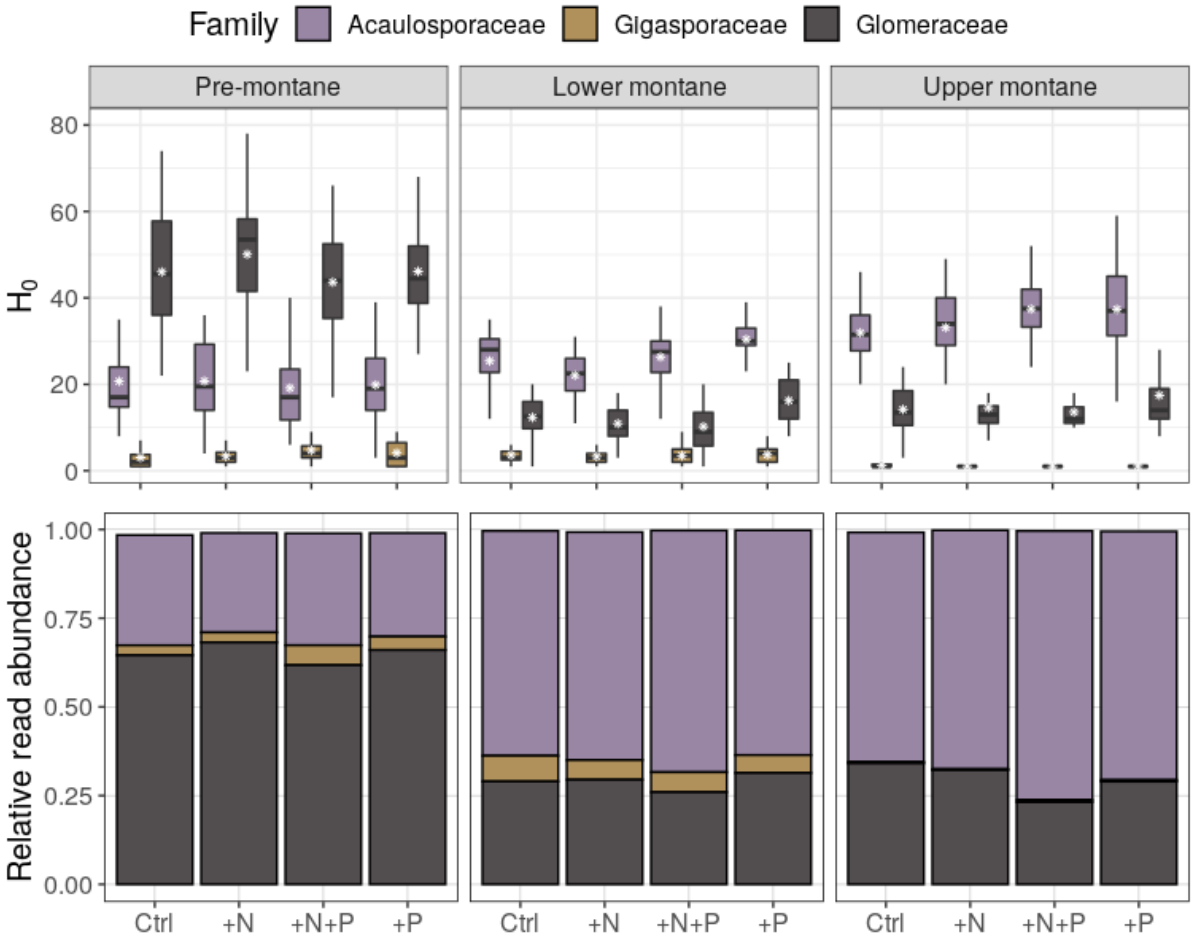

Figure 2.3 Diversity and relative read abundance of phylotypes assigned to main clades within Glomeromycotina

H_0 (Richness) of phylotypes and proportion of reads assigned to the most represented families across sites. White stars within boxplots represent the mean while the mid-horizontal line represents the median.

Turnover of the most represented OTUs within these families, however, was strong across sites. None of the aforementioned OTUs occurred in all three sites whereas c. 10% of these OTUs were shared between the lower montane and one of the other two sites (Fig. 2.4).

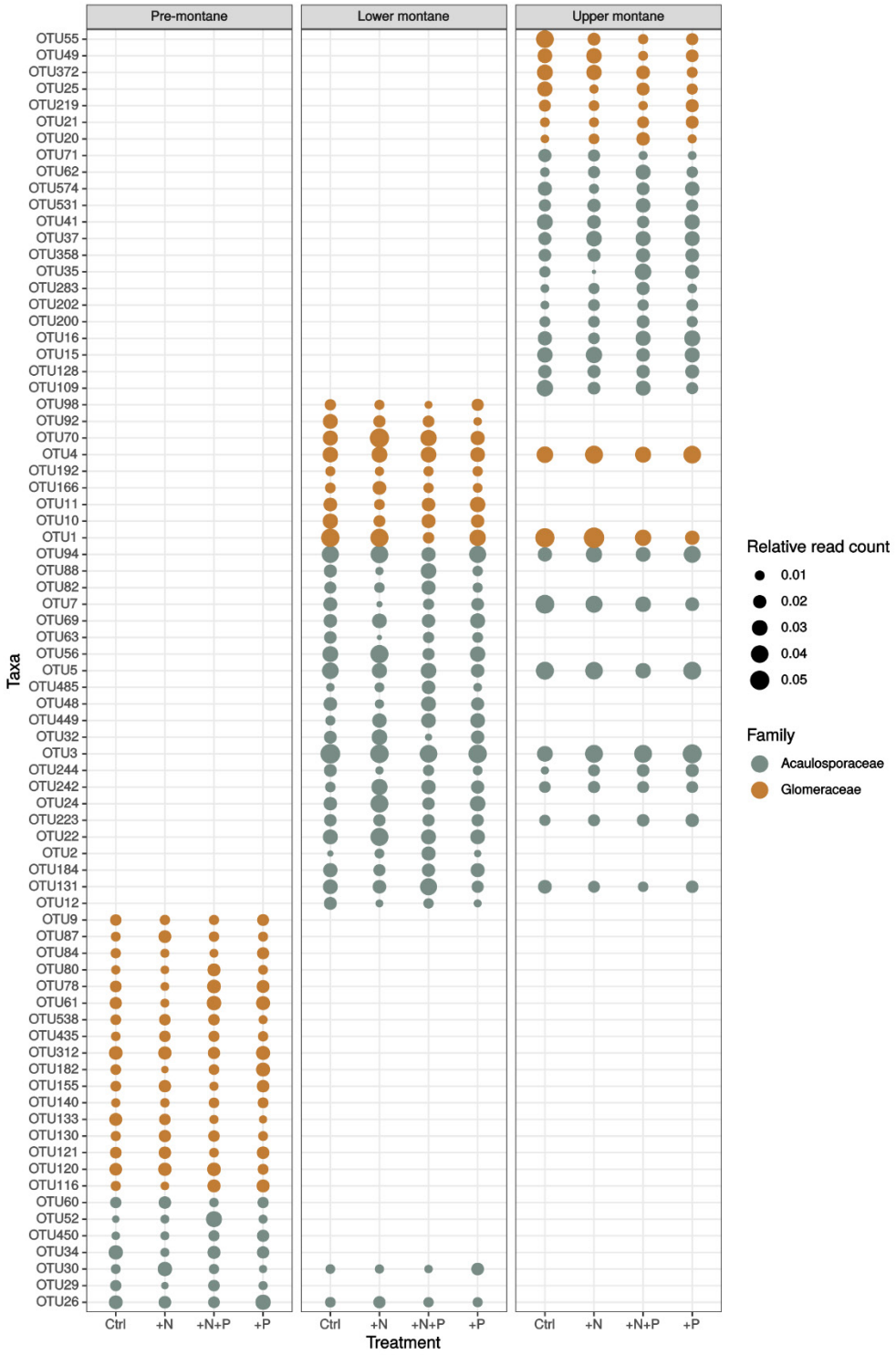

Figure 2.4 Turnover abundant OTUs across sites

OTUs were selected if their relative abundance was greater than 1% of the total. Taxa are ordered by family to emphasize their turnover across sites.

Effects of nutrient addition on AMF molecular diversity

Responses of diversity indices to fertilization regime were minimal and statistically insignificant when analyzing all sites together (Appendix A, Table S2.5). Closer inspection of effect sizes estimated for each site confirmed these differences were not biologically meaningful at any site (Fig. 2.5a). When the analysis was partitioned among families, no effect was observed with only one exception. Glomeraceae mean H_0 and H_2 decreased by 5.2 (± 2.4 SE) and 3.7 (± 1.7 SE) OTUs respectively (P = 0.02 and 0.02) as a response to the combined addition of N and P. The negative effect of the combined addition of N and P on Glomeraceae was consistent across sites (Fig. 2.5b).

Effects of nutrient addition on AMF community composition

Deviance tests indicate that nutrient addition consistently affected AMF community composition at every site (Table 2.1). Fertilization effects on community composition were dependent on the nutrient added and the ambient nutrient status at each site. While adding N did not elicit a shift in AMF community composition only in the pre-montane forest, adding P alone consistently elicited community shifts at all sites. Given that the most represented OTUs across sites are present in all fertilization regimes, the shifts detected by deviance tests are most likely driven by the appearance and disappearance of rare OTUs. Test results were robust to the inclusion of eigenvector maps, which also increased the fit of every model significantly. This suggests that, in addition to the fertilization effects, spatially structured factors also contribute to explain the observed variability in AMF community composition. RDAs on Jaccard dissimilarity index are congruent with this result, as these indicate that nutrient factors explained on average 3.87% variability in AMF community structure, while conditioned MEMs explained 15.5% (Fig. 2.6).

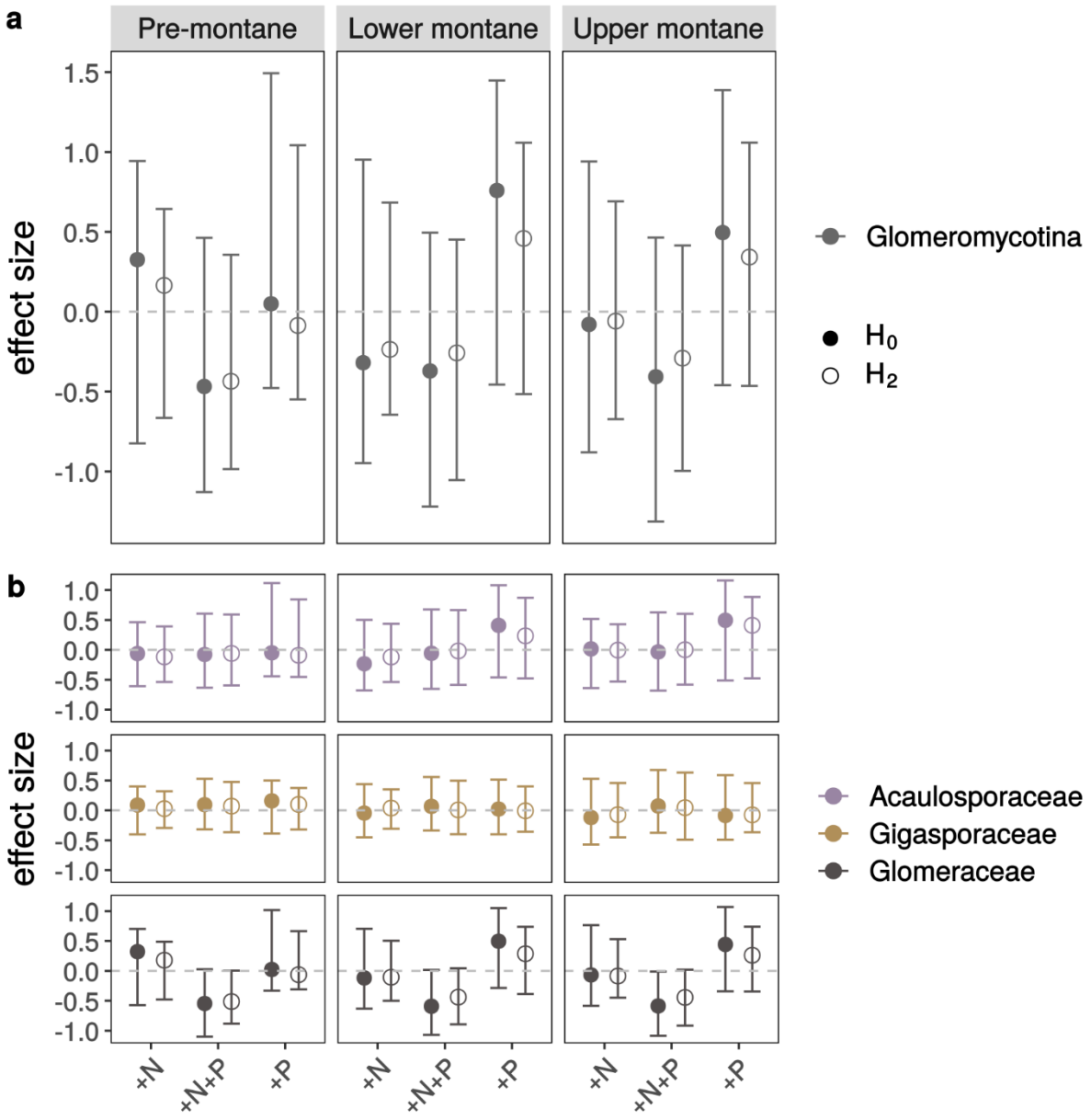

Figure 2.5 Estimated differences in mean H_0 (Richness) and H_2 (1/Simpson's dominance) of fertilized plots in relation to controls at each experimental site

Overall differences at the sub-phylum level are presented in (a) while (b) presents differences at the family level. The magnitude of the differences is presented in the square-root scale. Open and closed symbols represent point estimates and whiskers represent their 95% confidence intervals estimated by refitting the model 1000 times with parametric bootstraps of the original data. A 0.5 increase or decrease represents a difference of approximately 5 units.

Table 2.1 Deviance test results

Deviance test describe how each predictor contributed to improve the fit of the observed data to the model. MEMs stand for Moran's Eigenvector maps.

Site	Model	df	Deviance	*p*
Pre-montane	intercept	95		
	+ MEMs	86	4857.440	<0.001
	+ N	85	393.527	0.553
	+ P	84	593.784	<0.001
	+N:P	83	520.412	<0.001
Lower montane	intercept	88		
	+ MEMs	79	2862.472	<0.001
	+ N	78	260.035	0.030
	+ P	77	331.201	<0.001
	+N:P	76	297.538	<0.001
Upper montane	intercept	88		
	+ MEMs	80	2368.596	0.003
	+ N	79	329.737	0.003
	+ P	78	399.214	<0.001
	+N:P	77	215.143	0.054

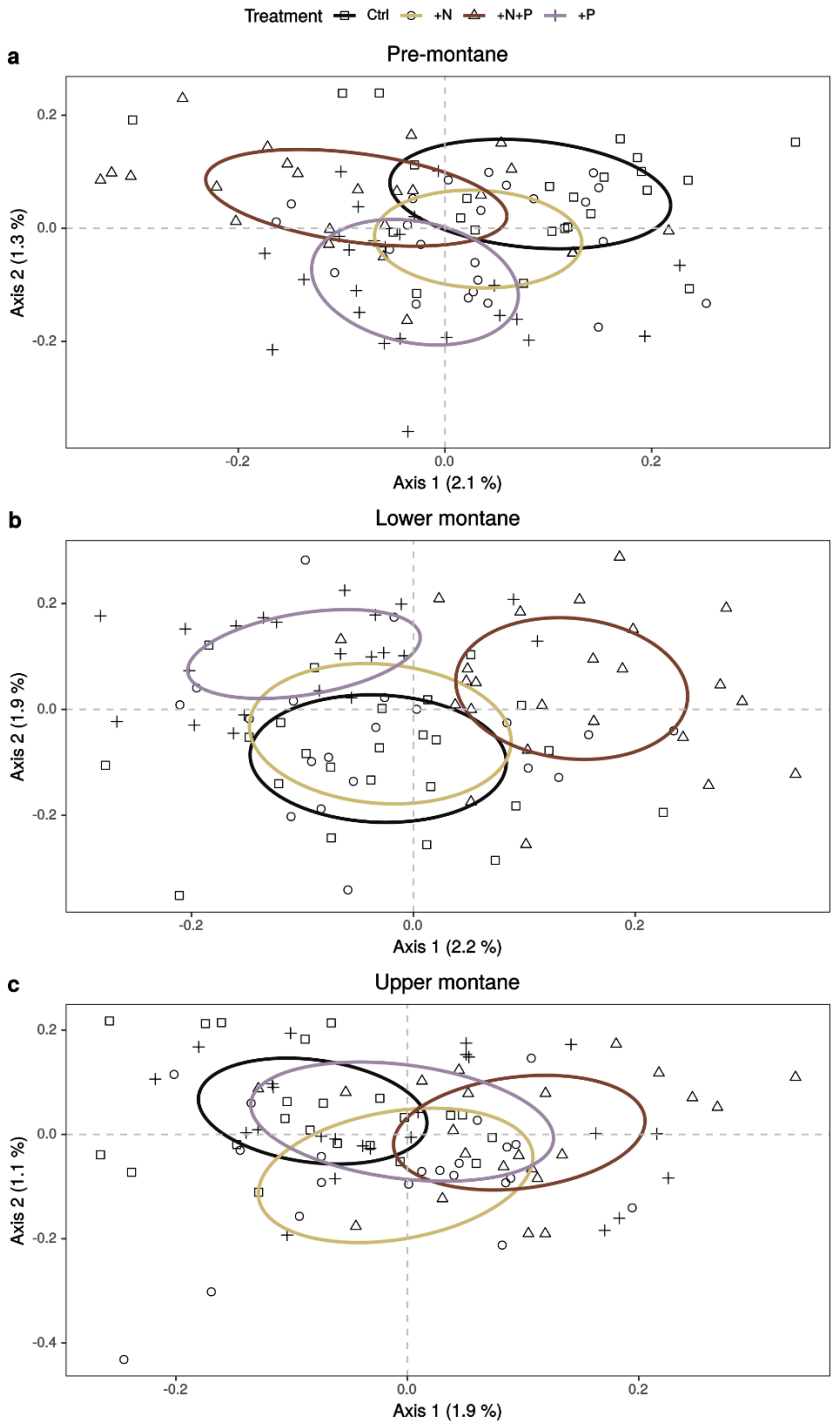

Figure 2.6 Constrained ordination plots depicting the influence of nutrient addition on AMF communities

Panels a–c present one ordination per site. Pairwise Jaccard distances were estimated from a normalized OTU table. Ellipses represent one standard deviation from group centroids. Two axes explained 4.3%, 4.5% and 3.9% variability in AMF community composition, after spatial dependencies were conditionally partialled out.

Sensitivity tests

Re-analysis of the dataset, using more traditionally employed statistical procedures, did not change results qualitatively (Appendix A Table S2.6 and S2.7). Rarefying to a minimum depth of 850 reads eliminated 3 AMF OTUs compared with VST. PERMANOVA on Jaccard distances found addition of P affects AMF community composition except for the upper montane forest site. In contrast the addition of N alone or in combination with P did not elicit shifts in AMF community composition.

2.4. Discussion

Our cross-site analysis indicates that tropical montane forests harbor highly diverse AMF communities that appear to be structured by site specific environmental conditions. We provide evidence that indicates seven years of moderate N and P fertilization rates have affected AMF community composition but not richness, a finding consistent among sites. Nutrient effects are indeed mild, but remain clear even when spatial dependencies in AMF community composition are accounted for. Our results further suggest that fertilization effects depend on site ambient nutrient status, since N addition did not affect AMF communities in pre-montane forests, while P shifted community composition independently of soil nutrient status. Furthermore, the composition of the regional OTU pool was site specific and the response to fertilization was clade-specific, suggesting differences among AMF clades in terms of their adaptation to different nutrient conditions. Overall, our results indicate that the rate of atmospheric nutrient deposition experienced by these forests constitutes a modest, yet consistent disturbance for AMF communities.

Both the phylotype pool and mean richness in our study sites are one of the highest so far reported for AMF, yet still fall within the boundaries of previous global AMF diversity assessments (Kivlin *et al.*, 2011; Davison *et al.*, 2015). Our observations that there was a substantial turnover of AMF taxa at different sites are also congruent with recent literature that found a strong influence of elevation on AMF beta diversity (Geml *et al.*, 2014; Kivlin *et al.*, 2017; Haug *et al.*, 2019). Given that metabarcoding studies are not consistent in the strategies adopted to arrive at OTU definitions (Lekberg *et al*., 2014; Hart *et al*., 2015), and that elevation is a compound variable that usually involves a number of inter-related climatic, topographic and soil variables, it is not possible to generalize this pattern to other areas in the Andes. Nonetheless, recent

reports of high AMF molecular diversity on both dry (Rodriguez-Echeverria *et al.*, 2017; Morgan & Egerton-Warburton, 2017) and wet (Bachelot *et al.*, 2017; García de León *et al.*, 2018) tropical lowland forests lend support to the idea that tropical Andean forests harbor highly diverse AMF communities. Acaulosporaceae higher abundance and richness at sites with acidic pH and low N availability is congruent with the characterization of members of this clade as stress tolerant (Oehl *et al.*, 2009; Veresoglou *et al.*, 2013; Liu *et al.*, 2015b). By contrast, high abundance of Glomeraceae at the site with the lowest C/N ratio among the set is in line with the association of this clade with higher N availability (Treseder *et al.*, 2018).

We found little support for our first hypothesis that predicted an overall negative effect of fertilization on AMF alpha diversity, which included sites with a slightly higher P availability. These results deviate from the short-term responses reported during an earlier assessment at the lower montane forest site (Camenzind *et al.*, 2014). As re-analyzing our data with traditional statistical approaches yielded qualitatively similar results, it is unlikely our observations are caused by a technical bias. Rather, these results could be attributed to temporal variability in the response of AMF communities to increased nutrient supply. Wide shifts in the response of AMF intraradical structures to fertilization over time have been observed in our study sites (Camenzind *et al.*, 2016). Alternatively, given that classic fertilization experiments have typically applied N and P at much higher rates (Egerton-Warburton *et al.*, 2007; Liu *et al.*, 2012; Sheldrake *et al.*, 2018), the rather moderate rate employed in this study could have allowed AMF communities to respond to the new nutrient condition without impacting taxonomic richness. Multiple examples of neutral responses of AMF richness as a function of fertilization dosage can be found in the literature (Alguacil *et al.*, 2010; Vályi *et al.*, 2015). Overall, these results support the notion that the intensity and duration of fertilization could be modulating the responses of AMF both in terms of abundance (Zhang *et al.*, 2018), alpha and beta diversity (Roy *et al.*, 2017).

In line with our second hypothesis, N and P addition did affect AMF community composition, with the effects of P addition the most consistent factor across sites. This is congruent with previous reports at both our study area and other tropical forests (Alguacil *et al.*, 2010; Camenzind *et al.*, 2014; Sheldrake *et al.*, 2018). It also suggests that AMF in this region are primarily involved in P for C transactions and that ambient nutrient status is important to consider when attempting to predict AMF root community

responses to fertilization, as adding N alone did not affect AMF community composition as consistently as P. The addition of P may select for taxa with better ability to hoard P in order to maximize carbon gains from the host (Whiteside *et al*., 2019). Our results also indicate that spatially structured ecological processes are influencing how AMF communities in these forests assemble. As this study was not designed to disentangle and quantify the relative importance of different ecological processes on AMF community composition, we can only speculate about this point. Previous studies have shown that at small to intermediate spatial scales, neutral and environmental drivers interact to determine the structure of AMF communities (Caruso *et al.*, 2012; Veresoglou *et al.*, 2019). In tropical forests, there is wide array of available hosts which are likely employing a variety of strategies to cope with nutrient limitations (Nasto *et al.*, 2014; Sayer & Banin, 2016; Baez & Homeier, 2018). Yet the degree to which individual tree species may influence the distribution and assemblage of AMF communities has yet to be firmly established in the tropics. For instance, a single AMF phylotype has been shown to associate with as many as 28 species of trees in one of our study sites (Haug *et al.*, 2013). What appears more likely, is that the composition of AMF communities inferred from mixed root samples is simultaneously reflecting the variability introduced by the host, fine scale edaphic factors, stochastic processes and priority effects. In order to identify the drivers behind these patterns, new field assessments that quantify environmental variation at smaller spatial scales are required.

We observed differential responses to fertilization of clades within Glomeromycotina in terms of taxonomic diversity, which lends some support to our third hypothesis. Differential trait expression (Chagnon *et al.*, 2013) might explain this contrasting response to some extent. Taxa within Acaulosporaceae are known to exhibit slow growth, both intra and extra radically (Hart & Reader, 2002). These traits have traditionally been associated with high carbon use efficiency. Following this logic, it is plausible that the negligible effect of fertilization on richness of this lineage is explained by their efficient use of carbon. By contrast, Glomeraceae consistent reduction in taxonomic diversity after N and P additions suggests that some members of this clade have greater carbon demands. As certain members of this clade tend to exhibit a fast colonization rate and greater investment in intra radical growth (Hart & Reader, 2005), it could be argued that they have a less efficient use of carbon and possibly provide

less P for C benefit to the host (Pearson & Jakobsen, 1993). If this is so, nutrient addition might promote their down regulation by the host or their competitive exclusion by those taxa that indeed make a more efficient use of available C (Kiers et al., 2011). Despite our observations fitting well with a differential trait expression framework, the highlighted traits might also vary at the species level (Maherali & Klironomos, 2012; Koch *et al.*, 2017). Since trait information only exist for a fraction of AMF isolates, at this stage we simply miss empirical information to clearly link AMF traits to nutrient requirements or function. This prevents us to unequivocally establish whether differential adaptations to nutrient supply are the basis for the patterns reported here.

In conclusion, AM fungal communities appear to have adjusted to moderate nutrient additions at all experimental sites by shifting their composition relative to control sites, while species richness remained stable. These changes are more subtle than predicted by studies using higher doses of experimental fertilization, yet its robustness and consistency clearly suggest that such responses to ongoing atmospheric deposition can also be expected across the tropical Andes. Regarding functional implications, selection of AMF clades that invest less in extra-radical mycelium might reduce C storage below ground and retention of surplus products of N mineralization (Velescu *et al.*, 2016). Changes in AMF community structure elicited by fertilization could also set feedback loops in motion (Bever *et al.*, 2012; Neuenkamp *et al.*, 2018). This could favor plants adapted to high nutrient availability and promote their dominance (Baez & Homeier, 2018). In the long-term, an increasing dominance of fewer plant hosts, the so called ‘homogenization’ of the mycorrhizal environment (Caruso *et al.*, 2012), could support less diverse AMF communities (Liu *et al.*, 2012; Alguacil *et al.*, 2012; Johnson *et al.*, 2015). In order to fully capture the functional consequences for these ecosystems we need to gain a better understanding of how AMF taxa functional roles differ in these diverse ecosystems, and on how fine scale structural heterogeneity shapes AMF communities in tropical forests. Future research needs to tackle how AMF community parameters vary at finer temporal, spatial and phylogenetic resolutions. Most importantly, complementary studies about AMF nutrient demands, host effects and feedbacks deserve further attention.

2.5. Acknowledgements

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3. Chapter 3 Non-mycorrhizal root associated fungi of a tropical montane forest are relatively robust to the long-term addition of moderate rates of nitrogen and phosphorus

This chapter is an author's typesetted version of the manuscript:

Dueñas JF, Hempel S, Homeier J, Suárez JP, Rillig MC, Camenzind T. **2021**. Non-mycorrhizal root associated fungi of a tropical montane forest are relatively robust to the long-term addition of moderate rates of nitrogen and phosphorus. An earlier version of this manuscript is available in bioRxiv since 11.08.2021.

3.1. Introduction

Tropical montane forests are increasingly recognized as important global carbon (C) sinks (Spracklen & Righelato, 2014; Duque *et al.*, 2021). Although the question remains as to how such C is partitioned above- and belowground, available estimates suggest the belowground portion is large (Moser *et al.*, 2011; Girardin *et al.*, 2013; de la Cruz-Amo *et al.*, 2020). In contrast to the tight nutrient cycling observed in lowland tropical rainforests, C, nitrogen (N), phosphorus (P) and base cations tend to accumulate in thick layers of organic matter along mountainous slopes (Wilcke *et al.*, 2002). While it is unclear what drives such accumulation, it is often attributed to reduced soil biological activity due to cooler temperature regimes (de la Cruz-Amo *et al.*, 2020), water saturation and recalcitrance of recently shed litter and accumulated humus (Werner & Homeier, 2015). As inorganic N and P availability is likely to increase in tropical mountains due to human induced increases in atmospheric deposition (Cusack *et al.*, 2016; Velescu *et al.*, 2016; Wilcke *et al.*, 2019), there is growing concern this will significantly reduce the stock of recalcitrant nutrients. Stated succinctly, the question arises if increased atmospheric deposition of N and P could reduce tropical montane forests C sink strength while at the same time increase nutrient runoff and the emission of greenhouse gases.

Experimental addition of N in tropical forests has led to variety of responses that range from positive to negative, depending on the process under examination and the elevation at which the forest is located (Cusack *et al.*, 2016). By contrast, P addition

has led to a consistent increase in the rates of N fixation and decomposition regardless of elevation (Camenzind *et al.*, 2018). Given these discrepancies, it is relevant to investigate how tropical soil microbial communities respond to increases in inorganic N and P, by extending our analysis to the broadest set of environmental conditions in which tropical forests occur.

Fungi are at the base of the soil food web and, together with bacteria, are thought to be largely responsible for the mineralization of organic bound nutrients in the soil (van der Heijden *et al.*, 2008; Fierer, 2017). Fungi are abundant in the upper organic layer of tropical montane forests (Krashevska *et al.*, 2010; Cusack *et al.*, 2011) and are also believed to be highly diverse in the tropics (Meiser *et al.*, 2014; Tedersoo *et al.*, 2014; Zhou *et al.*, 2016). Despite such prominence, research on the effects of increased nutrient availability on tropical fungal communities is rare and focuses heavily on specialized groups within the kingdom (e.g. mycorrhizal fungi, reviewed in Cusack *et al.*, 2016). Beyond mycorrhizal fungi, it is possible that other fungal groups are as important to determine the ecosystem response to increased atmospheric deposition of nutrients. For instance, a recent model estimated that saprobic fungi account for 35% of soil respiration, in contrast to the 5% represented by mycorrhizal fungi (Fatichi *et al.*, 2019). Given most fungal endophytes are thought to associate with the plant via recruitment form the soil (Rodriguez *et al.*, 2009; Hardoim *et al.*, 2015), monitoring how non-mycorrhizal root associated fungal (RAF) communities respond to N and P fertilization can shed light on how soil fungal communities are responding. In addition to this, monitoring RAF communities can offer insights into the occurrence and response of fungal pathogenic or mutualistic guilds, which are highly relevant to ensure the co-occurrence of hyper-diverse tropical communities (Bachelot *et al.*, 2015). Thus by targeting RAF communities, it is possible to increase our understanding of the response of several often neglected components of fungal communities to soil eutrophication.

Predicting the consequences of increased nutrient availability for RAF communities is complex due to our lack of a basic understanding of how RAF diversity and community structure relate to soil nutrient availability. Notwithstanding this situation, we can employ the existing ecological theory as a predictive framework. The resource ratio model postulates the balance of resources will determine whether co-existence is possible between a set of competing species (Cardinale *et al.*, 2009). If nutrient

addition leads to resource imbalances between the substrate and organisms (Peñuelas *et al.*, 2013; Li *et al.*, 2016), and such imbalances are impossible to bridge, differential abilities to obtain the most limiting resource could lead to extinctions. In the short term, N addition in tropical montane forests stimulated N mineralization but not microbial N retention (Baldos *et al.*, 2015); reduced microbial biomass; and increased the production of fine root necromass (Homeier *et al.*, 2012). In the long term, N and P additions decreased total organic C and the pool of inorganic N. Together, these responses might affect the composition of C pools belowground. That is, an increased production of leaves and fine roots with shorter lifespans could signal an increased proportion of recalcitrant C inputs, yet a reduction in the exudation of more labile C compounds from living roots. Alterations of the C pool composition, and the N:P ratio of the substrate are key, given fungal stoichiometry appears to closely track the stoichiometry of soils in montane tropical forests (Nottingham *et al.*, 2015). Indeed, non-mycorrhizal soil fungal communities have increased in biomass after the simultaneous addition of a C source together with a mineral nutrient in these forests (Krashevska *et al.*, 2010; Nottingham *et al.*, 2018b). Hence, it is reasonable to expect that asymmetric rates of nutrient addition will elicit the response of RAF communities. The direction and magnitude of such change, however, is still unclear.

To test these ideas, we surveyed the fine root fraction of an old growth tropical Andean forest subjected to long-term nutrient manipulation (Homeier *et al.*, 2012). We characterized the taxonomic and trophic guild structure of RAF communities by means of a meta-barcoding approach, and estimated community level attributes across fertilization treatments. To further our assessments of the effects of fertilization on RAF communities, we compared the relative read abundance of higher level taxonomic and guild categories across treatments with a differential abundance method that corrects for distortions in these parameters. We expected that an increase in the availability of organic and inorganic N and P in the soil will lead to 1) a reduction in RAF community diversity because fungal taxa for which the altered resource availability is detrimental will be suppressed; 2) shifts in community composition, due to the colonization of empty niches or the competitive dominance of taxa with more flexible coping mechanisms; and 3) major fungal lineages or trophic guilds will respond differently to fertilization in terms of adjusted relative read abundances, due to evolutionary fixed differences in their abilities to obtain limiting resources.

3.2. Materials and Methods

Experimental design and data collection

Samples were collected in August 2015, from a full factorial nutrient manipulation experiment established in 2007 (NUMEX, Homeier *et al.*, 2012). Experimental plots are deployed in an old growth forest located at ~ 2100 m. a. s. l. along the southern slopes of the San Francisco river valley – an eastern Andean valley located in southern Ecuador. The experiment lies within San Francisco biological reserve (3°58'S, 79°04'W), which borders with Podocarpus national Park. Based on historical estimates of aerosol deposition (Velescu *et al.*, 2016), the primary goal of NUMEX is to evaluate the ecosystem response to moderate increases in nitrogen and phosphorus deposition. Every six months, 5 kg ha^{-1} of P (as monosodium dihydrogen phosphate; NaH_2PO_4) and 25 kg ha^{-1} of N (as urea; CH_4N_2O) have been manually applied to the forest floor. The experiment is organized as a fully randomized factorial block design (Appendix B, Fig. S3.1). Each block consists of four 400 m2 (20 x 20 m) fertilized plots (i.e. +N, +P, +N+P) and one control plot of equal dimensions.

Within every experimental plot, six cores (40 cm depth and 3,5 cm diameter) distributed along two randomly placed orthogonal transects were collected. The upper 40 cm of soil where NUMEX is established correspond to a nutrient poor organic layer, which is also where the highest fine root density can be found (Wolf *et al.*, 2011; Moser *et al.*, 2011). Upon collection, twenty fine root pieces of approximately 2 cm length and < 2 mm diameter were separated from the upper 10 cm of the organic layer present in each core. Care was taken to maximize the morphological diversity of each mixed root subsample in the hope that this reflects the taxonomic diversity of trees present in the experimental plots (~45 species, Baez & Homeier, 2018). Upon collection, samples were brought back to the San Francisco research station where roots were rinsed in sterile water, preserved in 97% ethanol and stored at 4° C. Samples were transported to the Institute of Biology of Freie Universität Berlin, were they were stored at -20° C.

DNA extraction, amplification and sequencing

Isolation of DNA from mixed root samples followed the protocols delineated in Dueñas et al. (2020). Briefly, mixed roots samples were lyophilized and milled. DNA was isolated from the milled material using the MoBio soil kit following the manufacturer's protocol (MoBio Laboratories Inc., Carlsband, CA, USA).

Primer set fITS7 and ITS4 (Ihrmark *et al.*, 2012) was employed to characterize the variability of the internal transcriber spacer (ITS2) within the fungal rDNA operon. ITS2 amplification and sequencing were obtained from a 50 µl aliquot of each extract (n = 96) that was shipped to Macrogen Inc. laboratories in Seoul, Republic of Korea. Amplification products of the targeted lengths were gel purified and then denatured and ligated to a 5'–3' adaptor sequence. Libraries were then multiplexed and submitted to the sequencing platform (Illumina MiSeq, pair end 2 x 250 bp). Reads with less than 36 bp were discarded and adaptor sequences were removed by Macrogen Inc.

Bioinformatics and variant classification

Files of the two sequencing rounds were independently processed with packages Dada2 (Callahan *et al.*, 2016), ShortRead (Morgan *et al.*, 2009) and Biostrings (Pagès *et al.*, 2021) in order to define amplicon sequence variants (ASVs). Both workflows were based on Dada2 developer's tutorial for ITS sequences (v1.8). Briefly, primer oligos were removed from every sequence using Cutadap (v.2.1, Martin, 2011). Read ends were truncated if the quality score of base calls fell below two; and filtered out when reads were smaller than 50 bp or presented more than two erroneous or undefined base calls per sequence. Remaining reads were de-replicated and then subjected to the Dada2 sample inference algorithm. Denoised forward and reverse reads were then merged if they had an overlap of at least 12 bp between them. At the end of this process, an ASV abundance table was generated followed by a chimera removal step. Hereafter, the acronym ASV and the term 'variant' will be used interchangeably.

To allow comparisons to prior studies, ASVs were clustered into 97% OTUs using functions within the package DECIPHER (Wright, 2020). For all other analysis, ASVs were chosen as taxonomic units over traditionally employed OTUs because: 1) ASVs circumvent the need to specify a fixed similarity threshold to cluster units across fungal lineages with widely differing levels of intra-specific variability (Nilsson *et al.*, 2008); 2) ASVs are directly reproducible and independent of the dataset (Callahan *et al.*, 2017); and 3) ASVs do not mask biological diversity under arbitrarily defined representative sequences (Selosse *et al.*, 2016).

Taxonomic identity was assigned with the RDP naïve Bayesian classifier with a confidence threshold of 0.8 (Wang *et al*., 2007). Such a high confidence score was selected to avoid forcing the classification of variants potentially representing novel or unknown organisms (i.e. over-classification). We employed UNITE's SH database from February 2020 as a reference (DOI: 10.15156/BIO/786368, Nilsson et al., 2019). As UNITE databases employ the taxonomic framework proposed by Tedersoo *et al*. (2018), this framework was also adopted here. This taxonomic framework is mostly consistent with the one in use by the International nucleotide sequence database (INSD) but proposes a series of updates to the higher level taxonomic nomenclature of the kingdom fungi. The most relevant changes to the present study are the elevation of Basidiobolomycota, Glomeromycota, Kickxellomycota and Mortierellomycota to the status of phylum; as well as changes in several classes within phyla.

Unidentified sequences were subjected to a second classification attempt. The blast+ algorithm was employed (Camacho *et al*., 2009), restricting the query to version 5 of NCBI's database of eukaryote ITS reference sequences (O'Leary *et al*., 2016). Only the 10 best matches with an e-value < 1e-10 were recorded. Matches were sorted in decreasing order by the percentages of query coverage and identity. Ultimately, only the highest ranked match per query sequence was preserved as the best. If query cover of the best match was less or equal to 90%, only domain and phylum names were annotated. If query cover was greater than 90% and identity percentage was greater or equal to 97%, taxonomic names were inherited up to species level. If identity was >= 95%, taxonomic names were inherited up to genus level. From there on up to family when identity was >= 90%, to order when identity was >= 80% and to class when identity >= 70%.

Guild and trophic mode of variants was retrieved from FUNGuild database (Nguyen *et al*., 2016). The database was accessed in October 2020, retaining only those matches with probable or highly probable assignation confidence. Given many fungal taxa exhibited multiple possible trophic modes, inherited annotations were edited to generalize guild classification. Thus, taxa that were not exclusively classified as mycorrhizal, fell into the general category of root associated (RA). Within this general category, several alternative trophic modes were emphasized. Finally, growth morphology was also annotated (i.e. yeast, filamentous or dark septate endophyte).

Statistical analysis

Analysis was conducted in R version 3.6.3 using packages adespatial (Dray *et al.*, 2019), ALDEx2 (Fernandes *et al.*, 2014), DHARMa (Hartig, 2020), dplyr (Wickham *et al.*, 2018), emmeans (Lenth *et al.*, 2021), ggplot2 (Wickham, 2016), ggpubr (Kassambara, 2018), glmmTMB (Brooks *et al.*, 2017), lme4 (Bates *et al.*, 2015), phyloseq (McMurdie & Holmes, 2013), rslurm (Marchand *et al.*, 2021) and vegan (Oksanen *et al.*, 2018). Some analyses that required extensive computation resources were performed at the high performance computer cluster of Freie Universität Berlin (Bennett *et al.*, 2020).

Organization of the data, normalization and description of root associated communities

Given databases are strongly biased towards specimens from temperate ecosystems (Khomich *et al.*, 2018), many variants recovered here remained unclassified at the finer levels of the taxonomic hierarchy (i.e. family, genus or species). Therefore, variants recovered were either analyzed together or grouped according to the phylum to which these were assigned. It was assumed that taxonomic classification at the kingdom and phylum levels are robust to updates in the taxonomy or improvements of reference databases.

Unequal sequencing depth among samples was normalized by random subsampling without replacement (n = 100) to a minimum depth of 26 278 reads per sample. This is the minimum library size obtained here. Although rarefaction is an imperfect normalization method (McMurdie & Holmes, 2014), it remains a valid and widely applied method when analyzing presence-absence data (Weiss *et al.*, 2017).

Statistical inferences of community level attributes (i.e. richness and community composition) focused on well represented phyla. That is, Ascomycota, Basidiomycota and Mortierellomycota. These three phyla will be hereafter referred to as focal phyla. Glomeromycota was not included among focal phyla, since we have analyzed the response of Glomeromycota communities to the same experimental manipulations previously with more appropriate primer sets (Camenzind *et al.*, 2016; Dueñas *et al.*, 2020).

Effects of fertilization on alpha diversity of root associated fungal communities

Alpha diversity was characterized as the total number of fungal ASVs per sample (S_F), and as the total number of ASVs corresponding to the focal fungal phyla (S_P). These richness estimates were then used as response variables to investigate how taxonomic diversity changes in response to nutrient manipulation. Given richness estimates obtained here were overdispersed (i.e. variance was larger than the mean), and the variance was not homogeneous across treatments, data was modelled with generalized linear mixed effect models (Bolker *et al.*, 2009).

Fungal richness in sample *k* within block *i* and plot *j* (S_{Fijk}) was modelled as a function of fertilization with nitrogen (N_{ijk}, categorical, two levels), phosphorus (P_{ijk}, categorical, two levels) and their interaction. $Plot_j$ nested within $Block_i$ were specified as a nested random term with a random intercept. It was assumed that variance follows a negative binomial distribution and the link function was the natural log. The full model structure and associated assumptions are presented according to the notation suggested in (Zuur & Ieno, 2016) in Eq. 3.1:

$$
\begin{aligned}
&S_{Fijk} \sim NegativeBinomial(\mu_{ijk}) \\
&E(S_{Fijk}) = \mu_{ijk} \\
&var(S_{Fijk}) = \mu_{ijk} \times (1 + \mu_{ijk}/\theta) \\
&log(\mu_{ijk}) = N_{ijk} + P_{ijk} + N_{ijk} \times P_{ijk} + Block_i/Plot_j \\
&Block_i \sim Gaussian(0, \sigma^2) \\
&Plot_j \sim Gaussian(0, \sigma^2)
\end{aligned}
\qquad \text{(Eq.3.1)}
$$

, where θ is an overdispersion parameter allowing the variance to increase quadratically with the mean (Brooks *et al.*, 2017). Richness at the phylum level (S_{Pijk}) was modelled maintaining essentially the same structure, except for the inclusion of a fixed term representing focal phyla (Φ_{ijk}, categorical, three levels) and its interaction with fertilization factors. This term allowed to estimate whether different fertilization regimes affected the mean richness of focal phyla differently.

The magnitude and direction of fertilization effects on richness was determined by assessing the degree of overlap between the 95% confidence intervals (CI) of mean richness estimates between treatments and control (Nakagawa & Cuthill, 2007; Hector, 2015). Confidence intervals were computed by iteratively refitting models to parametric bootstraps of the original data (n=1000; Davison & Hinkley, 1997).

Fertilization effects on mean richness of focal phyla was assessed in the same way. In order to corroborate the inferences drawn from CI comparisons, *p* values were estimated from a Dunnett's *t* test, thus correcting such values for multiple comparisons (Dunnett, 1955).

Effect of fertilization on beta diversity of root associated fungal communities.

All analyses in this section were conducted using normalized presence-absence ASV tables. ASV read counts were not taken into account for this analysis given such counts cannot be assumed to represent the underlying abundance of marker molecules in the environment (Gloor *et al.*, 2017). In addition, given one sample failed (see Results section) and current implementations of restricted permutation designs only allow to conduct tests on balanced datasets, 15 samples were randomly excluded to achieve this balance (n=80).

Beta diversity was defined here as turnover of phylotypes across samples. To have an idea of how beta diversity of RAF communities in this site compares to fungal communities at other tropical forests, beta diversity was first summarized by the binary form of Jaccard dissimilarity index. This is an often employed dissimilarity index and was computed for both ASV and OTU tables. This was considered necessary to evaluate whether the estimates obtained employing ASVs are consistent to those obtained when employing OTUs. The strength and direction of the correlation between these two estimates was assessed through a Mantel correlation test (n=999). Similarly, to assess if distance between samples affected dissimilarity estimates, a Mantel correlation test was employed. Distance between samples was drawn from a Euclidean distance matrix of the UTM coordinates of each sample (coordinate reference system=WGS84, zone=17). The presence of a correlation between distance and dissimilarity would hint at the need to remove the trend imposed by distance (i.e. detrend) prior to testing for spatial autocorrelation.

Partial canonical redundancy analysis (RDA; Legendre & Legendre, 2012) was employed to model the variation in community composition of root associated communities as a function of fertilization. A total of four models were specified: one grouping all ASVs irrespective of their phylum assignation, and three additional ones corresponding to the focal fungal phyla examined in this study. ASV tables were specified as multivariate response variables while fertilization factors were specified

as constraining variables. That is, a two-way factor model with interactions (i.e. ~ *N*P*). To account for spatial dependencies, eigenvector based spatial analysis (Dray *et al.*, 2006; Bauman *et al.*, 2018) was employed. Spatial analysis allowed to identify positive autocorrelation signatures on the residuals of detrended ASV presence-absence tables (Borcard *et al.*, 2018). When significant positive spatial autocorrelation was indeed detected, the correspondent Moran eigenvector maps (MEMs) were included in each model as partial spatial covariates. If no positive autocorrelation was found, only the spatial coordinates of each sample in UTMs were included as partial spatial covariates. Spatial autocorrelation is often used as an indicator of the presence of spatially clustered ecological processes that are structuring fungal communities.

To test if fertilization or spatial terms explain more variance in community composition than would be expected by chance, ANOVA like permutation tests (i.e. anova.cca()) were performed for each model (Legendre *et al.*, 2011). Permutations were restricted within blocks to account for the experimental design structure. That is, free permutations (n = 9999) of data rows were allowed within blocks. Non metric multidimensional scaling (NMDS) was used to graphically represent dissimilarity between communities as a function of treatments. The NMDS algorithm was set to find an optimal four-dimensional solution employing 200 random starts. An additional run with default parameters starting from the solution arrived on the first set of iterations was used to plot the final ordinations.

Variance partitioning was employed to quantify the relative importance of covariate categories in structuring root associated fungal communities. Covariates were divided in three categories: fertilization (~ *N*P*), spatial (*Long+Lat or MEMs*) and environmental. Environmental variables refer to plot wise measurements of organic layer pH, tree species richness, C:N ratio and resin available P. Details of the methods employed to measure these environmental variables can be found in Dueñas *et al*. (2020). A goodness-of-fit coefficient for each of these categories in every model was calculated. Given some categories encompassed more covariates than others, the adjusted coefficient of determination (adjusted R^2) was calculated to correct for inflation of R^2 (Peres-Neto *et al.*, 2006).

Sensitivity tests

Phylotype tables recovered by meta-barcoding are typically composed of a large number of features with low read counts, which are often suspected of being artifacts (Bálint *et al.*, 2016). Thus to ascertain that the effects of experimental fertilization were not driven by its effects on rare ASVs only, all tests were repeated by segregating ASV tables by the frequency of occurrence of each feature. Frequent ASVs were defined as those present in at least 5 samples across the dataset. Consequently, rare ASVs were those observed in fewer than 5 samples.

Differential abundance

Although read counts derived from high throughput sequencing cannot be assumed to represent the underlying abundance of a taxon in the environment, with proper statistical treatment, such information can be used to test if experimental manipulation systematically changes the read count of a given taxon (Gloor *et al.*, 2017). Since conducting differential abundance testing at the level of ASVs is computationally prohibitive, it was investigated if the signal of an effect in read counts can be detected when grouping reads by their taxonomic assignment at phyla and orders, or by their trophic guild classification. In contrast to the analyses for community level attributes, all phyla were included in this analysis.

The package ALDEx2 (Fernandes *et al.*, 2014) was employed. This package uses Bayesian methods to estimate test parameters while modeling technical variance. ALDEx2 first models the precision of count estimates via Monte Carlo (MC) resampling. Here, 1000 MC resampling iterations were specified. Values generated in each iteration were transformed to relative abundance by a center log ratio transformation (CLR). CLR is defined as the logarithm base 2 of the ratio between the abundance of feature *i* in sample *j* and the geometric mean of abundances in sample *j* (Fernandes *et al.*, 2014; Gloor *et al.*, 2017). Then the package can model if the variation in relative feature counts (C_{clr}) can be attributed to fertilization or to technical variation by means of generalized linear models (glms). Fertilization factors were specified as explanatory terms via a model matrix ($C_{clr} \sim N*P$). A *t* tests of significance for each term in the model and a false discovery rate correction to the corresponding *p*-value (Benjamini-Hochberg) was estimated. Given a separate glm is fitted for each MC iteration, the mean of glm parameters across MC instances is reported.

3.3. Results

Sequencing results

A total of 95 samples were amplified and generated 17,338,558 reads. Sample 16F failed to amplify presumably because of low gDNA concentration (0.5 ng μl^{-1}). After bioinformatic processing 6,712,425 remaining reads were mapped to 7,083 ASVs. Taxonomic classification routines confirmed 6,333 of these ASVs were likely of fungal origin. The remaining 700 ASVs predominantly matched plants and protists, thus were excluded from the dataset. Normalization by rarefaction discarded 539 variants, yet a rarefaction curve indicated that the estimated ASV richness per sample was close to saturation for most samples at the chosen normalization threshold (Appendix B, Fig. S3.2).

Taxonomic and guild diversity

ASVs were placed within 10 phyla, 35 classes, 93 orders and 197 families. Classification efficiency declined with increased taxonomic resolution. While all fungal variants could be classified to phylum level, only 22.5% could be confidently identified to genus. In terms of read counts, Ascomycota and Basidiomycota were the most frequently recovered (66.3% and 30.5% respectively, Fig. 3.1a). By contrast, basal phyla together with phyla within subkingdom Mucoromyceta corresponded to only 3.3% of total reads. Beyond phylum level, Leotiomycetes and Sordariomycetes within Ascomycota accounted for the largest proportion of reads (Fig. 3.1b). By contrast, Agaricomycetes largely dominated the read proportion within Basidiomycota. Orders Helotiales, Sebacinales and Agaricales, concentrated an appreciable proportion of reads among orders (Fig. 3.1c). Archaeorhizomycetales was also present, albeit in low proportion.

There was a wide variation in total ASV richness among phyla, with lineages within subkingdom Dikarya showing the highest diversity range (Basidiomycota–Ascomycota: 1729–4001 ASVs, Fig. 3.2b). Basal lineages in general exhibited low to very low taxonomic richness, with Mortierellomycota and Glomeromycota the most diverse among these (144 and 297 ASVs, respectively). By contrast, Mucoromycota, Rozellomycota, Zoophagomycota, Basidiobolomycota, Chytridiomycota and Kickxellomycota ASV richness was overall low (range: 3–77 ASVs).

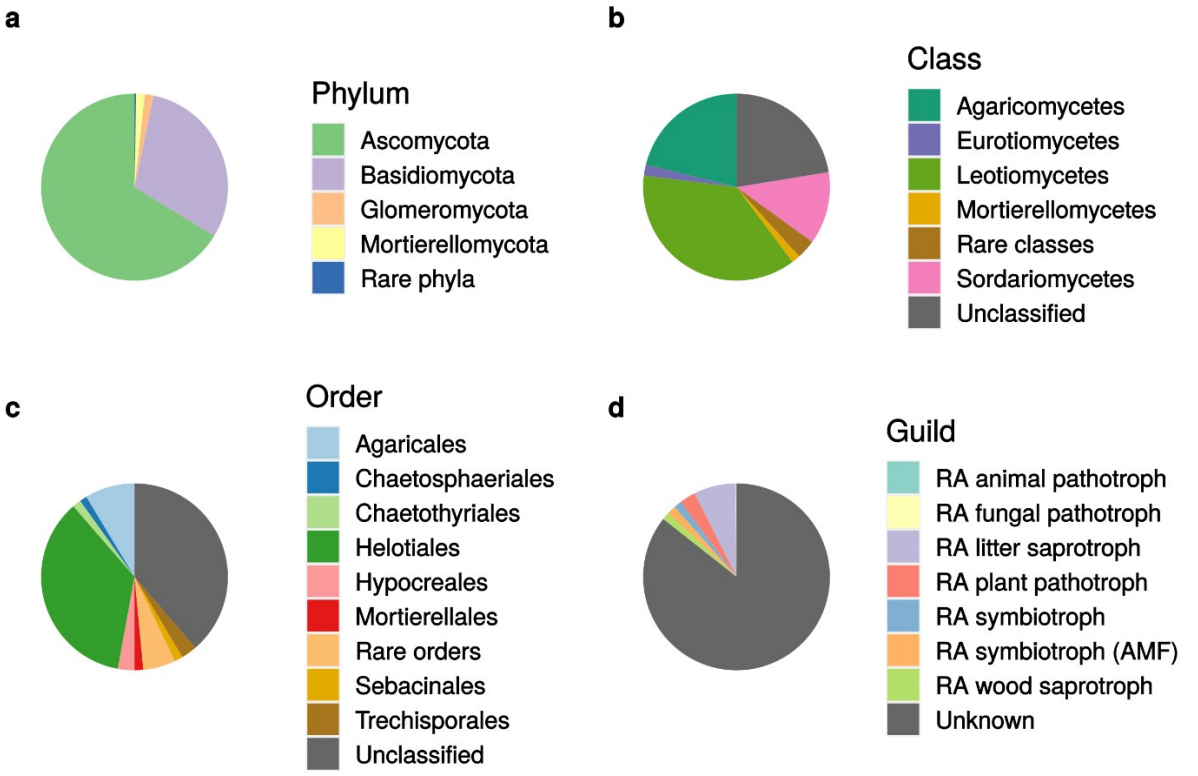

Figure 3.1 Relative abundance of amplicon sequence variants (ASVs) grouped according to their taxonomic classification at different levels (a,b,c), or according to their putative trophic guild (d)

RA stands for root associated and AMF for arbuscular mycorrhizal fungi. Read depth was not normalized prior to plotting. Low read count clades, defined as those with proportional abundance of less than 1% of totals, were aggregated in one category across plots.

Classification into trophic guilds was sparse. Only 1155 variants (18.3%) inherited a guild classification. Litter saprobes was the guild with the largest proportion of read abundance (Fig. 3.1d), followed by plant pathotrophs and wood saprobes. Guild variant richness tracked this pattern, with litter saprobes being the richest (467 ASVs) followed by wood saprobes and plant pathotrophs (78–204 ASVs, respectively). Symbiotroph read abundance was comparable to that of pathotrophs; however, more than half of those reads mapped back to AMF variants (297 ASVs). Beyond AMF, considerably fewer reads and variants (39 ASVs) were annotated as putatively symbiotrophic. Among these, 30 variants corresponded to genera confirmed as ectomycorrhizal: *Cortinarius, Tomentella, Tellophora, Inocybe, Endogone* and *Clavulina*. Remarkably, a few variants annotated within genera with a suspected ectomycorrhizal status were also recovered (9 ASVs, *Serendipita* and *Hymenoscyphus*).

Regarding growth mode and morphology, a small proportion of reads (2.3%) corresponded to variants annotated as dark septate endophytes (70 ASVs). The most prevalent and abundant taxa among DSE were *Pezoloma ericae* and the genus *Oidiodendron*. By contrast, yeasts were less abundant in terms of read proportion (0.6% reads), yet more diverse than DSE (94 ASVs).

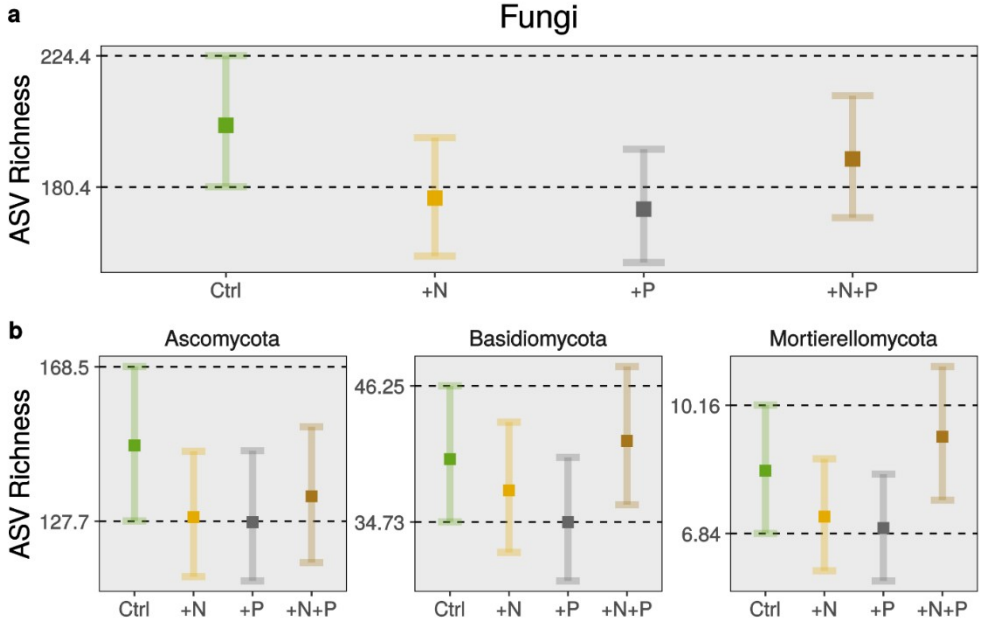

Figure 3.2 Effects of long term fertilization on taxonomic diversity of root associated fungal communities (n=95)

Panel a depicts generalized linear mixed model (glmm) estimates of mean ASVs richness per sample (excluding Glomeromycota variants). Panel b shows estimates of mean ASV richness per sample of the most frequently recovered phyla. Solid squares represent model mean estimates. Whiskers represent 95% confidence intervals estimated by refitting glmms 1000 times with parametric bootstraps of the original data. Breaks and horizontal dashed lines in y-axis correspond to CIs estimates of controls. Prior to calculate ASV richness per sample, read depth was standardized across samples by rarefaction.

Fertilization effects on alpha diversity

We observed that seven years of moderate fertilization rates did not elicit changes on RAF communities' alpha diversity (Fig 3.2a, Table 3.1). Model estimates showed that, in relation to control, mean ASV richness decreased by 12.1% (mean, 95% CIs: 176.7 ASVs, 157.2–196.9) after N addition and by 14% (172.9 ASVs, 155.1–193.1) after P addition. Combined addition of N and P reduced mean ASV richness by 5.6% (189.8 ASVs, 170.1–211.1). A considerable overlap of CI estimates between fertilization treatments and control indicate that the probability such reductions could have been observed by random variation of the data is larger than 5% (Fig. 3.2a). Dunnett's *t*

tests supported the CI assessment, confirming that no fertilization treatment elicited a statistically significant reduction in mean ASV richness at the kingdom level (Table 3.1).

Taxonomic level	Contrast	*t*	*p*
Fungi	+ N vs. Ctrl.	-1.639	0.251
	+ P vs. Ctrl.	-1.886	0.158
	+ N + P vs. Ctrl.	-0.730	0.780
Ascomycota	+ N vs. Ctrl.	-1.404	0.364
	+ P vs. Ctrl.	-1.500	0.313
	+ N + P vs. Ctrl.	-0.975	0.629
Basidiomycota	+ N vs. Ctrl.	-0.648	0.825
	+ P vs. Ctrl.	-1.338	0.401
	+ N + P vs. Ctrl.	0.361	0.946
Mortierellomycota	+ N vs. Ctrl.	-1.078	0.563
	+ P vs. Ctrl.	-1.355	0.392
	+ N + P vs. Ctrl.	0.731	0.780

Table 3.1 Dunnett's *t* test contrasting the difference in mean ASV richness between fertilization treatments and controls (n = 95)

Dunnett procedure corrects *p* values to reduce false positives due to multiple contrast with the control. Only focal fungal clades recovered in this study were tested with the exception of Glomeromycota.

Trends observed at the kingdom level remained consistent across focal phyla (Fig. 3.2b). That is, no fertilization regime changed mean ASV richness among these phyla. Dunnett's *t* tests supported the CI assessments, showing none of the differences elicited by fertilization treatments were statistically significant (Table 3.1). Assumption

validation plots and coefficients in the original scale for both models are presented in Appendix B, Figures S3.3–3.4 and Tables S3.1–3.2, respectively.

When the analysis was repeated segregating the data according to frequency of variants, trends remained similar for the kingdom level and for Ascomycota (Appendix B, Fig. S3.5a, b). Somewhat stronger mean richness reductions were observed amongst rare Basidiomycota and Mortierellomycota assemblages. However, Dunnett's *t* tests indicated only mean richness of rare Mortierellomycota decreased significantly after N addition ($p = 0.03$) or P addition ($p = 0.04$), but not after the addition of both nutrients. In stark contrast to the overall trend, mean richness of rare Basidiomycota variants was higher than the mean richness of frequent ones (Appendix B, Fig. S3.5b).

Fertilization effects on beta diversity

Beta diversity of RAF communities, as characterized by the Jaccard index, was large in this system (mean, ±SD: 0.92, ±0.03). This was the case irrespective of the resolution of taxonomic units employed (Appendix B, Fig. S3.6a). Dissimilarity of communities in terms of ASVs was strongly and positively correlated to the dissimilarity of communities constituted by OTUs (Mantel $\rho = 0.83$, $p < 0.001$). Overall, dissimilarity between communities constituted by ASVs was not correlated with distance (Mantel $\rho = 0.13$, $p = 0.395$). However, this pattern was inconsistent across treatments (Appendix B, Fig. S3.6b). While the correlation was weakly positive and significant among assemblages within Control, and +P plots, it was not significant when considering assemblages within the N addition treatments (Appendix B, Table S3.3).

Removal of samples to achieve a balanced design left a total of 5655 ASVs and 5 samples per experimental plot (n = 80). Spatial eigenvector analysis showed that the residuals of presence-absence tables were spatially independent in most cases (Appendix B, Table S3.4). Positive spatial autocorrelation among residuals was found only for assemblages consisting of frequent fungal variants and for assemblages consisting of all or frequent Ascomycota variants. Consequently, MEMs as spatial covariates were included only for these three cases. In all other instances, UTM coordinates were specified as spatial covariates.

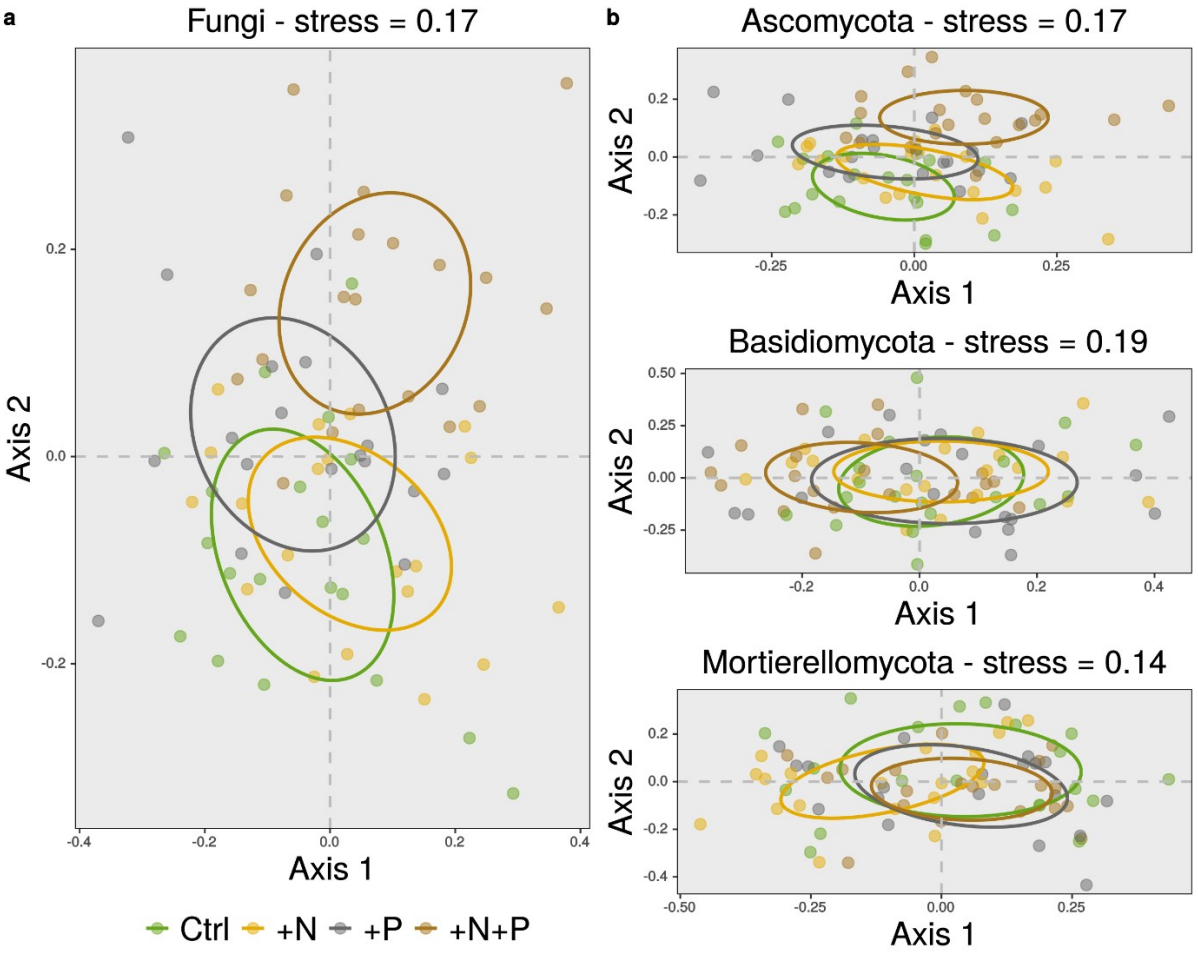

Figure 3.3 Non-metric multidimensional scaling (NMDS) of binary Jaccard dissimilarities (n=80)

The NMDS algorithm was set to resolve ordinations with 200 random starts. Four dimensions were necessary to arrive to a fair representation of the dissimilarity matrix in the ordination space. However, only the first two dimensions are illustrated here. Each colored point represents a fungal assemblage. Ellipses represent one standard deviation from the group centroid.

Permutation tests showed that both the addition of N and P explain a statistically significant portion of the variation in composition of root associated fungal communities (Table 3.2, Fig. 3.3). Test at the phylum level were rather inconsistent, with the exception of Ascomycota assemblages, for which the addition of N or P elicited significant community structure shifts. By contrast, assemblages consisting of Basidiomycota or Mortierellomycota variants responded weakly or not at all to fertilization with N or P. Specifically, composition of Basidiomycota communities shifted in relation to control only when P was added, while Mortierellomycota communities did not shift after any fertilization intervention. These results were robust to the inclusion of spatial covariates or when segregating data by variant occurrence

frequency (Appendix B, Table S3.5). Yet two notable differences arose when analyzing only rare ASVs. The addition of N did not shift the structure of any of the clades considered and, the addition of P did affect the structure of the two most frequent clades recovered in this study (i.e. Ascomycota and Basidiomycota).

Taxonomic level	Factor	Pseudo *F*	*p*
Fungi	+N	1.136	0.020
	+P	1.441	0.000
	+N+P	1.096	0.043
Ascomycota	+N	1.300	0.000
	+P	1.404	0.000
	+N+P	1.056	0.167
Basidiomycota	+N	1.046	0.224
	+P	1.173	0.007
	+N+P	1.114	0.053
Mortierellomycota	+N	1.164	0.164
	+P	1.381	0.071
	+N+P	1.328	0.099

Table 3.2 Pseudo-F tests assessing whether the proportion of variance in community composition explained by fertilization factors

Pseudo-*F* tests assessing whether the proportion of variance in community composition explained by fertilization factors is statistically significant. Spatial covariates were partialled out in all RDA models. Only the best represented fungal clades recovered in this study were tested with the exception of Glomeromycota. Values of *p* were calculated with permutation tests (n = 9999). Free permutations were only allowed within experimental blocks.

Variance partitioning showed that none of the covariate groups analyzed contributed to explain a considerable portion of RAF community composition variability (Fig. 3.4). Fertilization factors had relatively low explanatory power, explaining the largest portion of variability among Ascomycota assemblages (range: 0.3–0.8%). Similarly, spatial covariates were generally poor predictors of RAF community structure, again with the exception of Ascomycota assemblages (range: 0.3–1.2%). Plot wise environmental covariates, such as pH, plant available P, C:N ratio and tree species richness boosted the explanatory power of multivariate models in some cases. For instance, environmental covariates appear to be better predictors of Mortierellomycota assemblage structure than either fertilization or spatial covariates (1.6% versus 0.4% and 0.7% respectively).

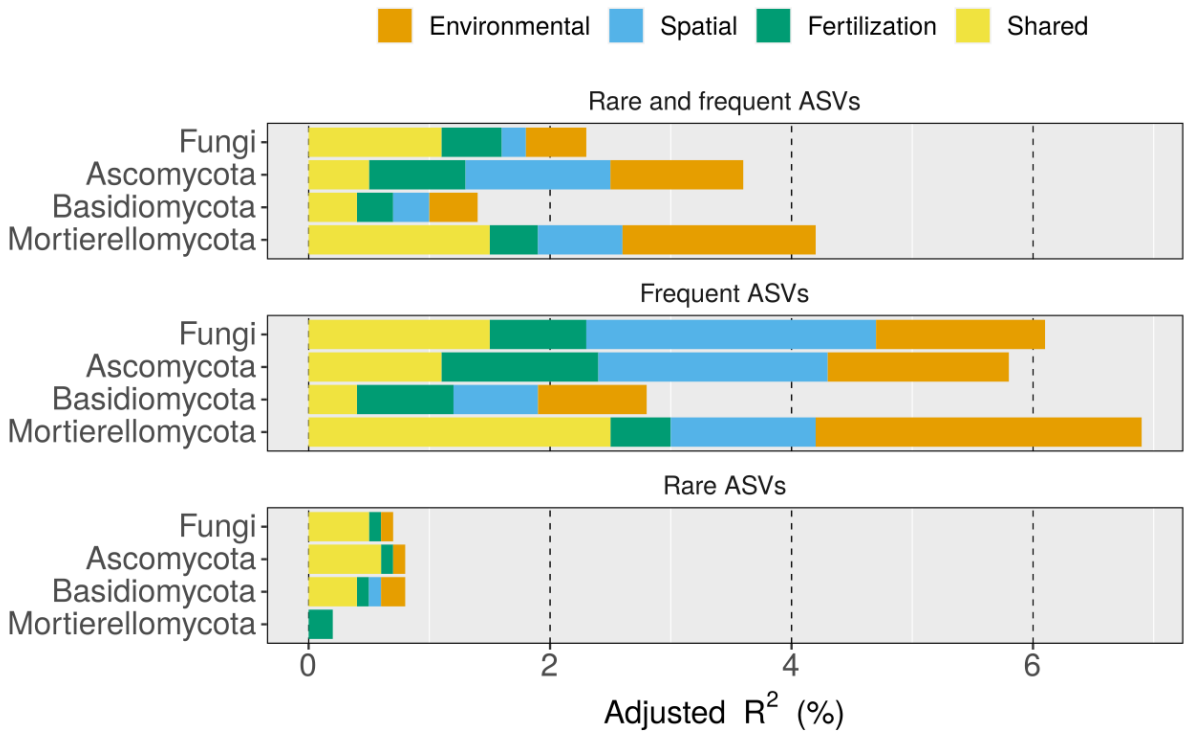

Figure 3.4 Proportion of variability in community composition explained by fertilization treatments among the focal fungal clades recovered in this study

The adjusted coefficient of determination (Adjusted R^2) was estimated with a variance partitioning function and then transformed to a percentage for plotting. 'Fertilization' represents the variance explained by fertilization covariates. 'Space' represents the variation explained either by spatial coordinates or Moran eigenvector maps. 'Environmental' represent the variance explained by additional environmental variables. That is, plot wise soil pH, plant available P, C:N ratio and tree species richness. The magnitude of variance explained by each covariate group was estimated excluding all other covariate groups.

The patterns just described varied widely according to whether ASVs were analyzed together or grouped according to phyla or frequency of occurrence. Regarding focal phyla, covariate groups cumulatively explained 1.4% of the variability in structure of Basidiomycota assemblages while these same predictors explained more than double of this proportion among Mortierellomycota assemblages (4.2%). Regarding occurrence frequency, covariate groups clearly explained a greater proportion of variance when assemblages where composed of frequent ASVs only. Conversely, predictor groups collectively explained less than 1% variability when considering assemblages exclusively composed of rare variants. The partitioning function found a large proportion of shared explained variability amongst the covariate groups analyzed.

Differential abundance

Relative abundance of reads amongst the great majority of phyla, orders or guilds did not change in response to fertilization (Fig. 3.5). Univariate tests indicated technical variation was as great as variation between treatments in almost all cases (Appendix B, Fig. S3.7). Few departures from this pattern were observed. For instance, the relative abundance of the order Glomerales was reduced while that of Sebacinales increased after the addition of P, yet univariate tests indicated such changes were marginally or non-significant after applying the B-H false positive correction ($p = 0.08$ and 0.18, respectively, Appendix B, Fig. S3.7).

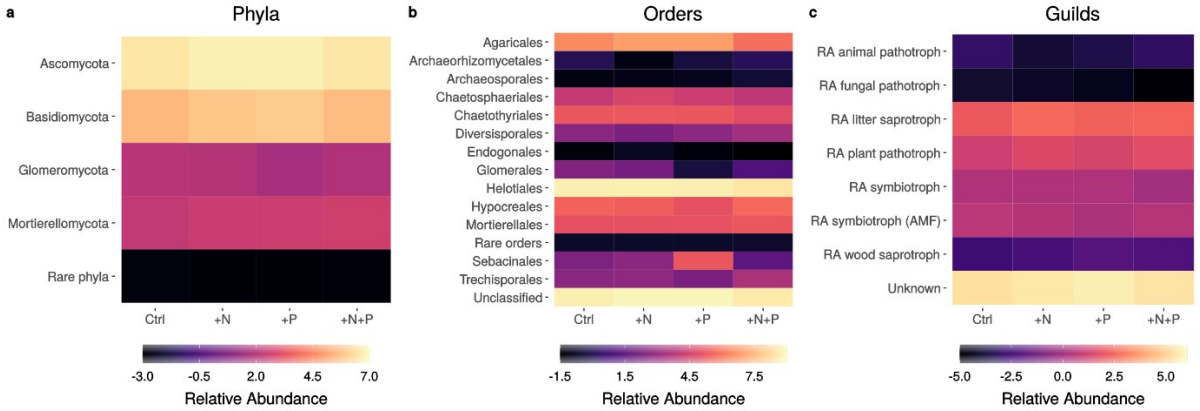

Figure 3.5 Heatmaps representing the change in mean relative read abundance of features as a function of fertilization treatments

Features refer to taxonomic clades and fungal guilds to which reads were mapped to. Prior to plotting, read abundance was transformed by the center $\log_2$ ratio transformation (CLR) to correct distortions in read counts. Lighter colors represent higher mean relative abundances, while darker colors represent lower relative abundances.

3.4. Discussion

We characterized, for the first time via a deep sequencing approach, the non-mycorrhizal fungi associated with the fine root fraction of an Andean tropical forest. Our assessment revealed that the fine roots of tropical montane trees are not only associated with AMF variants, but with a diverse set of fungal variants, among which many are primarily known as saprobes. We have also shown that the attributes of RAF communities remained mostly stable after a simulated chronic increase in the input of P and N to the soil. That is, mean richness reductions following fertilization were neither generalized among fungal lineages nor clearly distinguishable to what would be expected by chance in most cases. Regarding community structure, though we observed an overall shift following N and P additions, at the phylum level these shifts were most prominent among Ascomycota assemblages, but inconsistently observed among other phyla. Furthermore, the variability explained by fertilization factors was overall low. Given the inconsistent and rather weak responses observed, we suggest that tropical montane RAF communities are robust to moderate increases in the atmospheric inputs of N and P.

RAF communities in this system are dominated by phylotypes from sub-kingdom Dikarya. The overwhelming dominance of Ascomycota and Basidiomycota was expected, given it has been repeatedly reported for various neo-tropical lowland (Peay *et al.*, 2013; Barberán *et al.*, 2015; Schappe *et al.*, 2017), and montane forests sites (Geml *et al.*, 2014; Looby *et al.*, 2016; Nottingham *et al.*, 2018a). While at the stand level similar dominance patterns have been reported for RAF communities (Schroeder *et al.*, 2019), tree species level reports of communities dominated by Ascomycota (*Alnus accuminata*, Wicaksono *et al.*, 2017) or by Basidiomycota (*Oreomunnea mexicana*, Corrales *et al.*, 2017) also exist. Beyond the phylum level, our observation that Leotiomycete and Sordariomycete were abundant is consistent with a recent characterization of species within these classes as frequent components of soil fungal communities in mesic tropical forests (Egidi *et al.*, 2019). As far as we know, Sebacinales associating with trees from the region have never been reported. Yet their presence is unsurprising given their cosmopolitan distribution, broad host-spectrum and trophic flexibility (Riess *et al.*, 2014; Weiß *et al.*, 2016). We believe Sebacinales presence, as well as that of Archaeorrhizomycetales deserve independent

confirmation in order to establish if these play a beneficial role in the mineral nutrition of tropical montane trees (Weiß *et al.*, 2016; Pinto-Figueroa *et al.*, 2019).

A comprehensive characterization of trophic guild structure of RAF communities was not possible due to the large proportion of undescribed taxa and the lack of trophic guild information for tropical taxa. Yet we complemented the observations of early meta-barcoding studies of the fine root fraction of sub-tropical (Toju *et al.*, 2014), and neo-tropical trees (Schroeder *et al.*, 2019), which have reported the co-occurrence of symbiotrophs with a complex array of root associated guilds. The observation that the most frequent guilds in this fraction were saprobes, supports the idea that many members of RAF communities are horizontally transmitted and facultative (Rodriguez *et al.*, 2009; Hardoim *et al.*, 2015). An often given explanation to this pattern is that saprobic fungi can asymptomatically transition to an endophytic lifestyle, in order to 'escape' limiting conditions in other substrates (Baldrian, 2017; Nelson *et al.*, 2020). Regarding non-AMF symbiotrophs, the recovery of several ecto- and ericoid mycorrhizal lineages in our communities is in line with the idea that soil and climatic variables drive their distribution, rather than host availability (Peay *et al.*, 2015; Wicaksono *et al.*, 2017; Corrales *et al.*, 2018). However, it is worth noting that tree species suspect of broader mycorrhizal associations do occur in our experimental plots (e.g. *Bejaria aestuans* or *Graffenrieda emarginata*). Our study thus supports early assessments which reported morphological and molecular evidence of the presence of non-AMF mycorrhizal fungi in Andean forests (Haug *et al.*, 2004; Kottke *et al.*, 2004). The possibility that pathotrophs are as diverse and abundant as symbiotrophs in the fine root fraction is intriguing. Theory predicts that pathogen-mediated negative feedback balances the positive effects of mutualism thereby promoting the enormous plant biodiversity observed in the tropics (Bachelot *et al.*, 2015). Perhaps unlike seedlings (Bachelot *et al.*, 2017), adult trees have managed to escape negative feedbacks by establishing associations with a greater diversity of both mycorrhizal and non-mycorrhizal symbiotrophs. While these patterns must be interpreted with caution given the trophic guild of a fungal root associate is hard to predict from the identity of the taxa alone (Griffin & Carson, 2018), our attempt at characterizing fungal guild structure in this system has revealed a number of interesting patterns that await further confirmation.

RAF communities recovered in this study exhibited moderate richness levels. Providing context to this observation is challenging given the methodological inconsistencies of meta-barcoding studies (Bálint *et al.*, 2016). However, a rough comparison of mean richness estimates (i.e. using the same number of samples, and clustering ASV into OTUs at 97% similarity) obtained at various neo-tropical forests suggests RAF communities in tropical Andean forests are less diverse than fungal communities found in soil or at lowland tropical forests (Table 3.3). This is in line with the metabolic theory of ecology which posits that richness decreases monotonically with temperature (Zhou *et al.*, 2016), and fits well with the patterns revealed by the few fungal surveys conducted along elevation transects in wet tropical montane forests (Looby *et al.*, 2016; Nottingham *et al.*, 2018a). This comparison also suggest that RAF communities are populated by a subset of the taxa found in soil, which fits the patterns revealed by recent surveys in temperate forests (Goldmann *et al.*, 2016). We acknowledge, however, that until the richness of soil fungal communities at the same site, and at lower elevations, are thoroughly characterized, the question of how diversity estimates found here compare to other soil compartments and elevation belts will remain open (Looby & Martin, 2020).

Sample type	Site	Forest elevation	S_f	σ	Reference
Composite soil	Panama (BCI)	Lowland	183.44	58.09	Barberán et al. 2015
	Panama (Campo Chagres)	Lowland	449.67	68.85	Schappe et al. 2017
	Panama (Pipeline)	Lowland	434.67	67.95	Schappe et al. 2017
	Panama (Santa Rita)	Lowland	413.89	84.77	Schappe et al. 2017
	Costa Rica (Monteverde)	Montane	473.78	83.99	Looby et al. 2016
Mixed root	Mexico (Los Tuxlas)	Lowland	497.33	107.66	Schroeder et al. 2019
	Ecuador (San Francisco)	**Montane**	**107.78**	**14.29**	**This study**

Table 3.3 Estimates of mean fungal richness (Sf) and standard deviations (σ) across different types of wet neo-tropical forests

Estimates were obtained by randomly selecting nine samples from each site, as this was the minimum sample size of the studies compared. When appropriate, the random draw was selected from control plots only. All studies employed Illumina MiSeq platform and targeted

the ITS region. For the purpose of this comparison, phylotypes were clustered as OTUs at 97% similarity and singletons (i.e. phylotypes occurring in only one sample) were eliminated.

Contrary to our expectations, increased nutrient availability did not alter the mean richness of RAF communities, hence we did not find support for the resource ratio model expectations (Cardinale *et al.*, 2009). Fungal richness has been shown to increase after long-term N addition in temperate forests soils (Morrison *et al.*, 2016), and after long-term N and P additions to the organic layer of tropical forests (Kerekes *et al.*, 2013). While it would be tempting to attribute such shifts only to the high fertilization rates applied (≥ 100 kg N ha^{-1} yr^{-1}), comprehensive multi-site nutrient manipulation experiments (Leff *et al.*, 2015) and meta-analysis (Zhou *et al.*, 2020) indicate that, while there is considerable local variation, diversity indices of non-mycorrhizal fungal communities change little after fertilization treatments. Such neutral responses contrast with the clear richness reductions often observed among tropical forests AMF communities, even after moderate rates of N and P addition (Camenzind *et al.*, 2014; Sheldrake *et al.*, 2018; Dueñas *et al.*, 2020). Yet they are consistent with the neutral richness responses observed among tropical ectomycorrhizal fungal communities (Corrales *et al.*, 2017) after high N fertilization rates. Beyond the fertilization rate applied, what this evidence seems to indicate is that diversity of different fungal functional groups exhibit distinct sensitivities to soil eutrophication. Such sensitivity appears to depend on the ambient physico-chemical conditions of the soil. For instance, in the fertilization experiment surveyed by Kerekes *et al*. (2013), the largest richness gains were observed in the combined P and K addition treatment, which constitute two of the most limiting resources in lowland tropical forest soils. Similarly, Morrison *et al*. (2016) reported significant richness gains after N additions, in a system where N is the most limiting nutrient. Hence it appears that because Andean tropical forests soils have different nutrient limitations than temperate or lowland tropical systems, moderate N and P additions did not result in large richness increases or decreases.

We also hypothesized that nutrient imbalances caused by nutrient addition would elicit RAF community composition shifts. While we found some support for this prediction, community composition of most focal fungal phyla recovered here was not altered by the fertilization treatments. Moreover, our approach to measure changes in relative read abundance among focal phyla, orders and trophic guilds revealed that these remained stable across treatments. These results are in line with the notion that global

change factors, and in particular nutrient deposition, are not always detrimental to microbial communities (Zhou *et al.*, 2020). Indeed, the structure of fungal communities has been shown to respond in a variety of ways to increments in nutrient availability. While many studies in temperate and boreal forests report shifts in community composition, as well as changes in the abundance of certain lineages as a result of N addition (Edwards & Zak, 2011; Morrison *et al.*, 2016; Maaroufi *et al.*, 2019), reports of neutral responses in these indicators are not uncommon (Carrara *et al.*, 2018; Zak *et al.*, 2019). A similarly inconsistent response appears among the few studies conducted at tropical forests. Reports range from shifts in community composition and increases in PLFA markers as a result of N addition (Cusack *et al.*, 2011; Corrales *et al.*, 2017; Nottingham *et al.*, 2018b), to mild shifts in community structure or PLFA abundance after the experimental addition of N or P (Kaspari *et al.*, 2010; Kerekes *et al.*, 2013; Krashevska *et al.*, 2014). Our results thus add to this variability and highlight the fact that more tropical sites need to be assessed in the context of soil eutrophication in order for robust patters to emerge.

Many features of fungal communities can allow them to resist the effects of moderate increases in nutrient availability. Since deep sequencing approaches cannot distinguish between the active and the dormant fractions of fungal communities (Fierer, 2017), it is possible that many members of the community are able to persist in a dormant status (Blagodatskaya & Kuzyakov, 2013). Such portion of the community will still require to consume maintenance resources, yet contributes directly to the resistance of the system because it allows many taxa to persist despite an unfavorable shift in environmental conditions (Joergensen & Wichern, 2018). In addition to opt for dormancy, the active portion of fungal communities can adjust their enzymatic profile in response to long term nutrient additions without necessarily reflecting such functional shifts as major changes in richness or community composition (Edwards & Zak, 2011; Morrison *et al.*, 2018; Zak *et al.*, 2019). This is an important point, because it implies that a lack of change in fungal community attributes does not necessarily translate in functional stability. In addition to these possibilities, recent experimental evidence has shown that soil fungi exhibit extremely flexible stoichiometric homeostasis in relation to the stoichiometric ratio of culturing media (Camenzind *et al.*, 2021). If this ability is confirmed and widespread across the fungal kingdom, it will mean that non-mycorrhizal fungi have yet another possibility to bridge resource gaps

in time or space, thus allowing taxa to persist despite shifts in the nutrient supply. In summary, given fungi possess a unique variety of nutrient acquisition strategies and life history traits (Treseder & Lennon, 2015), a diverse fungal community is robust to moderate increases in nutrient availability given its constituents have the potential to compensate functionally or to become asynchronously active (Morrison *et al.*, 2018).

It is also possible that RAF communities are simply not structured by the same drivers affecting soil fungal or mycorrhizal communities. We observed high beta diversity, which was weakly correlated with distance between samples. This is remarkable, given the small spatial extent covered in this study (0.64 ha). Indeed, high turnover rates at local scales appear to be a generic feature of soil fungal communities (Kaspari *et al.*, 2010; Zinger *et al.*, 2019), as well as of leaf associated fungal communities (Meiser *et al.*, 2014) in neo-tropical forests. This suggests that drivers other than soil conditions are structuring fungal communities and in particular plant associated communities. For instance, RAF communities could be structured by the chemical and physical characteristics of the fine root fraction (Nguyen *et al.*, 2020). It is also reasonable to expect plant-fungal interactions to leave a signature in RAF community structure (Schroeder *et al.*, 2019). Both interpretations are in line with the strong correlations between tree and fungal community composition reported at some lowland tropical forests (Peay *et al.*, 2013; Barberán *et al.*, 2015). Although the root environment characteristics and host filters appear to be relevant for RAF communities, we cannot exclude that small scale variation in soil physico-chemical properties or even stochastic factors (i.e. ecological drift *sensu* Vellend, 2016) are playing a role in structuring these communities. Our results, however, seem to balance against the influence of soil environmental filters, given positive spatial autocorrelation was inconsistently observed amongst the communities studied here (Table S4). It is thus clear that more research on the multiple assembly rules that could drive RAF community structure is needed before we can arrive at solid conclusions about the relative importance of each one of these factors.

In conclusion, we found no evidence that indicates a seven year long moderate increase in nitrogen and phosphorus supply affects RAF community richness in this Andean forest. RAF community composition on the other hand is moderately affected by these treatments, driven mostly by changes observed among Ascomycota assemblages. RAF within Ascomycota thus appear to be the most responsive clade

to soil eutrophication. Altogether this indicates that the constituents of RAF communities, particularly those belonging to recently evolved, non-mycorrhizal fungal lineages, are quite adaptable to changes in the soil nutrient supply. This flexibility in turn is expressed as robustness of community level attributes to nutrient addition. As Kaspari *et al*. (2010) stated in a rather clever adaptation of Beijerinck's conjecture, it appears that in highly diverse tropical forests, fungi are "never everywhere—but plastic and adaptable". Our data support this idea and also suggests that the root environmental characteristics and plant top down control contribute to shape the structure of RAF communities. While these results are encouraging, we still need to tackle the question of how small scale gradients in soil and root nutrient availability structure RAF communities. Moreover, we need a better understanding of how biotic interactions shape these communities and how all this complexity affects the ecosystem level response to global change drivers (Carrara *et al.*, 2018). Yet our most pressing priority is to continue to close the wide gap in our understanding of tropical fungal communities in relation to temperate or boreal ecosystems. The global implications of climate change and the disproportionate contribution of tropical biomes to dampen or enhance these effects make this a crucial task.

3.5. Acknowledgements

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4. Chapter 4 Finding a suitable base for soil microbial diversity: should we account for differences in bulk density in comparative microbial community studies?

4.1. Introduction

Soil microbes are a highly diverse group of organisms that include host-associated or free-living archaea, bacteria, fungi and protists (Orgiazzi *et al.*, 2016; Geisen *et al.*, 2019). Soils are also diverse bodies composed of organo-mineral particles, liquids and gases (Minasny *et al.*, 2010). When discounting the temporal aspect of pedogenesis, soils can be described as three dimensional, opaque and porous bodies with distinguishable physical and chemical attributes. The surfaces along the pore spaces, the liquid phases within them, and the contact zones of soil particles with roots or the galleries left by fossorial fauna offer a wide range of micro-habitats for soil microbes (Thakur *et al.*, 2020). Thus, the variety of substrates and the architectural and structural complexity of soils arguably plays a major role in generating the enormous levels of microbial diversity and abundance found in them (Rillig *et al.*, 2017; Erktan *et al.*, 2020). At the same time, these same features pose enormous challenges for studying soil microbial communities.

Microbial ecology and macro-ecology have flourished in the last decades. This is reflected by the noticeable increment in the number of publications that compare community attributes of soil microbial groups locally (Alteio *et al.*, 2021) or across large spatial scales (Chu *et al.*, 2020; Dickey *et al.*, 2021). Both disciplines have in common an increasing integration and exploitation of sophisticated molecular and modeling tools that broaden the scope of inference (Geisen *et al.*, 2019). In particular, the application of high-throughput sequencing techniques has become an indispensable standard. While it is undeniable that our understanding of soil microbes has benefited from these additions, some of the nuanced aspects of surveying soils are prone to being lost amidst all this sophistication. Relative to the extensive efforts devoted to assess and overcome biases occurred during laboratory and bioinformatics processing (Porter & Hajibabaei, 2018; Alteio *et al.*, 2021), less attention has been paid to how physical soil sampling strategies could introduce bias. Excellent points have been made regarding the need for a greater harmonization and clearer reporting

of field protocols (Dickie *et al.*, 2018). Large and balanced datasets are necessary for an accurate estimation and comparison of microbial attributes such as richness and abundance across large spatial extents (Hermans *et al.*, 2019; van den Hoogen *et al.*, 2019). Finally, it is clear that compliance with metadata reporting standards has still a long way to go (Jurburg *et al.*, 2020; Rodríguez-Ezpeleta *et al.*, 2021). We would like to add to this list a concern that so far seems to have largely escaped the attention of the microbial ecology research community. We refer specifically to the observation that large scale, amplicon based, microbial community studies often do not account for differences in soil bulk density when extrapolating and comparing results. We argue that the impact of these differences is important to consider, particularly when estimates obtained from a few grams of soil are extrapolated to whole regions, ecosystems or biomes.

The present piece is a discussion on the potential extent of this problem. To illustrate our discussion, we first recall the concept of bulk density and discuss how it can influence or reflect the extent, heterogeneity and structure of microbial habitats. We then characterize how soil collection campaigns are typically implemented and on which basis microbial community attributes are reported. The characterization is based on the sampling strategies employed by a set of 13 recent broad scale amplicon based microbial ecology studies (Tedersoo *et al.*, 2014; Hermans *et al.*, 2016; Terrat *et al.*, 2017; Karimi *et al.*, 2018, 2020; Delgado-Baquerizo *et al.*, 2018, 2019, 2020; Bahram *et al.*, 2018; Egidi *et al.*, 2019; De Gruyter *et al.*, 2020; Zhang *et al.*, 2020; Bastida *et al.*, 2021). We focus on this set given we think the issue discussed here is especially relevant in the specific context of large-scale surveys, because they inherently will include soils that differ widely in bulk density. Based on these elements, we explain why we think comparisons of microbial community attributes representing whole ecosystems or biomes should account for differences in bulk density. We finish with a short discussion of the possible reasons why this issue is underappreciated in microbial ecology.

4.2. What is bulk density?

Bulk density is a physical parameter that relates soil dry mass to volume and is usually expressed in g cm^{-3} (Throop *et al.*, 2012). Bulk density is a dynamic property, which is used as an index of soil compaction but can also characterize a series of soil qualities directly relevant to how the microbial habitat is structured. Namely the capacity of

water and gases to move through the soil column (i.e. infiltration rates, hydraulic conductivity and aeration) or the availability and extent of microbial habitats. This latter property refers to the fact that bulk density is inversely related to the pore space fraction in soils (Nimmo, 2013). Yet perhaps the greatest utility of bulk density is as a basis for calculations and extrapolation. Bulk density enables to transform estimates expressed on a mass basis to a volume or an area basis (Sparling & Schipper, 2004; Throop *et al.*, 2012). These conversions are important to consider when estimates need to be compared.

Although the relation of bulk density to soil microbes and the process they drive is primarily studied from the perspective of changes in land use management (Beylich *et al.*, 2010; Hartmann *et al.*, 2012), the reality is that bulk density changes naturally as a function of seasonality, soil texture, plant cover, the presence of rocks, organic matter and the activities of soil organisms (Sparling & Schipper, 2004; Throop *et al.*, 2012). Available 'snapshot' estimates show bulk density can substantially vary across space and as a function of depth (Hengl *et al.*, 2017). In addition to spatial variation, bulk density can vary within the same ecosystem at different time points (Mora & Lázaro, 2014).

4.3. Fixed depth as the guiding standard of soil sampling protocols

A soil survey typically involves the collection of several soil samples at a fixed depth within a pre-defined sampling unit. Our survey revealed the basic sampling plot area ranged from 4 x 10^2–1 x 10^7 m^2 whereas the targeted depth ranged from 5–30 cm. Only related studies (i.e. studies that re-analyzed the same datasets or shared co-authors) showed consistency in the depth surveyed, and in most studies it was unclear whether sampling included the organic layer of soils. As variation within a sampling unit is not of interest, local soil spatio-temporal variability is usually 'averaged' by mixing several individual soil samples (5–40 cores per sample). We found only one instance in which individual cores were processed separately and estimates were averaged at the level of data after the laboratory and bioinformatics process.

Samples then undergo a process of intense fractionation and mixing, whereby soils are air dried and sieved to remove large particles such as gravel, rocks or coarse organic matter. We found most studies use a 2 mm diameter sieve sterilized between

samples, but also encountered reports of manual removal of larger particles and debris. After homogenization, the resulting fraction is often divided into two portions: one for soil property characterization and the other dedicated to study microbial communities. We also found instances in which soil collection for physico-chemical characterization was performed separately. Most studies we surveyed favored chemical characteristics of soil such as pH or nutrient pools. The most commonly measured physical parameters were texture and particle size distribution. Only one study measured bulk density. Few studies report the volume or fresh weight of soil at the time of collection, but in some cases sample volumes could be inferred by the number, diameter and depth of the cores used. The mass of processed soil that is subjected to nucleic acid extraction was universally justified on the grounds of commercial extraction kit recommendations (0.25–10 g).

4.4. Possible confounding effects of bulk density variation in soil microbial community surveys

From the previous section it is apparent that there is considerable variability across studies in terms of soil collection strategies. While we surveyed a minor portion of the literature, experience and previous work tells us that many more variants of this baseline collection strategy can be found (Dickie *et al.*, 2018; Pollock *et al.*, 2018). This lack of consensus clearly represents a challenge for meta-analysis and cross study comparisons. Yet our main motivation here is to assess how natural differences in bulk density can affect extrapolation and comparison of estimates within studies.

The alpha diversity index most frequently reported in our survey was taxonomic richness. Although richness is never reported in relation to mass, in our opinion any estimate obtained from subsamples of soil mass collected at a standard soil depth should be expressed on this basis. So strictly speaking what is obtained is an estimate of species density per mass of soil (i.e. number of phylotypes g^{-1}). Estimates are then compared with the goal to establish an association between microbial alpha diversity and some large-scale environmental gradient. At this point it is interesting to consider if estimates are directly comparable. The fact that DNA was extracted from an equivalent mass of soil at a standard depth gives the impression that an equivalent sampling efforts have been employed. So as long as the samples are processed by

the same laboratory and bioinformatics approaches, then comparisons are trustworthy.

However, this may be a misconception. Consider comparing an old growth tropical montane forest organic soil and a temperate grassland soil, and let us assume the latter has twice the bulk density of the former (0.6 g cm^{-3} for the forest and 1.2 g cm^{-3} for the grassland, Fig 1a). Let's imagine both areas are part of a large scale monitoring program in which microbial richness is compared to establish a relation of microbial alpha diversity with temperature or pH. Ten samples are taken with a soil corer of a given diameter from each area to a standard 10 cm depth. If DNA is extracted from 1 g subsample of dry soil from each sample in these two different soils (i.e. a total of 10 g for each ecosystem), then the extraction is representing radically different areas sampled; in this example half the area of the grassland soil. We calculated this difference using the following equations:

$V_s = m/\rho$ (eq.1)

$A = V_s/h$ (eq. 2)

, where V_s represents the volume of fine soil accounting for bulk density, *m* the mass of fine soil from which DNA was extracted, *ρ* represents the bulk density of the fine soil fraction, *A* represents the area covered by the sampling and *h* represents the length of the core (i.e. depth sampled). Since the area difference is most likely due to differences in the content of coarse organic matter and percentage of pore volume (Fig 4.1b), this introduces a heavy bias in comparing among these samples in terms of how much potential microbial habitat is available at 10 cm depth. Basing the diversity estimate on mass thus would need to be corrected by the bulk density in the field to arrive at a fairer comparison, at least in terms of general area sampled.

A different issue regarding microbial habitat availability arises when we consider that the fine soil fractions in these biomes have different textural compositions. The organic-rich soil fraction in the forest is composed of mineral particles (silicates and oxides) mixed with organic matter (Wilcke *et al.*, 2002), while the grassland fine soil fraction may have a sandy loam texture (Lehmann *et al.*, 2020). If we consider the forest soil is proportionally dominated by small diameter particles while the grassland soil is dominated by sand, then the inhabitable surface might be inherently different between these two soils (Fig 4.1c). In the forest soil there will be an abundance of

small pores while in the grassland soil the pore space will be overall reduced. This is because larger particles occupy more volume and tend to generate larger pores.

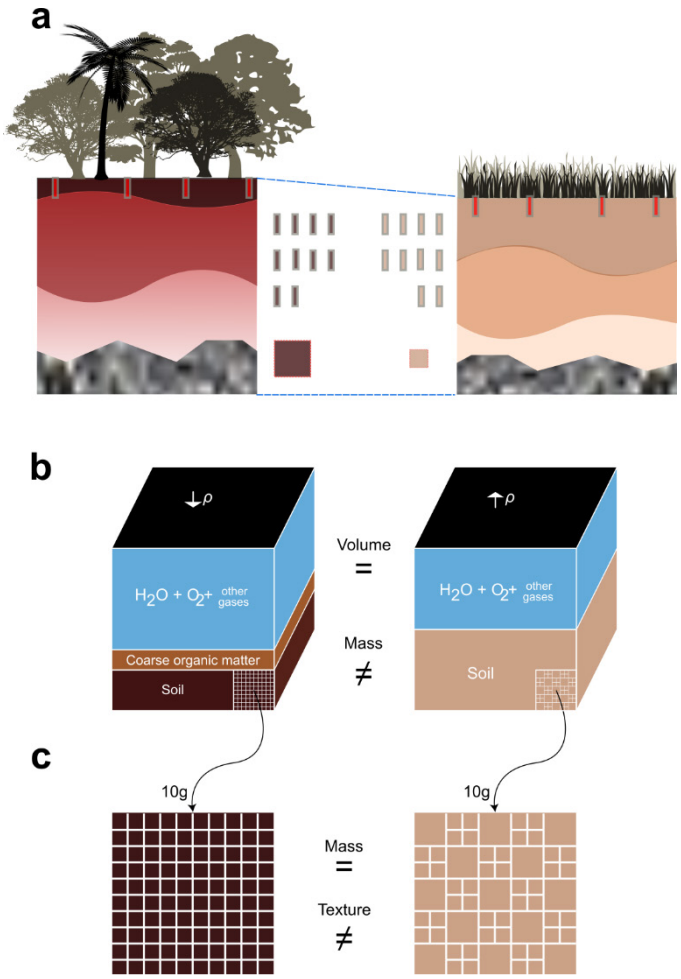

Figure 4.1 Examples of possible confounding effects introduced by natural variation in bulk density and texture across biomes

Panel **a** shows a situation in which despite following a consistent collection protocol, the effective area sampled in an ecosystem with lower bulk density is always greater than another with higher bulk density if comparisons are based on a mass-based subsample used for DNA analysis (e.g. 1 g). Red rectangles represent cores, and the square below is the total area sampled. Panel **b** shows a schematic representation of the differences in mass of fine soil present in equivalent volumes of 'intact' soil at different ecosystems. Panel **c** shows an idealized arrangement of soil particles in the same mass of soil with different textures. Squares of different sizes represent soil particles (e.g. sand vs silt) and white lines represent pores between them. It is clear that despite the same mass of soil per ecosystem is collected, samples differ in their potential microbial inhabitable area due to textural differences.

Finally, a less obvious and potentially not as important an issue: we must remember that bulk density changes also with time, so if we were to return to survey the same ecosystem, then most probably the fixed depth protocol will not guarantee recovery of

an equivalent mass of soil. This is because soil is not a static body, but the expression of many interacting variables. Thus if we return to measure one of our ecosystems after an extended dry period, the standard depth sampling method will recover 'deeper soils', because the soil column might have 'shrunk' (Mora & Lázaro, 2014). This shift would manifest as an increase in bulk density between sampling times. If some sort of depth dependent gradient exists that affects taxon richness or composition, it is possible that samples taken at different time points will capture different portions of this gradient.

4.5. Bulk density correction and the basis of soil microbial biodiversity estimates

For other soil properties, such as soil organic C content, correcting for soil bulk density is the norm (i.e. expressing values on area units). The same can be said for virtually any elemental pool estimate. We argue that it should be no different for biological diversity, since this represents an estimate of the biodiversity 'pool'.

However, fundamentally this is a question of 'habitable area', because this would be the most direct equivalent of area for plant and animal diversity surveys. Habitable area is approximated better by area-based estimates than by mass-based estimates; however, clearly, area-based estimates are still an imperfect approximation of truly inhabitable space.

Pore volume, which can be derived from bulk density, and particle density measures, would be a better basis for comparison, but is unlikely to be broadly adopted since it requires additional measurements. However, generally speaking pore volume would be a reasonable approximation of the space in soil that can be colonized by microbes.

4.6. Obtaining bulk density data

So why is the natural variation in bulk density mostly ignored in comparative microbial community studies? One reason might be that bulk density estimation is time consuming, requires dedicated sampling and equipment (e.g. metal rings of known volume), and careful collection and transport of samples to obtain an unbiased estimate (Throop *et al.*, 2012). So if the magnitude of the confounding effect is not large, it will not warrant the additional effort to account for it. In local scale studies, comparisons of microbial community attributes are likely carried out within the same

ecosystems. So we can assume in these situations bulk density will not vary as widely as across ecosystems. Clear exceptions to this statement are cases in which experimental treatments induce shifts in bulk density (e.g. tillage, compaction, or addition of materials with different density, such as biochar), when bulk density shifts drastically as a function of season or time (e.g. for swelling soils), or when we are comparing community attributes across soil layers.

In other domains of soil sciences, a clearer consensus has been reached regarding the need for a methodological shift in order to control for the impacts of bulk density variation across space and time. The practice of collecting a soil subsample of equal mass using fixed depth as a standard has been shown to introduce bias on elemental pool or water content estimations, which can potentially lead to erroneous conclusions about a range management actions (Wuest, 2009; Smith *et al.*, 2020; Juvinyà *et al.*, 2021). The proposed solution is to adjust estimates based on empirical curves that relate element mass per area (e.g. kg C m^2) to the cumulative mass of soil collected per area (kg m^2) (Rovira *et al.*, 2015; Haden *et al.*, 2020). This is known as the 'cumulative coordinates' or 'equivalent mass' method. As this adjustment method is conceptually similar to rarefaction curves (Gotelli & Colwell, 2011), perhaps it can be adapted to adjust microbial diversity estimates in reference to an equivalent mass of soil per area. However, it must be noted that while the equivalent mass method effectively corrects the shortfalls of the fixed depth method, it requires additional work at the moment of sampling. Given bulk density changes rapidly with depth, the equivalent mass method requires to sample consecutive and smaller depth intervals (5 cm) within the soil column. In each of these intervals, the mass of soil, the volume and mass of the accompanying coarser particles and an estimate is required to build the empirical curve. This means that the cost of accounting for this bias make this alternative impractical for the time being.

A lower cost alternative to deal with the issue would be to use third party information to adjust measurements. There are now high resolution maps of global scale bulk density estimates based on thousands of field samples and very robust modelling approaches (Hengl *et al.*, 2017; Batjes *et al.*, 2020). However, the uncertainty of these estimates might still be too high in some regions (Hengl *et al.*, 2017). By contrast, highly accurate estimates might be available at a higher resolution from well-established national soil mapping efforts (Batjes *et al.*, 2020). In case none of these

options exists, new methods to determine bulk density *in situ* employing sensors might be available for field applications in a near future (Lu *et al.*, 2016).

4.7. Conclusion

Our analysis intends to show that natural differences in soil bulk density are important to consider when microbial community parameters are compared across ecosystems or over time. Contrary to what one might expect, this problem is not only relevant to comparisons across studies that may use different soil collection standards. Within-study comparisons might also be confounded if soils within the areas or times being studied vary widely in terms of bulk density. In our sample of the microbial ecology literature, we could confirm differences in bulk density are not accounted for when extrapolating and comparing estimates across large spatial scales and it is rarely disclosed whether the organic layers of soil are included in the sampling. This contrast with the literature that compares microbial biomass estimates at global scales, where we found several examples in which differences in bulk density were accounted for when extrapolating and comparing estimates (Fierer *et al.*, 2009; Serna-Chavez *et al.*, 2013; van den Hoogen *et al.*, 2019). More fundamentally, what we are addressing is a question of to what extent we are capturing microbial habitat with our current collection protocols. Clearly, current sampling methods ignore many soil structural features that possibly affect the extent of microbial habitat surveyed and consequently the estimates we obtain. Yet these are the most practical approaches we have so far. We thus need to start thinking about ways to overcome these shortfalls if we aim to provide a representation of microbial communities that better captures reality.

4.8. Acknowledgements

We would like to acknowledge Francisco Dueñas-Serrano for drawing parts of the figure that appears in this chapter.

5. Chapter 5 General Discussion

The goal of this dissertation was to gain insight into the response of root associated fungal communities to increases in the atmospheric depositions of N and P in eastern Andean forests. In **Chapter 2** we reported a consistent effect of P additions on AMF community composition and a consistent reduction in Glomeraceae richness after the combined addition of N and P. The study presented in **Chapter 3** found that while P additions alter the composition of non-AM root associated fungal communities (RAF), the response was not consistent across major fungal lineages. Finally, **chapter 4** draws attention to the fact that current sample collection standards in microbial ecology neglect the natural variations in soil density, potentially introducing bias to comparisons of microbial community attributes. The present section discusses these findings and situates them in the context of broader fungal ecology. For simplicity, this discussion will be restricted to studies that have employed sequencing techniques to assess fungal diversity and community composition.

5.1. Distribution and diversity of root associated fungi

Elucidating the factors that control the distribution of mycorrhizal guilds is one of the fundamental questions in mycorrhizal ecology. A recent paper argues that climate variables, such as temperature and precipitation, control the distribution of main mycorrhizal guilds across the world (Steidinger *et al.*, 2019). This idea was originally postulated by David J. Read (1991), who suggested that biomes where N mineralization and litter decomposition are hindered by climate conditions are prime habitats for ecto- and ericoid mycorrhizal fungi. Conversely, ecosystems where N mineralization and litter decomposition proceed much faster due to warmer and unseasonal climates are dominated by AMF. Another pattern that has also gathered much attention in recent years, is the positive correlation between the cover of EM vegetation within a biome and its positive correlation with the belowground soil organic carbon content (Averill *et al.*, 2018; Soudzilovskaia *et al.*, 2019). This correlation has not been convincingly identified for biomes where AMF are dominant. In **Chapter 2** we presented results that counter these patterns. We showed that while AMF diversity decreased with elevation, AMF communities were nonetheless present across a 2000 m elevation gradient of tropical montane forests. These findings indicate that the AM symbiosis tolerates cooler temperatures and can be present in sites characterized by

low mineralization rates of C or N in the tropics. The results presented in **Chapter 2** are also in line with previous studies of montane tropical forest that showed that diverse AMF communities can be found all the way from lower montane forest to páramo grasslands at 4000 m above sea level (Camenzind *et al.*, 2014; Haug *et al.*, 2019). Furthermore, in **Chapter 3** we show that while some ectomycorrhizal and ericoid mycorrhizal taxa are present at the lower montane forest site, the relative abundance of either one of these mycorrhizal guilds is lower than the AMF abundance. As it is clear that tropical montane data are misrepresented in global databases, it is important to expand sampling efforts to include other sites across the Andes and other major mountain ranges across the tropics.

Regarding AMF beta diversity, Davidson *et al.* (2015) showed that the phylum Glomeromycota exhibited low levels of endemism across continents and biomes, and speculated that this pattern is due to a natural capacity for long distance dispersal of AMF taxa or assisted dispersal by humans. In **Chapter 2** we show that local AMF communities in tropical Andean forest exhibit high turnover rates of abundant taxa (i.e. high beta diversity). We also found that Acaulosporaceae read abundance increased with elevation, whereas the opposite trend was observed for Glomeraceae (Haug *et al.*, 2019). These patterns suggest that even when AMF are capable of long distance dispersal, a strong signature of environmental filtering is present both at the family and at finer taxonomic levels in tropical montane systems. Interestingly, the shifting pattern of AMF family abundances has been independently observed by other studies in the area (Camenzind *et al.*, 2014; Haug *et al.*, 2019). Members of Acaulosporaceae have been characterized as stress tolerant, and exhibit reduced intra- and extra-radical growth (Hart & Reader, 2002; Oehl *et al.*, 2009; Chagnon *et al.*, 2013), whereas members within Glomeraceae favor extensive root colonization over extra-radical growth and are characterized as nitrophilic (Hart & Reader, 2002; Powell *et al.*, 2009; Treseder *et al.*, 2018). Although tropical montane forest soils can certainly be characterized as stressful, due to the acidic pH, low temperature and availability of mineralized nutrients, the high extra-radical abundance observed at higher elevations (Camenzind *et al.*, 2016) questions the generality of these traits among the cited AMF families. These results indicate that we still know very little about the variability of life history traits within AMF families and point to an interesting paradox that needs to be clarified with further research.

Information regarding the distribution of RAF clades and guilds is generally scarce (Jumpponen *et al.*, 2017). Previous research has shown that richness of endophytes and parasitic fungi tends to increase towards the tropics (Tedersoo *et al.*, 2014; but see Větrovský *et al.*, 2019). Aside from these generalities, very few studies have assessed the patterns of diversity and composition of RAF in tropical forests (Luo *et al.*, 2014; Bonfim *et al.*, 2016; Schroeder *et al.*, 2018, 2019). This scarcity highlights the contribution of **Chapter 3**, where a detailed assessment of the taxonomic and guild structure of RAF communities is presented. Our research shows that these communities are dominated by Ascomycota amplicon sequence variants (ASVs), a large portion of which were classified as Helotiales, a fungal order encompassing a prominent group of endophytes in temperate forests (Jumpponen *et al.*, 2017). Hence, it would be interesting to conduct studies that further assess the trophic mode and function of Helotiales associated to roots in tropical montane forests.

5.2. Responses to nutrient additions

The relationship between AMF diversity and the availability of N and P in unmanaged soils is mostly based on research in temperate regions, which largely focuses on grasslands (Johnson *et al.*, 2015; Ceulemans *et al.*, 2019; but see Lilleskov *et al.*, 2019). These studies indicate that AMF diversity is inversely related to the concentration of soil N or P, and strong AMF richness declines occur when N deposition rates exceed the critical threshold of ~7.7 kg N ha^{-1} yr^{-1}, or when P concentration increases beyond 7.23 mg P kg^{-1} soil (Ceulemans *et al.*, 2019). In line with this evidence, recent global-scale meta-analyses show that increased N availability lead to significant AMF richness reductions, if soil P availability and fertilization dosages are high or when fertilization persists over long periods of time (Han *et al.*, 2020; Ma *et al.*, 2021). The results presented in **Chapter 2** are consistent with these patterns to a certain extent, as significant richness reductions were only observed after concurrent N and P addition across all our experimental sites. However, we observed clade specific responses and found no evidence that indicates the effects of nutrient addition worsened with time. On the contrary, the effects we observed after N or P addition were milder relative to the results obtained by Camenzind *et al*. (2014), who found a significant reduction in AMF richness across all fertilization regimes after only 2 years since the fertilization intervention started. These discrepancies could have arisen due to seasonal differences, or the use of a different sequencing platform.

However, it is clear more research on the temporal dimension of nutrient pollution is needed to clarify these issues. The consistent effect of P additions on AMF community composition presented in **Chapter 2** is congruent with previous studies in tropical forests (Alguacil *et al.*, 2010; Camenzind *et al.*, 2014; Sheldrake *et al.*, 2018) and points to an important role of P pollution for tropical systems, regardless of elevation.

There is simply no prior research on the community level response of RAF clades to fertilization in the tropics. Since we assumed in **Chapter 3** that the pool of fungi available to colonize roots is recruited from soil, it is possible to discuss these results based on the literature that examines how fertilization affects soil fungi. To our knowledge, there is only one nutrient manipulation experiment in the Neotropics where the response of litter fungal communities to fertilization has been systematically measured (Gigante Peninsula, Republic of Panama, Kaspari *et al.*, 2010, 2017; Kerekes *et al.*, 2013). High throughput sequencing assessments within the context of this experiment revealed that richness decreases (non-significantly) after N addition, but does not respond to the addition of any other single nutrient (P or K). In contrast, the addition of multiple nutrients, such as the combined addition of P and K, or the simultaneous addition of several micronutrients, increased fungal diversity and elicited compositional changes (Kerekes *et al.*, 2013; Kaspari *et al.*, 2017). Assessments of soil fungal communities in fertilization experiments conducted outside the tropics reveal similar patterns. In the context of a large scale study of N and P fertilization to temperate grasslands, researchers did not find a negative, or positive, effect of fertilization on richness of Ascomycota or Basidiomycota. Yet community composition of these clades was significantly affected by the addition of both N or P (Leff *et al.*, 2015). In **Chapter 3** we showed that despite the fact that fertilization reduces richness of several fungal clades, these reductions were not statistically significant. In contrast, P addition altered the composition of RAF communities at the kingdom and phylum levels. Thus our findings appear to be in line with prior results and again point to a stronger response of fungal communities to P addition in tropical montane forests.

Kaspari *et al*. (2017) proposed a series of compelling hypotheses to explain the responses of fungal communities to fertilization in Panama. Some of these hypothesis can also serve to explain the results observed in tropical Andean forests. First, it was proposed that N addition negatively impacts fungal richness through indirect effects. For instance, by reducing soil pH (Chen *et al.*, 2016; Zhou *et al.*, 2020) and increasing

Al toxicity. Since Al toxicity affects P uptake (Kaspari *et al.*, 2017), the negative effect of Al toxicity will be mitigated by the concurrent addition of P, or when P is naturally more abundant. Though tropical montane forests soils have low P availability, P content is higher in relation to lowland tropical forests soils (Wilcke *et al.*, 2002, 2008; Unger *et al.*, 2010). Moreover, there is so far no evidence of acidification of the soil organic layer after the addition of N in the context of the Ecuadorian nutrient manipulation experiment (Krashevska *et al.*, 2014; Velescu *et al.*, 2016), and the concentration of Al in the organic layer of these forests is relatively low (Wilcke *et al.*, 2002, 2008). Hence the indirect deleterious effects elicited by N addition have so far not been observed in the Ecuadorian soils, therefore not reducing fungal richness significantly. Second, given tropical soils are highly heterogeneous (Townsend *et al.*, 2008), single nutrient additions are unlikely to homogeneously and consistently alleviate the deficiency of resources across soil. In other words, patches of low resource concentration will continue to exist, thereby providing a niche for organisms adapted to low resource utilization and thus decreasing the chances of competitive exclusion by organisms adapted to high nutrient availabilities.

5.3. Limitations of modern approaches to study microbial communities

Our intention at the beginning of **Chapter 3** was to assess the response of fungal pathogens and beneficial mutualists to increases in the availability of nutrients. Unfortunately, the paucity in research on tropical fungal taxa and the fact that a large proportion of fungal phylotypes presented in **Chapter 3** are undescribed hampered our efforts. To put this into context, we could only assign a putative trophic guild to 14.37% of the fungal reads recovered, which is significantly less than the ~40% achieved by a study of the effects of N and P addition on functional guilds in temperate grasslands (Lekberg *et al.*, 2021). Such low assignation success does not allow to conduct a robust analysis on the effects of N and P additions on root associated pathotrophs or facultative saprobe communities.

High throughput sequencing techniques hold the promise to revolutionize microbial ecology. In **Chapter 4** we posit this premise is true, provided we find an appropriate basis to express microbial community attributes. The implications of comparing microbial community attributes across large spatial extents are discussed in light of

the fact that physical attributes of soil, such as bulk density or texture, vary extensively across biomes or ecosystems (Hengl *et al.*, 2017). Consequently, soils differing in their physical properties will offer drastically different extents of micro habitats and environmental conditions for microbial organisms which could lead to differences in richness or composition. In order to mitigate the effects of this potential bias in downstream inference, we propose microbial richness estimates should be standardized on the basis of area, and not mass as it is currently done. We think expressing estimates on area basis allows a better assessment of the sampling effort applied across samples and reflects the extent of surveyed microhabitat more accurately. The issues discussed in **Chapter 4** are mostly relevant when microbial communities are compared across ecosystems or biomes. Thus considering the recent explosion in studies that aim to establish relationships between microbial community attributes and environmental gradients over large spatial scales, we argue it is important to open the discussion about these caveats.

5.4. Conclusion

The results presented in this dissertation suggest that root associated fungal communities in eastern Andean forests are more sensitive to increased P than to N deposition. These responses are generally in line with studies that indicate P is more limiting than N for microbes and the processes they drive in tropical montane forest (Velescu *et al.*, 2016; Dietrich *et al.*, 2017; Camenzind *et al.*, 2018). However, there is also an increased awareness that microbial responses to fertilization cannot be fully understood from the perspective of limitation of individual nutrients (Kaspari *et al.*, 2017; Camenzind *et al.*, 2018) or from employing models conceived for plant communities (Kaspari *et al.*, 2017). Regarding tropical montane fungal communities, more research is needed to assess clade specific responses to C addition (Krashevska *et al.*, 2010; Nottingham *et al.*, 2018), as well as calcium, manganese, sulfur and zinc, which are also scarce elements in the organic layer of tropical montane soils (Wilcke *et al.*, 2002).

Despite the fact that root associated fungal communities are directly or indirectly involved in many ecosystem processes (C fixing, decomposition, soil respiration, N immobilization, forest regeneration) that might shift as a result of increased atmospheric deposition, a detailed understanding of how these key fungal communities respond to this disturbance in tropical montane forests is still lacking.

This dissertation has contributed to change this situation to a certain extent, but has also made apparent that high throughput sequencing approaches do not constitute a panacea to remedy all our knowledge deficiencies about fungi in this special region. It is clear that the capacity of these techniques to reveal the effects of environmental change on fungal communities hinges on the work of taxonomists and the overall improvement of the representation of tropical taxa in reference databases. Moreover, a focus shift away from mycorrhizal clades is needed in order to uncover the ecologies of a large portion of tropical fungal lineages that still remain in the shadows. More broadly, we need to reflect about the extent to which these modern techniques are capturing the underlying microbial environment and how we can improve our sampling strategies to ensure the diversity estimates we derive reflect microbial communities more accurately.

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Contributions to the publications

I. **Chapter 2: Dueñas JF, Camenzind T, Roy J, Hempel S, Homeier J, Suárez JP, Rillig MC**. **2020**. Moderate phosphorus additions consistently affect community composition of arbuscular mycorrhizal fungi in tropical montane forests in southern Ecuador. *New Phytologist* **227**: 1505–1518. doi: 10.1111/nph.16641

Own contribution: I performed the laboratory work, analyzed the data and wrote the manuscript. I also corrected the manuscript according to the suggestions of co-authors, the editor and three anonymous reviewers.

II. **Chapter 3: Dueñas JF, Hempel S, Homeier J, Suárez JP, Rillig MC, Camenzind T**. **2021**. Non-mycorrhizal root associated fungi of a tropical montane forest are relatively robust to the long-term addition of moderate rates of nitrogen and phosphorus. In preparation.

Own contribution: I performed DNA extractions, conducted bioinformatics and statistical analysis and wrote the first full draft of the manuscript. I corrected the manuscript according to the suggestions of co-authors and two anonymous reviewers.

III. **Chapter 4: Dueñas JF, Rillig MC**. Finding a suitable base for soil microbial diversity: should we account for differences in bulk density in comparative microbial community studies? In preparation.

Own contribution: I contributed with the conception of the idea, performed the literature research, created the figure, and co-wrote the first full draft of the manuscript.

Curriculum Vitae

To comply with data protection laws, the CV of the author is not published in the electronic version.

Appendix A – Supplementary Material for Chapter 2

Figure S2. 1 Study area, site location and experimental design schema

(a) Experimental work occurred at three sites covered by evergreen tropical Andean forests. Sites are located between the provinces of Zamora-Chinchipe and Loja in South Ecuador. At the lowest site, experimental plots are located on the south-east facing slopes of Bombuscaro river valley (4°7'S 78°58'W). Mid elevation experimental plots lie on the north facing slopes of San Francisco river (3°58'S, 79°04'W). The highest elevation site lies on the slopes of Cajanuma range (4°7'S 79°11'W).

(b) Experimental design scheme depicting the arrangement of plots within blocks in the experimental site of San Francisco river valley. Figure S2.1 is taken from Homeier *et al*. (2013). Reproduced and modified with permission from the author.

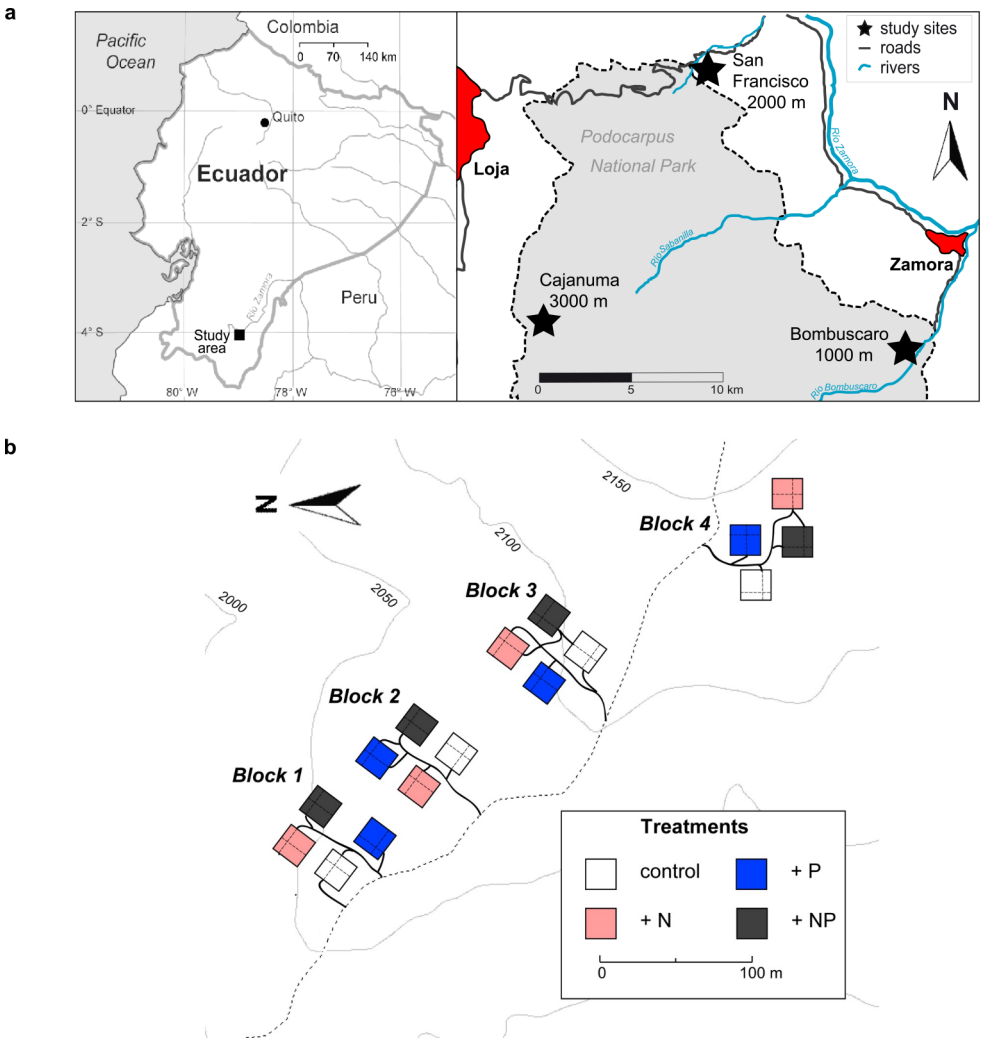

Table S2. 1 Experimental site detailed environmental properties

Biotic and abiotic properties of each site are conditioned by geologic, climatic and topographic factors. Each site is covered by a different forest type within the larger category of montane tropical forest.

Forest type[a]	Plot elevation range[b]	Parent material[b]	Soil type[c]	Organic layer thick-ness[c]	Mean annual temperature[d]	Soil temper-ature[c]	Common tree families[b]
Evergreen pre-montane rainforest	990 – 1100 m	Jurassic weathered granitic rock	Dystic Cambisol	0 – 5 cm	~19.4° C (11.5° – 30.2°)	~16° C	Fabaceae, Melasto-mataceae, Moraceae, Myris-ticaceae, Rubiaceae and Sapotaceae.
Evergreen lower montane forest	2020 – 2120 m	Paleozoic metamor-phic schist and sandstones with some quartz veins	Stagnic Cambisol	10 – 30 cm	~15.7° C (7.9° – 29.4°)	~13° C	Euphorbiaceae, Lauraceae, Melastomataceae and Rubi-aceae.
Evergreen upper montane forest	2900 – 3050 m	Paleozoic metamor-phic schist and sandstones with some quartz veins	Stagnic Histosol	10 – 40 cm	~9.4° C (3.1° – 18.8°)	~ 4° C	Aquifoliaceae, Clusiaceae, Cunnoniaceae, Lauraceae and Melastomataceae.

[a,b] Homeier *et al*. 2013

[c] Martinson *et al*. 2013

[d] Moser *et al*. 201

Table S2. 2 Summary of the commands and packages used for statistical analysis

Statistical procedure	Command	Package
Build up and manipulation of phyloseq object	phyloseq(), merge_phyloseq() otu_table() tax_table() sample_data() subset_samples() prune_samples() subset_taxa() filter_taxa() psmelt()	phyloseq
Rarefaction and species accumulation curves	rarecurve() specacum()	vegan
Variance stabilizing transformation	phyloseq_to_deseq2() estimateSizeFactors() estimateDispersions()	DESeq2
Taxonomic diversity estimates	specnumber() diversity()	vegan
Aggregate OTU abundance table by order	group_by() summarize()	dplyr
Linear mixed effect models	lmer() anova() summary() bootMer() fixef() coef()	lme4 lmerTest
Multivariate generalized linear models	mvabund() decostand() meanvar.plot() manyglm() anova.manyglm()	mvabund vegan
Ordination plots	vegdist() metaMDS() envfit() plot() dbrda() gg_ordiplot() ggplot() ggarrange()	vegan ggordiplots ggplot2 ggpubr
Re-sampling without replacement (Rarefaction)	rrarefy()	vegan
Permutational multivariate analysis of variance	vegdis() adonis()	vegan
Geo spatial statistics	coordinates() proj4srings() spTransform() readOGR()	sp, rgdal
Moran eigenvector maps	listw.candidates() listw.select()	adespatial

Figure S2. 2 Density distribution of sequencing depth across sequencing runs

Before applying the VST, density profiles of sequencing depth per sample showed a left skewed distribution across sequencing runs.

(a) The difference in sequencing depth between samples spanned several orders of magnitude (median: 19,463 reads, range 17–115,691).

(b) Transformation led to a shrinkage in the variability of sequencing depth (253, 14–656).

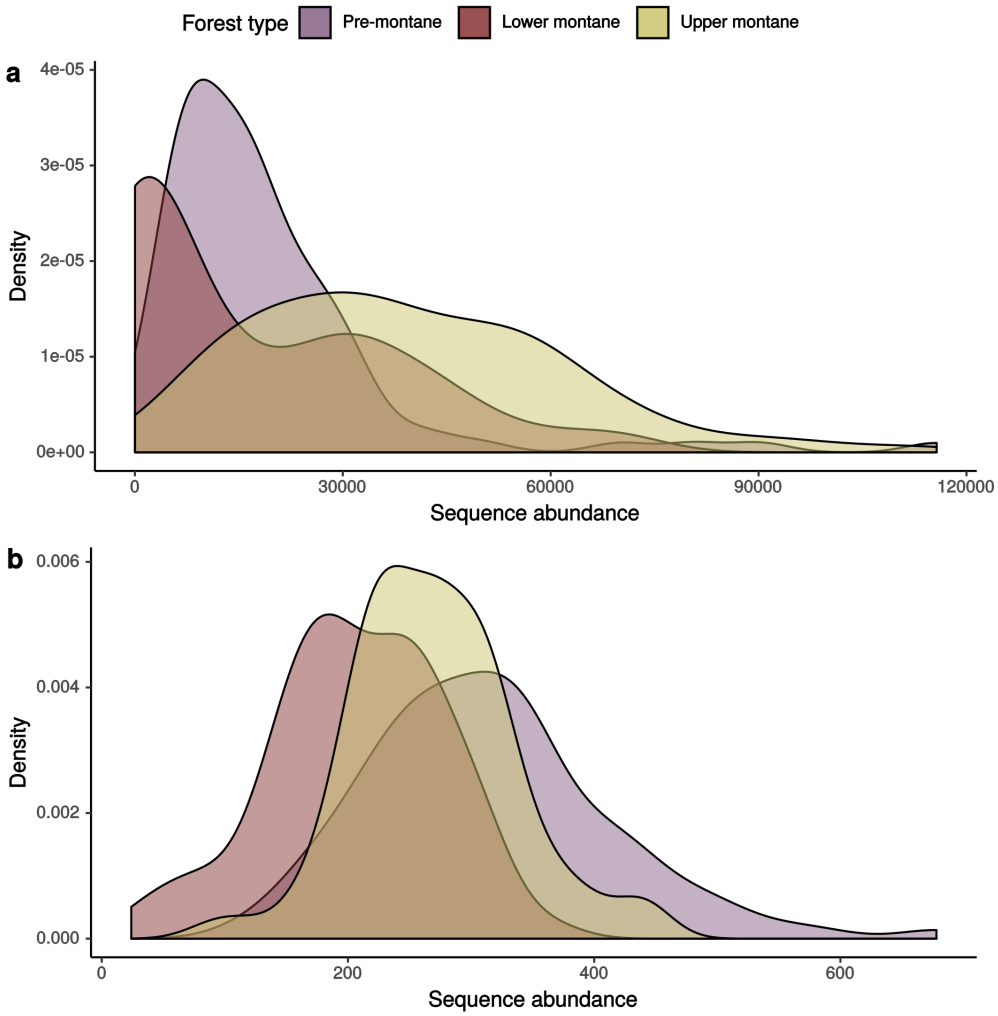

Figure S2. 3 Rarefaction curves per sample and per treatment estimated for non-transformed (a—d) and variance stabilized data (e—h)

Solid colored lines represent asymptotic functions of OTU accumulation as sequencing depth increases. Red vertical lines represent the median sequencing depth of both non transformed and transformed data. Lilac represents samples from control plots, brown from N fertilized plots, yellow from N and P fertilized plots and black from P fertilized plots.

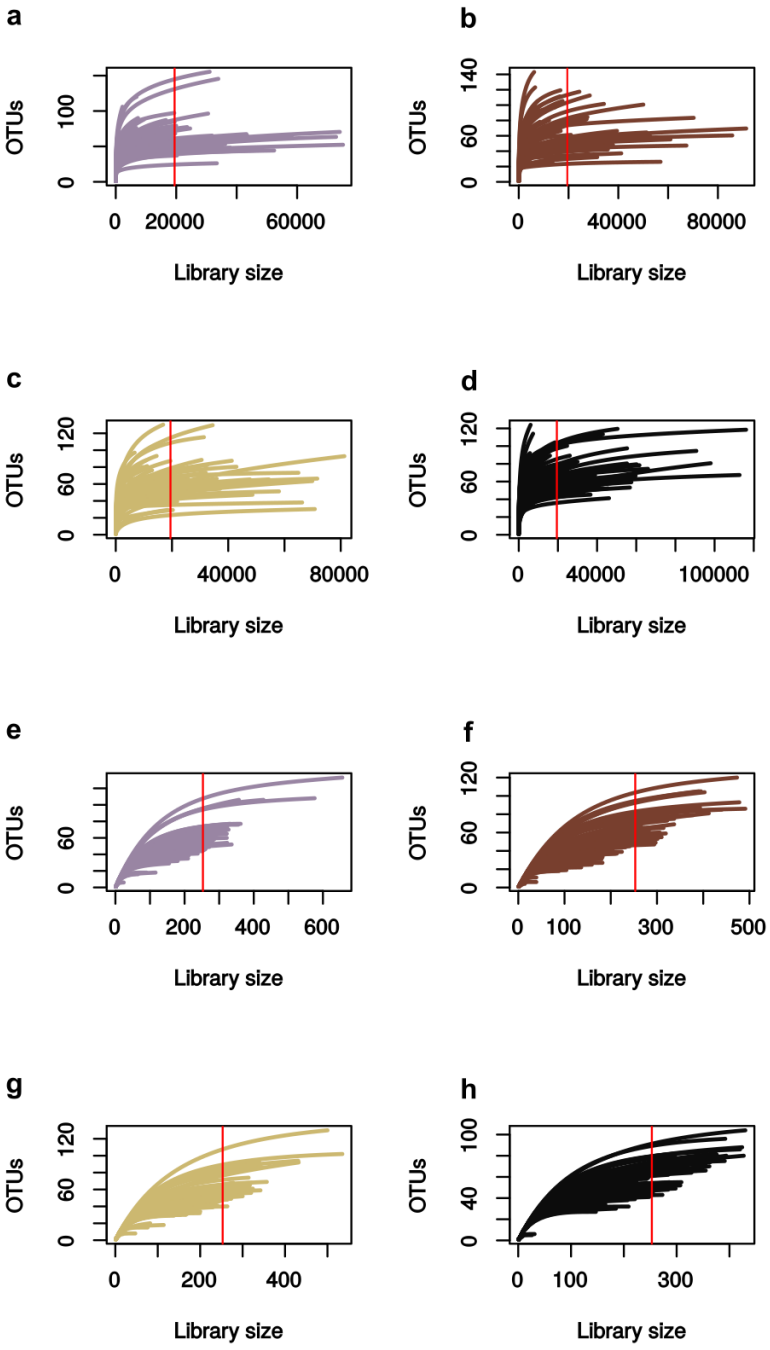

Figure S2. 4 Relationship between mean OTU presence and its variance

This relationship was observed at the (a) pre-montane, (b) lower montane and (c) upper montane datasets. Different symbols represent the four different treatments. The cloud of points has been slightly faded to emphasize the consistency of the relationship across datasets and treatments.

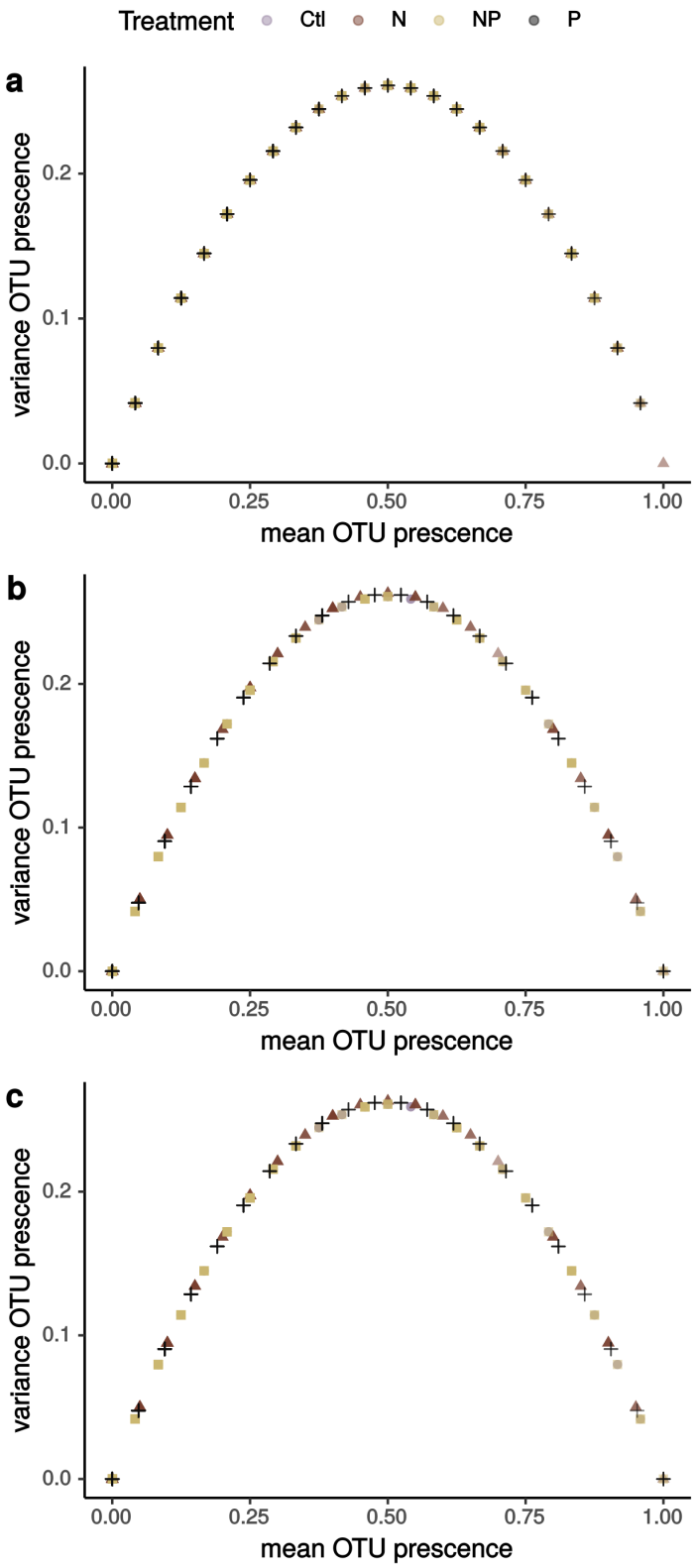

Figure S2. 5 Taxon accumulation curves estimated from variance stabilized data

Lines represent asymptotic functions of OTU accumulation as sample size increases. The area around the curve represents the standard error of the estimated asymptote.

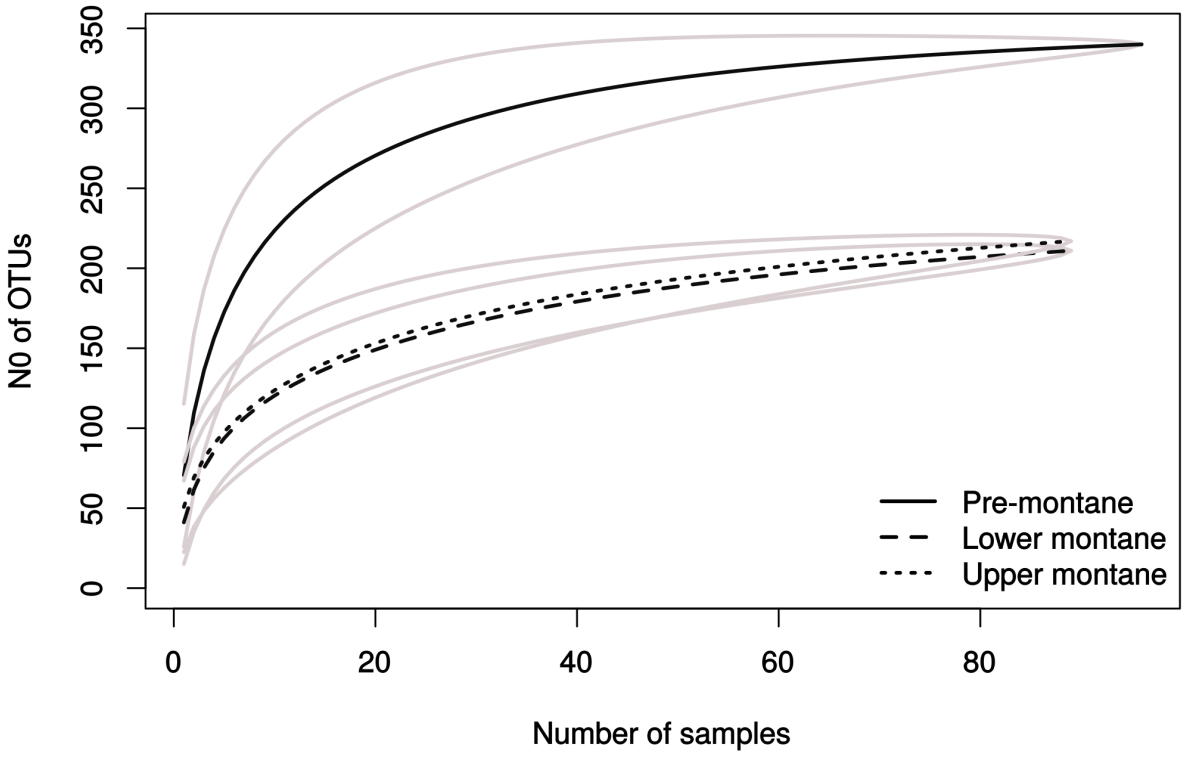

Table S2. 3 Contribution of each random term component to explain residual variability in H_0 (Richness) and H_2 (1/Simpson's dominance)

The contribution of each random term component to explain residual variability in H_0 (Richness) and H_2 (1/Simpson's dominance) was assessed by comparing nested models via likelihood ratio tests (LRT). The order of models is in accordance to the percentage of residual variance explained by the random term components. Response variables were square root transformed before model fitting. Degrees of freedom represent the number of parameters estimated for each of the models.

Response variable	Fixed terms	Random terms	df	log like-lihood	Devi-ance	χ^2	*P*	Var	SD	%
H_0	N x P	Site	6	-433.434	866.867			0.65	0.80	32.58
	N x P	Site/Block/Plot	7	-432.000	863.999	2.868	0.090	0.10	0.32	5.14
	N x P	Site/Block	8	-432.000	863.999	0.000	1.000	0.00	0.00	0.00
		Residual						1.23	1.11	62.28
H_2	N x P	Site	6	-375.973	751.946			0.35	0.60	28.73
	N x P	Site/Block/Plot	7	-374.187	748.374	3.572	0.059	0.07	0.27	6.05
	N x P	Site/Block	8	-374.187	748.374	0.000	1.000	0.00	0.00	0.00
		Residual						0.80	0.90	65.22

Table S2. 4 Means and standard errors of environmental factors

Chemical parameters of the soil organic layers are based on four samples per study treatment. Tree species richness (*S*) gives the mean number of trees species with a minimum diameter of 10 cm at breast height registered per treatment.

		Pre-montane		Lower montane		Upper montane	
Treatment	Parameter	Mean	SE	Mean	SE	Mean	SE
Ctrl	pH	4.18	0.20	2.65	0.03	2.66	0.14
	C:N	16.27	0.76	23.49	0.54	26.68	1.12
	P (µmol g^{-1})	0.17	0.07	0.30	0.08	0.49	0.23
	Tree *S*	16.50	3.23	12.25	0.48	18.00	2.48
+N	pH	3.79	0.15	2.65	0.06	2.69	0.14
	C:N	16.03	0.71	21.68	0.54	23.79	1.23
	P (µmol g^{-1})	0.35	0.05	0.28	0.09	0.49	0.16
	Tree *S*	19.50	1.85	14.75	2.06	15.25	0.95
+N+P	pH	4.01	0.33	2.83	0.27	2.56	0.11
	C:N	15.55	0.31	21.45	0.24	24.07	1.99
	P (µmol g^{-1})	0.32	0.03	1.17	0.17	1.30	0.23
	Tree *S*	17.75	1.11	16.00	1.35	16.75	0.25
+P	pH	4.03	0.18	2.63	0.08	2.65	0.08
	C:N	16.76	1.83	23.65	0.58	24.78	2.04
	P (µmol g^{-1})	0.35	0.06	0.70	0.16	1.32	0.28
	Tree *S*	16.75	0.85	13.50	1.50	16.25	0.25

Table S2. 5 Estimates and statistical tests derived from linear mixed effect models fitted with data normalized with variance stabilizing transformation

Estimated intercepts and slopes are in the square root scale. Test statistics and *P* values were calculated by estimating denominator degrees of freedom with Kenward-Roger approximation method. H_0 (Richness) and H_2 (1/Simpson's dominance).

Taxonomic Rank	Hill number	Treatment	Estimate	SE	ddf	*t*	*P*
Glomeromycotina	H_0	Control	7.114	0.594	2.260	11.970	0.004
		N	-0.013	0.240	42.442	-0.053	0.958
		P	0.436	0.238	41.085	1.834	0.074
		N:P	-0.423	0.338	41.808	-1.251	0.218
	H_2	Control	6.189	0.445	2.320	13.900	0.003
		N	-0.038	0.198	42.443	-0.191	0.850
		P	0.239	0.196	41.112	1.223	0.228
		N:P	-0.333	0.279	41.819	-1.195	0.239
Acaulosporaceae	H_0	Control	4.995	0.449	2.348	11.137	0.004
		N	-0.103	0.207	43.383	-0.497	0.622
		P	0.293	0.203	42.057	1.441	0.157
		N:P	-0.057	0.291	41.875	-0.197	0.845
	H_2	Control	4.367	0.386	2.382	11.304	0.004
		N	-0.098	0.186	43.484	-0.528	0.600
		P	0.197	0.182	42.223	1.079	0.287
		N:P	-0.022	0.261	41.899	-0.083	0.934
Glomeraceae	H_0	Control	4.608	1.050	2.038	4.387	0.047
		N	0.058	0.171	43.061	0.340	0.735
		P	0.326	0.167	41.540	1.950	0.058
		N:P	-0.591	0.239	41.761	-2.470	0.018
	H_2	Control	4.040	0.883	2.034	4.576	0.043
		N	0.005	0.136	43.033	0.037	0.971
		P	0.165	0.133	41.497	1.239	0.222
		N:P	-0.479	0.190	41.748	-2.513	0.016
Gigasporaceae	H_0	Control	1.516	0.278	2.444	5.450	0.020
		N	0.000	0.146	34.696	-0.002	0.999
		P	0.045	0.140	34.561	0.321	0.750
		N:P	0.086	0.202	35.209	0.425	0.674
	H_2	Control	1.441	0.231	2.506	6.246	0.014
		N	0.015	0.128	34.958	0.117	0.907
		P	0.012	0.122	34.834	0.095	0.925
		N:P	0.050	0.177	35.528	0.281	0.780

Table S2. 6 Estimates and statistical tests derived from linear mixed effect model fitted with data normalized by rarefying to 850 read minimum depth

Estimates and statistical tests derived from linear mixed effect models fitted with data normalized by rarefying to 850 read minimum depth. Estimated intercepts and slopes are in the square root scale. Test statistics and *P* values were calculated by estimating denominator degrees of freedom with Kenward-Roger approximation method. H_0 (Richness) and H_2 (1/Simpson's dominance).

Taxonomic Rank	Hill number	Treatment	Estimate	SE	ddf	*t*	*P*
Glomeromycotina	H_0	Control	6.02	0.389	2.341	15.465	0.002
		N	-0.091	0.18	33.869	-0.509	0.614
		P	-0.122	0.176	32.517	-0.695	0.492
		N:P	-0.236	0.251	32.856	-0.937	0.355
	H_2	Control	2.872	0.099	16.413	29.036	0
		N	-0.122	0.137	43.214	-0.895	0.376
		P	-0.227	0.134	41.754	-1.694	0.098
		N:P	0.028	0.192	41.8	0.145	0.886
Acaulosporaceae	H_0	Control	4.162	0.378	2.376	11.011	0.004
		N	-0.074	0.181	43.243	-0.41	0.684
		P	0.007	0.178	41.827	0.041	0.968
		N:P	-0.001	0.254	41.832	-0.004	0.997
	H_2	Control	2.206	0.129	3.367	17.151	0
		N	-0.075	0.103	43.178	-0.729	0.47
		P	-0.093	0.101	41.721	-0.915	0.365
		N:P	0.087	0.145	41.806	0.6	0.552
Glomeraceae	H_0	Control	3.924	0.832	2.045	4.714	0.04
		N	-0.052	0.146	43.104	-0.355	0.724
		P	-0.116	0.143	41.606	-0.805	0.425
		N:P	-0.431	0.205	41.779	-2.103	0.042
	H_2	Control	2.136	0.239	2.205	8.957	0.009
		N	-0.089	0.087	42.789	-1.018	0.314
		P	-0.139	0.085	41.148	-1.629	0.111
		N:P	-0.009	0.122	41.622	-0.074	0.941
Gigasporaceae	H_0	Control	1.427	0.217	3.118	6.578	0.006
		N	-0.016	0.161	25.712	-0.1	0.921
		P	0.018	0.163	27.472	0.111	0.912
		N:P	0.056	0.227	28.697	0.247	0.807
	H_2	Control	1.246	0.12	3.896	10.363	0.001
		N	0.046	0.109	26.226	0.426	0.674
		P	0.025	0.11	27.723	0.23	0.819
		N:P	-0.064	0.153	29.015	-0.422	0.676

Table S2. 7 Two-way PERMANOVA results based on Jaccard dissimilarity matrices

Original abundance table dataset was rarefyed to a minimum read depth of 850 reads. Pseudo F tests of PERMANOVAs indicate that adding N does not elicit changes in AMF community composition at any site. By contrast, adding P elicits shifts in AMF community structure at the pre-montane and upper montane forest.

Site	Treatment	Pseudo F	R^2	Pr(>F)
Pre-montane	MEMs	1.25	0.12	<0.01
	N	1.21	0.01	0.11
	P	1.52	0.02	0.01
	N:P	0.89	0.01	0.72
	Residuals		0.85	
Low. montane	MEMs	1.2	0.08	0.02
	N	1	0.01	0.46
	P	1.44	0.02	0.03
	N:P	0.85	0.01	0.76
	Residuals		0.88	
Upp. montane	MEMs	2.08	0.11	<0.01
	N	0.68	0.01	0.86
	P	1.45	0.02	0.10
	N:P	1.38	0.01	0.12
	Residuals		0.85	

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Homeier J, Leuschner C, Bräuning A, Cumbicus NL, Hertel D, Martinson GO, Spannl S, Veldkamp E. 2013. Effects of nutrient addition on the productivity of montane forests and implications for the Carbon cycle. In: Bendix J, Beck E, Bräuning A, Makeschin F, Mosandl R, Scheu S, Wilcke W, eds. Ecosystem services, biodiversity and environmental change in a tropical mountain ecosystem of south Ecuador. Berlin, Germany: Springer, 315–329.

Martinson GO, Corre MD, Veldkamp E. 2013. Responses of nitrous oxide fluxes and soil nitrogen cycling to nutrient additions in montane forests along an elevation gradient in southern Ecuador. *Biogeochemistry* **112**: 625–636.

Moser G, Leuschner C, Hertel D, Graefe S, Soethe N, Iost S. 2011. Elevation effects on the carbon budget of tropical mountain forests (S Ecuador): the role of the belowground compartment. *Global Change Biology* **17**: 2211–2226.

Appendix B – Supplementary Material for Chapter 3

Figure S3. 1 Map of the Ecuadorian nutrient manipulation experiment (NUMEX)

Each colored symbol represents a mixed root sample within an experimental plot. Colors represent the different fertilization regimes to which plots were subjected to. Symbols indicate the spatial position of the four blocks. The inset details how plots are arranged within blocks, and the spatial distribution of samples along the two orthogonal transects within each plot. The experimental area amounts to 6400 m^2. The shortest distance between samples was 1.01 m while the longest was 328.80 m. The terrain polygon layer was obtained from Open Street Maps.

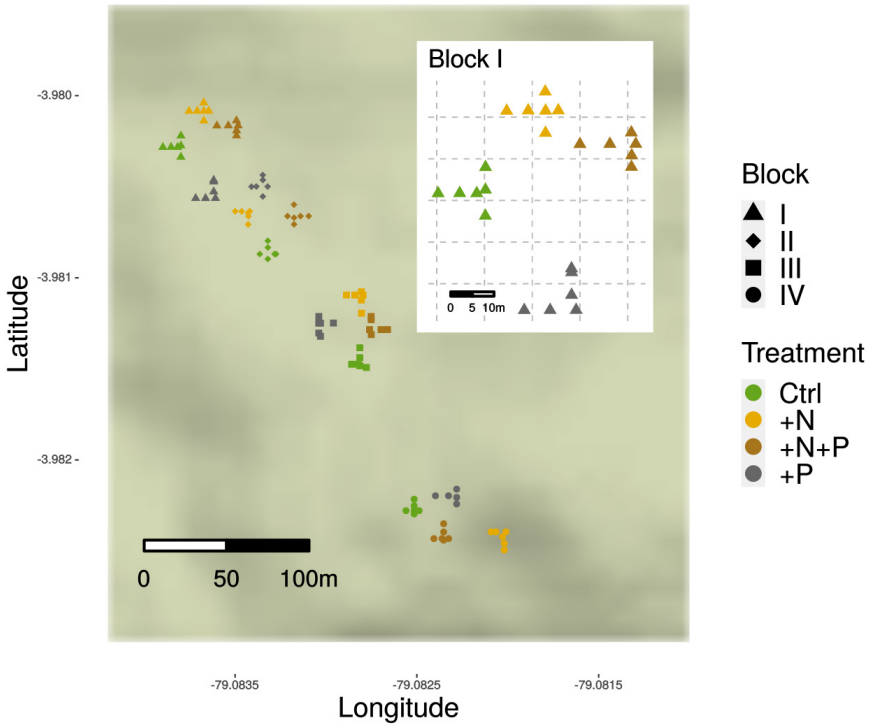


Figure S3. 2 ASV richness accumulation curve as a function of the number of reads per sample

The vertical line signals the minimum read count achieved in our dataset (26 278 reads). This is the cutoff value employed subsequently for normalization of sample read depth. Horizontal lines indicate the rarefied ASV richness estimated for each sample at the chosen normalization depth. Normalization was carried out by random sampling without replacement (n = 100). Unless otherwise stated, all results presented in the main text or below were estimated using the

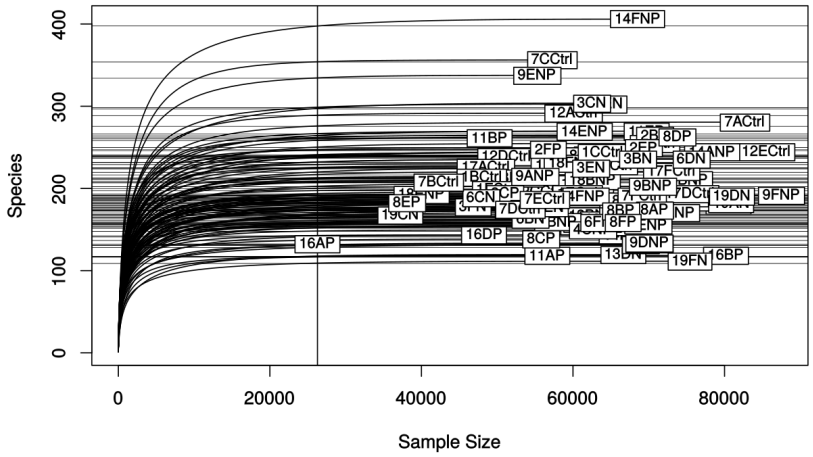


Figure S3. 3 Assumption validation of first glmm (Table S3. 1) with the DHARMa R package

Both figures show that data fit well model assumptions, residuals are not overdispersed, and the negative binomial distribution was a fair choice to model the variance of richness estimates.

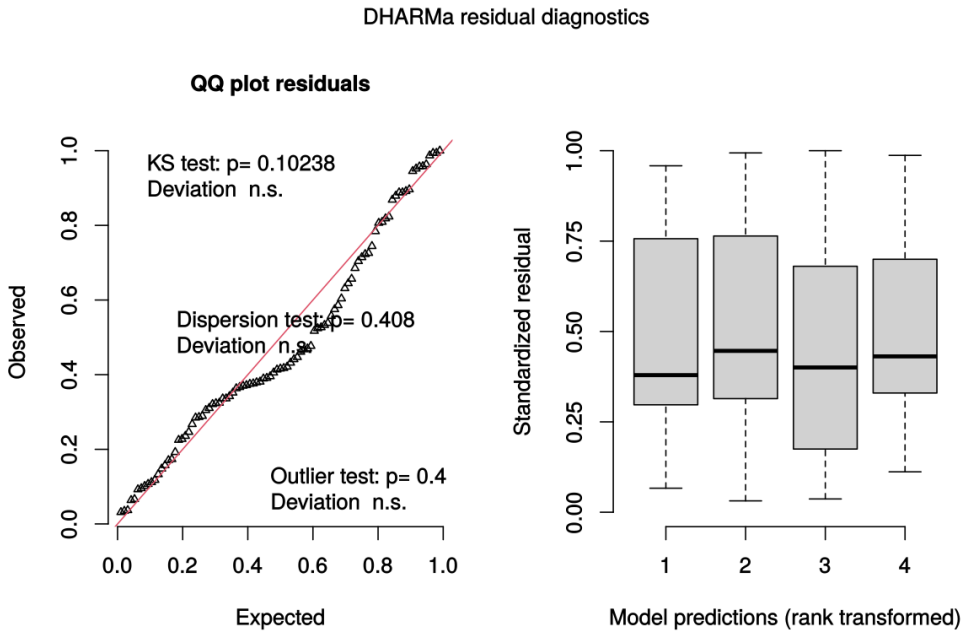


Table S3. 1 Coefficients of the first generalized linear mixed effect model where fungal ASV richness was modelled as a function of fertilization treatments

The intercept estimate corresponds to the mean ASV richness for control plots. Estimates and standard errors are presented in the natural logarithmic scale, as this was the specified link function for the negative binomial probability distribution. *Z* represents the Wald-*Z* statistic, while *p* represents the probability of observing a theoretical *Z* value as large as the observed. σ represents the square root of the estimated random effect variance. The term 'Block' was dropped from the random effect specification, as it led to model overfitting.

Effect	**Group**	**Term**	**Estimate**	**Std. error**	***Z***	***p***
fixed	NA	Intercept	5.396	0.049	109.21	<0.01
fixed	NA	+N	-0.116	0.072	-1.610	0.107
fixed	NA	+P	-0.185	0.073	-2.535	0.011
fixed	NA	+N:+P	0.178	0.104	1.710	0.087
random	Plot	σ	0	NA	NA	NA

Figure S3. 4 Second glmm (Table S3. 2) assumption validation with the DHARMa R package

Both figures show that data fit well model assumptions: residuals are not overdispersed, and the negative binomial distribution was a fair choice to model the variance of richness estimates.

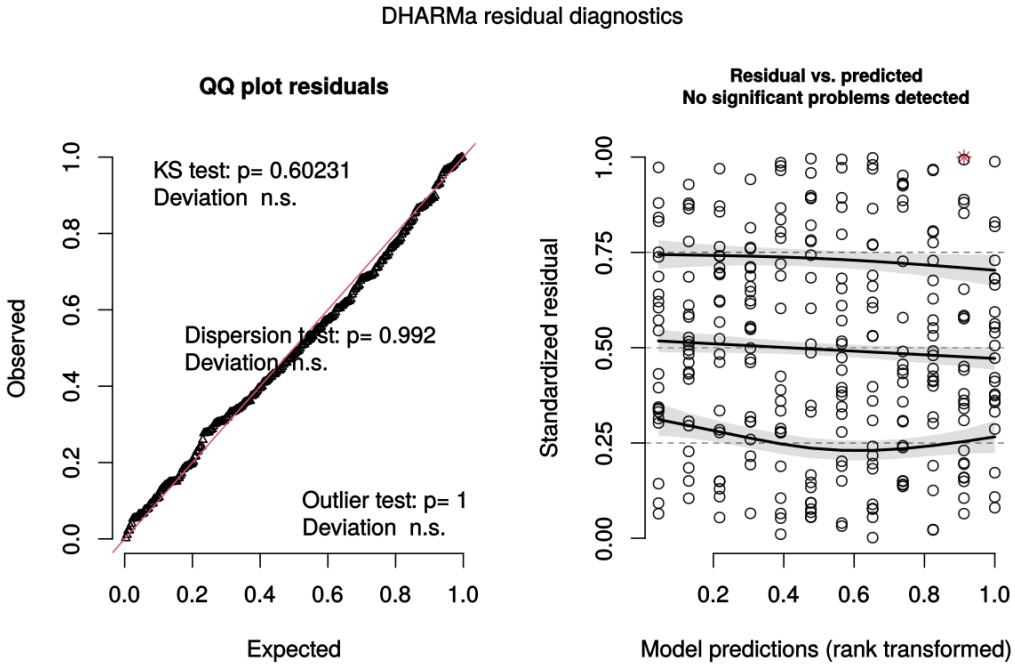


Table S3. 2 Coefficients of the second generalized linear mixed effect model where ASV richness of focal fungal phyla was modelled as a function of fertilization treatments

Estimates along the row labelled as 'intercept' correspond to the mean ASV richness of Ascomycota in control plots. Estimates and standard errors are presented in the natural logarithm scale, as this was the specified link function for the negative binomial probability distribution. *Z* represents the Wald-*Z* statistic, while *p* represents the probability of observing a theoretical *Z* value as large as the observed. σ represents the square root of the estimated variance for each component of the nested random term.

Effect	Group	Term	Estimate	Std. error	*Z*	*p*
fixed	NA	Intercept	4.995	0.069	72.338	0.000
fixed	NA	+N	-0.137	0.098	-1.404	0.160
fixed	NA	+P	-0.148	0.099	-1.499	0.134
fixed	NA	Basidiomycota	-1.305	0.077	-16.867	0.000
fixed	NA	Mortierellomycota	-2.860	0.099	-28.792	0.000
fixed	NA	+N:+P	0.190	0.139	1.365	0.172
fixed	NA	+N:Basidiomycota	0.069	0.110	0.628	0.530
fixed	NA	+N:Mortierellomycota	-0.014	0.143	-0.094	0.925
fixed	NA	+P:Basidiomycota	0.005	0.112	0.045	0.964
fixed	NA	+P:Mortierellomycota	-0.045	0.146	-0.307	0.759
fixed	NA	+N:+P:Basidiomycota	0.059	0.157	0.379	0.705
fixed	NA	+N:+P:Mortierellomycota	0.253	0.203	1.241	0.214
random	Plot:Block	σ	0.093	NA	NA	NA
random	Block	σ	0.000	NA	NA	NA

Figure S3. 5 Comparison of observed differences in ASV richness as a function of fertilization between frequent and rare variants (n=95)

Variants which occurred in at least five samples or more were considered frequent. The horizontal bar in each box represents the median, while the white star represents the mean. Dots above each box represent outliers.

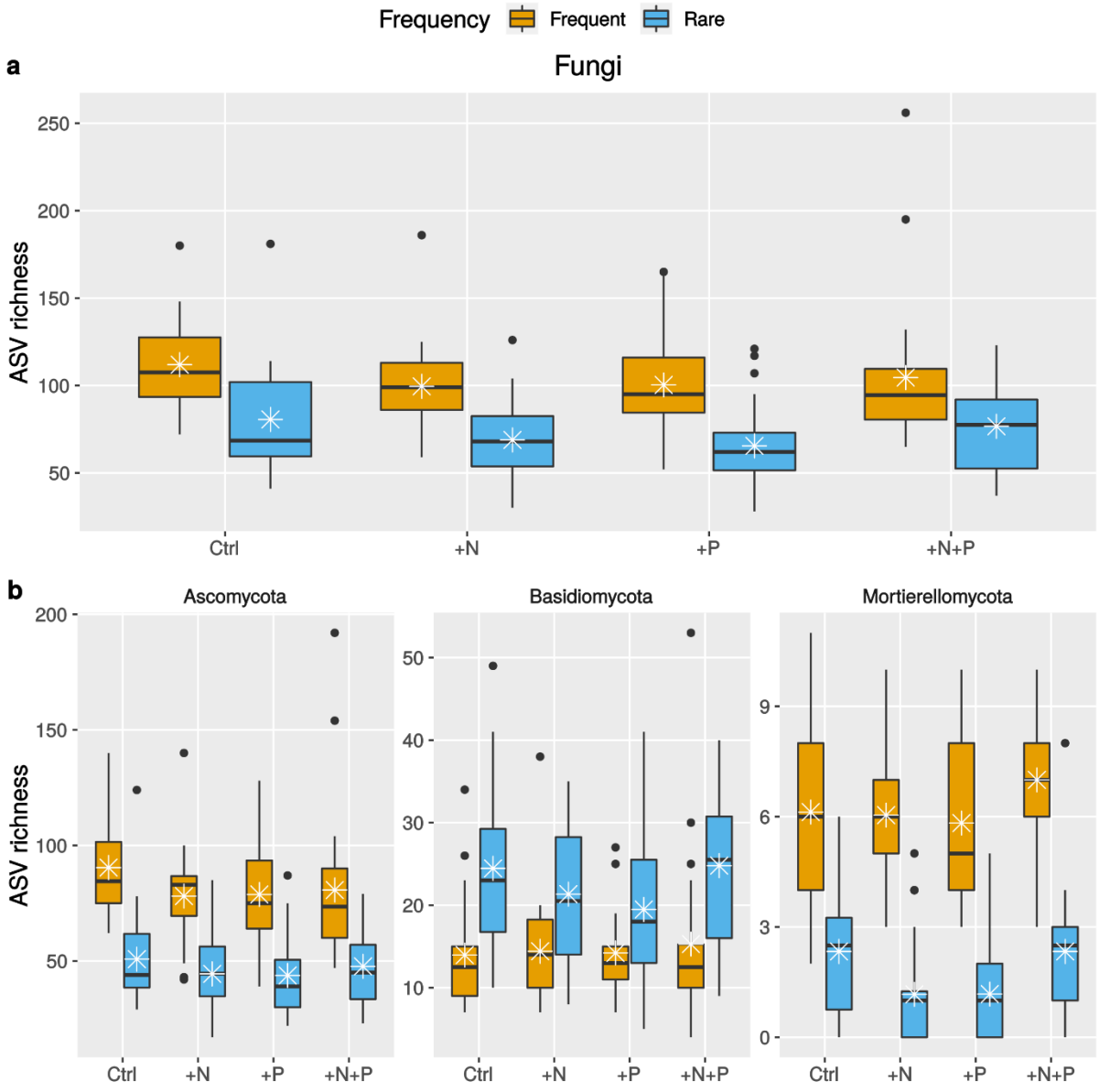


Figure S3. 6 Beta diversity of RAF communities

Beta diversity, defined as turnover of features across communities, is represented here by the Jaccard dissimilarity index. Communities composed by ASVs or by variants grouped within their corresponding species were used to visualize these relations. Panel **a** plots a correlation between beta diversity estimates of the finer levels of taxonomic resolution. Panel **b** plots the relation between beta diversity and Euclidean distance between samples. Red lines indicate the correlation was significant. Correlation parameters can be found in Table S3.3.

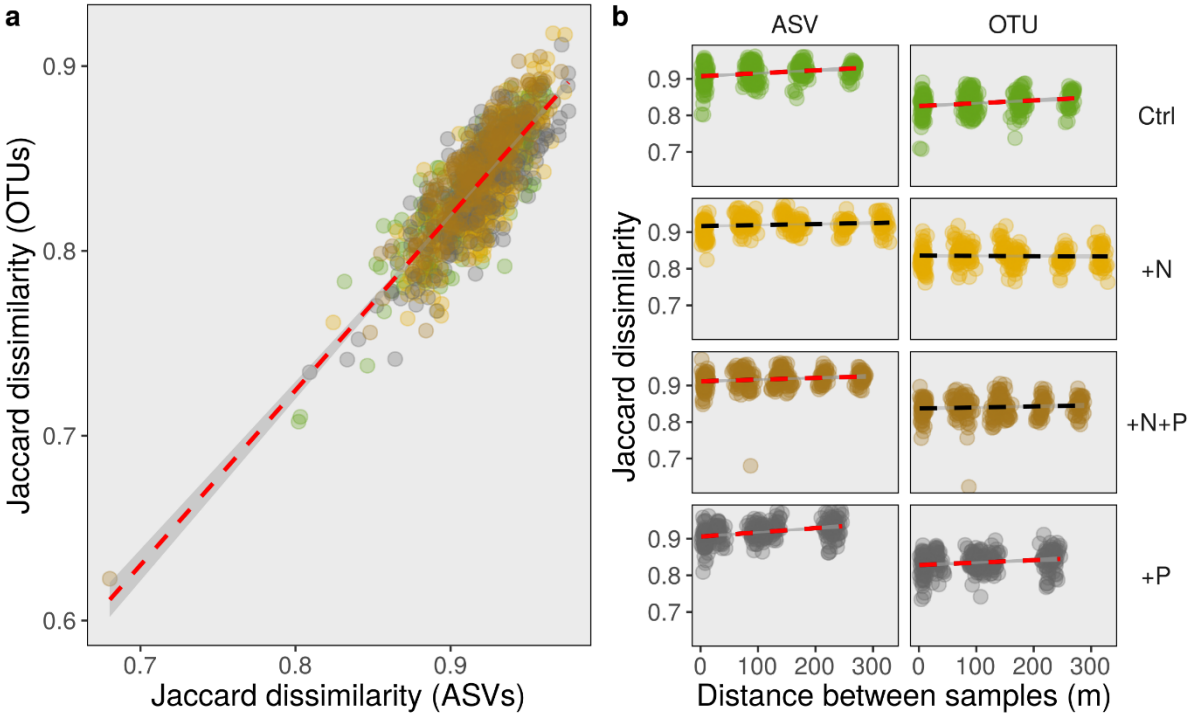


Table S3. 3 Mantel correlation tests

Jaccard dissimilarity matrices were correlated to Euclidean distance matrices. The default correlation type was employed (Pearson). Permutation test were free (n=999). OTUs are clustered at 97% similarity.

Taxonomic unit resolution	**Treatment**	**Mantel *ρ***	***p***
ASV	Control	0.246	0.002
	+N	0.129	0.07
	+N+P	0.157	0.027
	+P	0.355	0.001
OTU	Control	0.217	0.004
	+N	-0.019	0.554
	+N+P	0.085	0.151
	+P	0.188	0.027

Table S3. 4 Neighborhood matrices tests to draw spatial eigenvectors

Residuals were extracted from multiple linear models where ASV tables were modelled as a function of spatial coordinates. Spatial coordinates were expressed in UTMs. Permutation tests were employed to select optimal neighborhood matrices. Optimal matrices maximize the proportion of variance explained (adj. R^2) by Moran eigenvector maps when significant positive correlations are found. Three graphical agglomerative criteria were tested: Gabriel graph (Gabriel), relative neighborhood (Relative) and minimum spanning tree (MST). Binary, linear and concave weighting parameters were also tested.

		All ASVs			Frequent ASVs only	
Taxonomic level	**Neighborhood criterion**	**adj. R^2**	***p***		**adj. R^2**	***p***
Fungi	Gabriel - binary	0.014	0.073		0.031	0.020
	Gabriel - linear	NA	NA		0.034	0.008
	Relative - binary	0.005	0.554		NA	NA
Ascomycota	Gabriel – binary	0.018	0.047		0.026	0.036
	Gabriel – linear	0.019	0.032		0.032	0.008
	Gabriel – concave down	0.020	0.028		0.027	0.039
Basidiomycota	Gabriel - binary	0.007	0.511		0.012	0.588
	Relative - binary	0.000	0.853		0.012	0.563
	MST - binary	0.014	0.219		0.022	0.341
Mortierellomycota	Gabriel - binary	0.007	0.791		0.018	0.663
	Relative - binary	0.010	0.728		0.024	0.622
	MST - binary	0.005	0.780		0.010	0.749

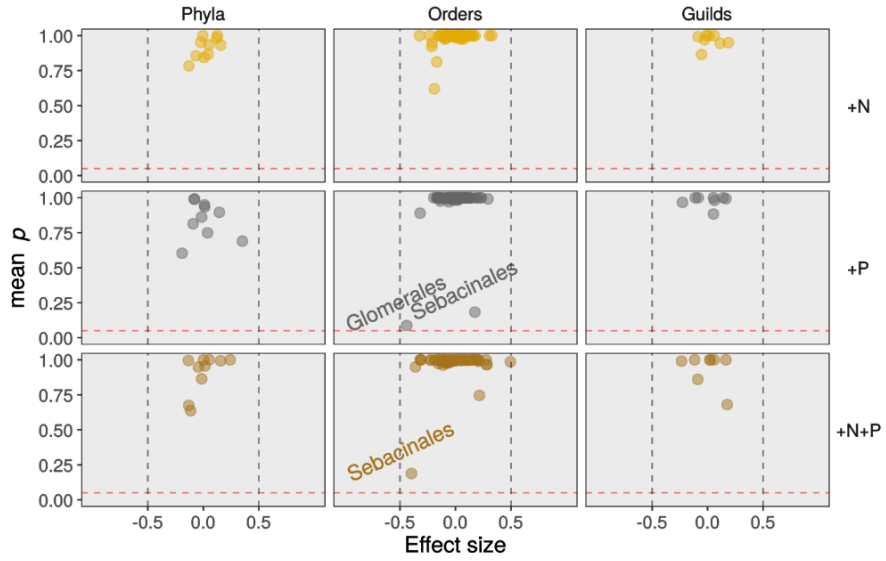
Table S3. 5 Sensitivity tests of the effects of long term fertilization on the structure of root associated fungal (RAF) communities

Pseudo-*F* tests assessed the statistical significance of the proportion of compositional variance explained by fertilization treatments. Tests were repeated twice dividing the dataset in frequent and rare variants. Frequently observed (i.e. presence-absence matrices of those ASVs occurring in at least 5 samples) and rare variants were modelled as a function of fertilization factors. Only the best represented fungal clades recovered in this study were tested with the exception of Glomeromycota. Values of *p* were calculated with permutation tests (n = 9999).

		Frequent ASVs only			Rare ASVs only	
Taxonomic level	**Factor**	**Pseudo *F***	***p***		**Pseudo *F***	***p***
Fungi	+N	1.521	0.001		1.007	0.243
	+P	1.377	0.005		1.145	0.000
	+N+P	1.197	0.031		1.023	0.301
Ascomycota	+N	1.752	0.000		1.078	0.062
	+P	1.553	0.001		1.171	0.007
	+N+P	1.188	0.038		1.061	0.112
Basidiomycota	+N	1.154	0.217		1.004	0.296
	+P	1.275	0.058		1.134	0.002
	+N+P	1.376	0.026		1.014	0.396
Mortierellomycota	+N	1.332	0.157		0.882	0.535
	+P	1.516	0.088		1.152	0.154
	+N+P	1.488	0.104		1.058	0.346

Figure S3. 7 Graphical illustration of the results of univariate tests of the effect of fertilization treatments on the relative read abundance of taxonomic clades and trophic guilds

Data was modelled by generalized linear models (glm). Abundance was calculated by aggregating ASV read counts according to their phylum (n=10), order (n=91) or guild (n=8) classification. Read counts were then transformed to log_2 ratios using the center log ratio procedure. Plots in the grid show the relation between the standardized effect size (median difference in relation to control / maximum difference within treatment) and the mean *p* value. Expected *p* values were corrected by the Benjamini-Hochberg procedure in order to minimize the probability of false positives due to multiple testing. The dashed horizontal line references a *p* value of 0.05.



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